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A RAPID METHOD FOR DETERMINATION OF ALPHA  
HYDROXY ACIDS IN SEAWATER AND BIOLOGICAL FLUIDS AT TRACE LEVELS

by

RYAN SCHWIDERSKI

A THESIS

Presented to the Faculty of the Graduate School of the  
MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

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Approved by

Shubhender Kapila Advisor  
Paul K. Nam  
Virgil J. Flanigan



## ABSTRACT

Alpha hydroxy acids (AHAs) – carboxylic acids with a hydroxyl substitution on the alpha carbon are an important class of molecules. Hydroxy Methyl Thiobutanoic Acid (HMTBA) is an  $\alpha$ -hydroxy analog of essential amino acid methionine; it finds extensive use as a feed supplement for avian and bovine species. Efficacy of alpha hydroxy acid uptake is important for nutritional studies and such studies rely on accurate and precise methods for determination of these chemicals in biological samples. Several methods for AHAs determination have been reported, however, the methods are tedious, requiring multiple sample preparation steps. Experiments reported in this thesis were aimed at development of a method that is simple, accurate and precise. The method involved lyophilization of sample matrices (bovine blood serum or seawater), extraction of the residues with solvents such as acetone or methanol, and reconstitution of the extracts in a suitable solvent system. The solvent system was dependent on the instrumental method used for final determination. The reconstituted extracts were then analyzed with liquid or the gas chromatography interfaced to mass spectrometry (HPLC-MS, GC-MS) or directly with electrospray ionization mass spectrometry (ESI-MS). Validation experiments with fortified synthetic seawater and bovine blood serum samples showed that analyte recoveries were consistently in the 90-100% range and analytes were readily monitored over a 0.01 – 50 parts per million concentration range. Improved selectivity was achieved through S – methylation of HMTBA to the sulfonium chloride cation. The cation was readily detected through direct introduction into ESI-(MS)<sup>2</sup> and monitoring for a fragment ion at m/z 103 resulting from molecular ion m/z 165 with the loss of dimethyl sulfide as the neutral species.

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## 1. INTRODUCTION AND REVIEW OF LITERATURE

### 1.1 ANIMALS AND NUTRITION

Nutrition is defined as the sum of the processes by which an animal or plant takes in and utilizes food substances (1). Plants produce nutritive molecules, such as proteins, carbohydrates, and fats, through the uptake of carbon dioxide from the air, inorganic chemicals from the soil, and energy derived from sunlight in a process called photosynthesis. This process starts the nutrient cycle, which corresponds to the passing of energy from organism to organism up the food chain. The nutrient cycle is brought full circle through the death and decay of all plants and animals, which in turn facilitates the release of nutrients back into the soil that ultimately sustains plant life. Humans fit into the food chain as an omnivore consuming both plants and animals, and rely largely on agriculture to fulfill their nutritive needs (1).

**1.1.1 Amino Acid Requirements of Mammalian Species.** The amino acid requirements of most mammalian species are met through direct absorption of hydrolyzed amino acids from the GI tract or transamination of precursor molecules -  $\alpha$ -keto acids. Ruminants can meet some of the amino acid requirements through conversion of nonprotein nitrogen (NPN) to amino acids and proteins by rumen microflora (2). Ruminant protein requirements are divided into two categories the fermentable, or total digestible nitrogen (TDN), and ruminally undegradable protein (RUP) (3). Microbial fermentation results in deamination of amino acids with the release of ammonia and organic acids. The rumen microorganisms can utilize the ammonia and acids as energy sources and/or precursors for the synthesis of new amino acids and proteins (2). Thus

microbial metabolism alters amino acid composition of the dietary proteins. Proteins that escape rumen degradation are known as the RUP and are directly available to the animals, such proteins are often referred to as the “by-pass” protein (2, 3, 4). The by-pass characteristic is dependent on the type of feed used and the preconditioning steps used during feed preparation.

The essential amino acid requirements particularly that of methionine can be met via the supplementation of amino acid hydroxy analogs, such as the hydroxy analog of methionine; hydroxy methyl thio butanoic acid (HMTBA). The hydroxy acid supplement can be utilized efficiently through its conversion to the keto acid and subsequent transamination to an amino acid. An amino acid is classified as an essential amino acid if the animal cannot successfully synthesize the  $\alpha$ -keto acid precursors of the amino acids. Examples of amino acids that are not synthesized by animals are Lysine, histidine, leucine, isoleucine, valine, methionine, threonine, tryptophan, and phenylalanine. Literature shows that these nine amino acids are essential for most all animal species (4, 5, 6). Therefore, protein requirement of animals depends on the essential amino acid requirements, while the bioavailability of an essential amino acid depends on digestibility of the proteins present in the feed and absorbability of a particular essential amino acid (4, 7).

### **1.1.2 Supplementation of Synthetic Amino Acids and Non-protein Nitrogen**

**Sources.** High-protein fish meal is a good source of proteins for livestock, but it is too costly to be used as a regular supplement (8). Therefore, amino acids obtained through microbial fermentation or chemical syntheses have been produced; these large scale production routes yields crystalline amino acids that are added to animal feed. Large

scale production of amino acids has been shown to be cost effective. Fermentation is a better process because it can provide a product comprised only of the L-amino acids, which can be used directly by the animal. However, due to extensive deamination of crystalline essential amino acids efficacy of crystalline amino acid as supplements in ruminants and aves is low (2, 4, 8, 9).

**1.1.3 Supplementation of Amino Acid Analogs.** Amino acid analogs include  $\alpha$ -keto acids and  $\alpha$ -hydroxy acids can be used as supplements. The amino acid analogs can be used for supplementation only if these can be enzymatic converted to L-amino acids (4). Bioavailability of the amino acid analogs is also dependent on their survival and subsequent absorption in the GI track (10, 11). In ruminants, absorption depends on the rumen by-pass and the active transport through the intestine membrane (4, 5). Common reactions that are known to compete with bioconversion reaction include catabolic oxidation of the  $\alpha$ -keto acids as well as their direct use as an energy source. Analogs that are not absorbed are excreted through the GI tract, while absorbed analogs that cannot be metabolized are excreted through the urinary tract.

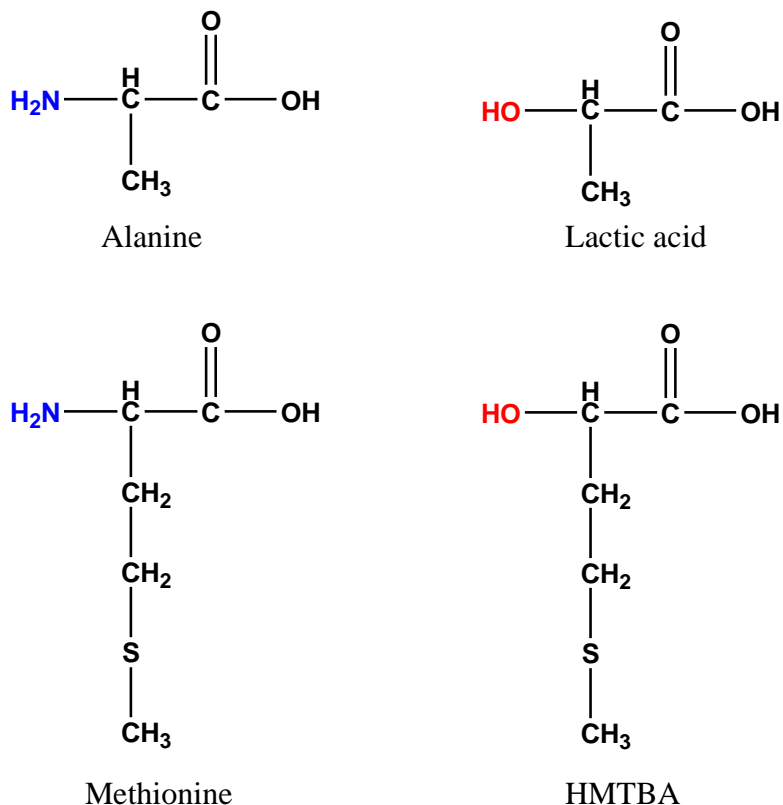


Figure 1.1 Structures of methionine, HMTBA, alanine and lactic acid.

**1.1.4 Use of HMTBA as Methionine Supplement.** Different approaches for protection of methionine from degradation in rumen have been attempted. In one method of L-methionine hydrochloride was encapsulated in a pH sensitive polymer matrix. Polymer matrix was stable around neutral pH and allowed transport of encapsulated amino acid through the rumen to the abomasum, where the polymer degrades because of the acidic environment. However, the approach was not commercially viable because of problems with the bioavailability of the encapsulated L-methionine and the high cost of the polymer coating (12, 13).

Studies have shown that the most effective means of methionine supplementation is through the use of HMTBA in ruminants and poultry. Monsanto Company (St. Louis, MO) introduced a commercial form of D,L-HMTBA as the calcium salt under a trade name name, MHA® (Methionine Hydroxy Analog) in 1956. In 1979, a liquid form of D,L-HMTBA was introduced under a new trade name Alimet®, which consisted of 88% (w/w) HMTBA and 12 % water. Alimet® is manufactured by Novus International, Inc., St. Charles, MO (14). The biological efficacy of HMTBA supplementation has been somewhat controversial with some researchers questioning the actual bioavailability of HMTBA, while others have reported that the introduction of HMTBA of cattle does not effectively increase the production of milk (15, 16). However, of late a consensus has emerged that HMTBA supplementation is in fact a good source of methionine in some animal species (9, 17, 18, 19, 20, 21). Studies with Carbon-14 labeled HMTBA have shown that HMTBA is more resistant to degradation in rumen fluid than methionine (20). Radioactive labeled methionine was detected in the blood, milk, and urine of the cows demonstrating that HMTBA was in fact bio-transformed to methionine (20). Patterson and Kung have reported the benefits of using HMTBA feed supplementation with cows by showing that both MHA® and Alimet® showed greater resilience in ruminal fluid compared to methionine (21). It was shown that while only 5% of the L-methionine added as supplement survived through the rumen nearly 60 to 80% of HMTBA added as the supplement survived passage through the rumen. Results also showed that supplementation of HMTBA increased L-methionine in tissues (21).

## **1.2 ANALYTICAL METHODS FOR ALPHA HYDROXY ACID DETECTION**

Alpha hydroxy acids have been a focus in an array of industrial applications from the food industry to the cosmetic industry. Common applications in the cosmetic industry include a number of skin care products such as wrinkle removing creams, facial cleansers, anti-aging products, etc. Also, AHA's are found throughout the food industry with common applications in candy, energy drinks, fruit drinks, and most items that include fruits. Fruits are the most common natural sources of AHA's, for this reason AHA's are commonly referred to as the fruit acids. AHA's have also become significant as animal feed supplements. The most common commercially synthesized AHA is the HMTBA, which has been used extensively over the past 30 years. The widespread use of AHA has led to a need for improved methods for monitoring these acids in a number of difficult matrices.

**1.2.1 HPLC Methods Used for the Detection of Alpha Hydroxy Acids.** A variety of methods have been developed for the detection of AHA. Due to the polar nature of these molecules commonly used methods employ High Pressure Liquid Chromatography (HPLC), but methods using Capillary Electrophoresis (CE) and Spectrophotometry have also been used.

Couch and Howard describe a HPLC based method for the detection of glycolic acid in cosmetic products. The method involved suspension of cosmetic products in a water: acetonitrile mixture. The extract was concentrated and basified to ensure deprotonation of the carboxylic functionalities and passed through a strong anion exchange (SAX) resin cartridge. Glycolic acid was retained on the anion exchange column through ionic interaction and was eluted with an acidic solution. The SAX cartridge



proved to be an efficient means for the elimination of background noise through selective retention of glycolic acid. The method avoided the use of heated columns or ion-pairing reagents and permitted good recoveries of glycolic acid from the samples comprised of lotions and creams. Average recoveries from the cosmetic media were reported to be ~100.0% (22).

A method reported by Nicoletti et al. describes a procedure for the detection of a number of AHAs extracted from cosmetic products. The method employed a narrow bore HPLC column and yielded an enhanced sensitivity and higher efficiency. In addition use of narrow bore column lowered the mobile phase volume utilized for separation; it also reduced sample volume required for analysis. The authors describe a procedure that does not employ tedious sample preparation or clean ups steps that are often needed for reliable sample analysis. Despite the simplified sample preparation described by the authors the method required the use of ion-pairing reagents for chromatographic separations. The method was considered a reliable, reproducible, and effective means for the determination of a variety of AHAs in cosmetic products with recoveries between 96 and 100%; the relative standard deviation of the method was reported to be 3%. (23).

A method reported by Tanabe et al. also afforded the convenience of a greatly reduced sample clean up procedure but relied on the use of ferric perchlorate as a complexing agent for lactic acid. The authors described a procedure that focused on the extraction of lactic acid and pyruvic acid from heparinized blood. They speculated that the method can be used as a general procedure for the detection of a number of alpha keto and AHAs. The method was used for detection of a limited number of analytes however the sensitivity of the method was generally was lower than that of other methods (24).

Other drawbacks were the use of expensive, non-conventional columns such as, strong cation exchange (SCX) and SAX columns that are not widely used for routine analysis (24).

### **1.2.2 Other Methods Used for the Detection of Alpha Hydroxy Acids.**

Methods that employ CE, modified HPLC techniques, spectrophotometers, and thin layer chromatography (TLC) are not used for routine analysis for a number of reasons; these include the use of expensive reagents, reduced method sensitivity, reduced method reliability, reduced reproducibility, and complicated sample and instrument preparation procedures.

Reportedly reliable methods for the detection of AHAs in cosmetic products and fruits have been produced. These methods allow for a very simplified sample preparation by requiring only dilution steps. A method described by Dutra et al. describes a CE technique for the determination of AHAs in cosmetic products. Their method used indirect ultraviolet detection with potassium phthalate as a background electrolyte. The authors also employed cetyltrimethylammonium bromide (CTAB) as an electrophoretic modifier to optimize analyte resolutions and to decrease analyte retention time. They reported that recoveries of 99 to 100% could be achieved with an analysis time of 3 minutes with good sensitivity showing limits of quantitation ranging from 0.4 to 1.0ppm. Combining the speed of analysis with the greatly reduced sample preparation time, this method could be truly considered rapid. Also, a method very similar to the previously discussed one was developed by Vorarat et al. included the use of the long-chain cationic surfactant CTAB, but opted for direct ultraviolet detection instead of indirect detection. Their method had similar results when compared to the previously mentioned one but the

direct detection suffered in sensitivity with limits of detection ranging from 2.5 to 5.0ppm. Even though these methods employ a very easy sample preparation they are not used nearly as often as HPLC methods because CE is not commonplace in today's laboratories (25, 26).

Modified HPLC techniques have gained use in recent years with an increased availability of technologies and techniques. Yokota et al. describe an HPLC technique that utilizes a post column addition of a ruthenium complex to take advantage of chemiluminescence detection. The method was developed for hydroxy acids and dicarboxylic acids to take advantage of the reactive characteristics of hydroxy acids. This method is possible because the ruthenium complex reacts with oxalic acid, which is produced from the oxidation of the hydroxy acid. The use of ultraviolet irradiation to promote the reactions was also described. The method showed good precision with relative standard deviations ~ 5% but was not able to match detection limits that were achieved by other methods with detection limits close to 2 nmoles (27).

A method developed by Matulis and Guyon describe a spectrophotometric technique used for the determination of citric acid. The technique relied on the AHAs ability to have a bleaching effect on the blue hue of a reduced molybdenate solution. The study showed consistent results but with many major drawbacks in comparison to already established methods for AHA detection. The study used a relatively complicated sample preparation and was not established for analytes extracted from a matrix. Also, the method was shown to have relatively poor detection limits with the lowest concentration of analyte studied at 50ppm. In comparison to other methods this would not be suitable for trace analysis (28).

**1.2.3 Methods Used for the Detection of 2-Hydroxy-4-(Methylthio) Butanoic Acid (HMTBA).** Due to HMTBA's widespread use as a supplement in animal feed, there is a need for a rapid, reliable, and precise method for its determination. However, a review of the literature showed that very few methods for such determination have been established. Published methods are based on gas chromatography with flame photometric detection (GC-FPD) and HPLC.

The method employing GC-FPD relied on the derivitization of the HMTBA with a silyating reagent, N, O-bis(trimethylsilyl) acetamide (BSA). The method was established for the determination of HMTBA extracted from cow's milk, urine, and tissue. Relatively complicated sample preparation steps were needed these often led to reduced recoveries and poor precision. The analysis yielded good detection limits with varied matrices 50ppb for milk, 80ppb for the urine, and 200ppb for the tissue samples. However, the method did show great variation from sample to sample throughout their study with recoveries ranging from 70 to 140% in the milk analysis, 80 to 150% in the tissue analysis, and 50 to 120% in the urine analysis. The inconsistency in the analyte recoveries for this method leaves it in need of further study and improvement to be used for routine analysis (29).

An HPLC method developed by Ontiveros et al. describes a procedure for the detection of HMTBA in an animal feed matrix. The method employs the use of a reversed phase amine column and ultraviolet detection. The method also takes advantage of a relatively simple sample preparation with only an extraction of the feed with an aqueous solution of 10% acetonitrile and the addition of base and acid before dilution for HPLC analysis. Good results were found with the analysis showing recoveries between

96 and 99% along with good precision showing relative standard deviations between 3 and 4%. The method also afforded relatively good sensitivity with a detection limit close to 1.5ppm. Even though the described method showed good results, an analysis that could provide greater sensitivity with a procedure that has a simpler sample preparation and a more simple instrumental approach was warranted (30).

## 2. STATEMENT OF OBJECTIVES

The objective of this work was to develop a rapid, accurate and precise method for determination of alpha hydroxy acids in general and HMTBA in particular at trace levels (parts per million – parts per billion) in particular in biological and environmental matrices such as blood serum and seawater. To meet the overall objective a series of experiments were carried out to:

1. Optimization HMTBA recovery from saline solutions and bovine blood serum.
2. Evaluation and optimization liquid chromatography separation of AHAs.
3. Evaluation and optimization of ESI-MS parameters.
4. Esterification of AHAs with different alcohols.
5. Evaluation and optimization of gas chromatography separation of AHAs esters.
6. Selection of predominant ions for quantification of AHAs esters.
7. Evaluation and optimization of HMTBA S-methylation.
8. Evaluation and optimization of ESI-MS-MS parameters for S-methyl HMTBA.

### 3. EXPERIMENTAL

A systematic approach was used to characterize methods for the detection of alpha hydroxy acids in two matrices i.e. synthetic seawater and bovine blood serum. Methods were developed for three different types of instrumentation that include reverse phase High Performance Liquid Chromatography with UV detection (HPLC), Gas Chromatography with Mass Spectrometry (GC-MS), and direct injection Electrospray Ionization with Mass Spectrometry (ESI-MS). The methods were developed with accuracy, precision, ease, and speed as our main criteria for evaluation. A method that is quick, accurate, and precise with relative ease of performance was sought and achieved. Details of experiments and instrumental parameters carried out to achieve the stated objectives are given in the sections that follow.

#### 3.1 MATERIALS

Tartaric acid, Malic acid, and Mandelic were all purchased from Sigma Aldrich, St. Louis, MO. The methanol, acetonitrile, acetone, anhydrous methanol, anhydrous ethyl alcohol, 2-propanol, 1-propanol, iso-octane, ethyl acetate, hexane, and ammonium hydroxide were all purchased from Fisher Scientific, St. Louis, MO. Hydrochloric acid gas was obtained from Scott Specialty Gas, Plumsteadville, PA. Nitrogen gas was obtained from Ozarc Gas, Rolla, MO. D,L-HMTBA (Alimet®) was procured from Novus International, Inc., St. Louis, MO. The salt used for the production of synthetic seawater was Instant Ocean, which is manufactured by Spectrum Brands, Inc., Atlanta, GA. The 25cm x 4.6mm i.d., 5µm particle size C-18 column for the HPLC analysis and

the DB-5MS 30m x 0.25mm, 0.25 $\mu$ m film thickness column for the GC-MS analysis were purchased from P.J. Cobert Associates, Inc., St. Louis, MO. The nano-pure water used in the experimentation was generated using a Synergy 185 filtration system from Millipore Corporation, Billerica, MA.

### **3.2 RECOVERY OF HMTBA FROM SALT SOLUTION AND BOVINE BLOOD SERUM**

Approaches for quantitative recovery of HMTBA residues from a relatively clean and simple matrix – synthetic seawater and a more complex biological matrix (bovine blood serum) were evaluated and optimized. The two matrices posed different challenges in extracting a hydrophilic analyte from the water based matrices. Details of approaches evaluated during the study are discussed below.

**3.2.1 AHA Extraction Procedure from a Synthetic Seawater Matrix.** Alpha hydroxy acids (AHA) were spiked to a solution of used synthetic seawater. The synthetic seawater was drawn from an existing mature reef aquarium with the salinity at 3.65% or specific gravity of 1.025. The AHAs were spiked to 1mL of salt solution at concentrations ranging from 10 parts per billion up to 1 part per million. The solutions were then prepared for lyophilization by placement in a -30°C freezer and allowed to freeze for 4 to 6 hours. Once frozen, the sample were moved to the freeze dryer and allowed to concentrate for 12 to 24 hours. Extraction was then carried out with Acetone (3 x 2mL). Each extraction step included brief vortex stirring and a 15 minute sonication followed by a 15 minute centrifugation. The extracts were then dried under a gentle stream of nitrogen gas (4.8 Grade). Depending on the next step the dried residues were



either picked up in a 50% solution of methanol in water for HPLC analysis or prepared for derivitization.

**3.2.2 AHA Extraction Procedure from Bovine Blood Serum Matrix.** Alpha hydroxy acids were spiked to a solution of bovine blood serum. The serum was provided to us from colleagues at the University of Missouri at Columbia. The AHAs were spiked to 1mL of blood serum at concentrations ranging from 50 parts per billion up to 2 part per million. The solutions were then prepared for lyophilization by placement in a -30C freezer and allowed to freeze for 4 to 6 hours. Once frozen, the sample were moved to the freeze dryer and allowed to concentrate for 12 to 24 hours. Extraction was then carried out with Methanol (3 x 2mL). Each extraction step included brief vortex stirring and a 15 minute sonication followed by a 30 minute centrifugation. The extracts were then dried under a gentle stream of nitrogen gas (4.8 Grade). Depending on the next step the dried residues were either picked up in a 50% solution of methanol in water for HPLC analysis or prepared for derivitization.

### **3.3 ANALYTICAL METHODS USED FOR DETECTION**

Three different methods were developed for the detection of alpha hydroxy acids. Methods for HPLC, GC-MS, and ESI-MS were developed and carried out for a number of alpha hydroxy acids. The HPLC and GC-MS methods encompassed all AHAs while the ESI-MS method was used only for 2-Hydroxy-4-(Methylthio) butanoic acid (HMTBA). The other AHAs studied include Tartaric acid (TarA), Malic acid (MalA), and Mandelic acid (ManA). The HMTBA was supplied from Novus International Inc. out of St. Louis, Missouri in the form of Alimet®. Alimet® is a highly viscous, brown

liquid that is 85% HMTBA. All other AHAs were obtained from Sigma Aldrich out of St. Louis, Missouri.

**3.3.1 Reversed Phase High Performance Liquid Chromatography.** The instrument used for liquid chromatographic analysis was a Hitachi 2000 series (Hitachi Instruments, Inc., San Jose, CA). The system consisted of a piston pump (L-2100), a column oven (L-2300), an autosampler (L-2200) with a 50 $\mu$ L injection loop, and a diode array detector (DAD, L-2450). The separations were carried out using a reversed phase C18 column (25cm x 4.6mm i.d., 5 $\mu$ m particle size). The data was recorded using EZChrom Elite version 3.1.3 software.

The dried residues from sample preparation were used for the analysis. The dried residues were reconstituted in a 50% solution of methanol in water. A volume of 1 or 2mL was generally used. The solutions were then agitated using vortex stirring for 30 seconds followed by filtration through 0.22 $\mu$ m membrane filters and transferred to 2mL autosampler vials. The samples were then placed in the autosampler for analysis.

The separation was carried out using DAD detector with the monitoring wavelength at 210nm. The mobile phase used was a combination of water with 0.1% trifluoroacetic acid and acetonitrile. An injection volume of 10 $\mu$ L was used for each sample with a mobile phase flow rate of 1.0mL per minute.

**3.3.2 Direct Injection Electrospray Ionization.** The instrument used of the ESI-MS analysis was a Varian 1200L Quadrupole MS/MS (Varian, Inc., Palo Alto, CA). It was fitted with a solvent pump (ISCO SFC-500 Microflow Pump) for the introduction of a 50:50 mixture of methanol and water. The flow rate was set at 250 $\mu$ L per minute with injections using a 5 $\mu$ L sample loop. The system was run in MS/MS mode with the

collision energy at -15eV with the Q2 pressure at approximately 1.00 mtorr. The MS/MS analysis was done at m/z 165 and the daughter ion monitored was at m/z 103. The detector voltage was held at 1500V with the needle voltage at 3500V. The nebulizing gas was nitrogen and the drying gas was held at 100°C.

If ESI-MS analysis was to be done the sample extracts were transferred to 10mL ampoules for derivitization. After the samples from the blood serum and seawater were extracted and transferred to the ampoules they were brought to dryness under a gentle stream of nitrogen while being heated at 30-40°C. The ampoules were then fitted with stir bars and an alcohol, hydrochloric acid solution was added. The alcohol with hydrochloric acid solution was prepared by taking a known mass of alcohol and bubbling hydrochloric acid gas through the solution until enough had been added to produce a mixture that was approximately 10-12M. The molarity of the solution was determined by using a w/w ratio of the components. Immediately after the alcohol with hydrochloric acid solution was added to the reactions the ampoules were sealed using a torch and heated to 70-75°C for 1 hour. After heating the reactions were placed in a freezer to cool for at least 4 hours. Once sufficiently cooled the reactions were dried under a gentle stream of nitrogen while heating to 35-40°C. Once dried the reactions were prepared for analysis.

The samples were prepared by reconstitution in a water solution containing 0.01% ammonium hydroxide. The reactions were agitated using a vortex stirrer for 30 seconds and filtered into 2mL autosampler vials using 0.22µm membrane filters. They were then allowed to sit at room temperature for at least 1 hour for the seawater samples and at least

4 hours for the serum samples. Injections were done in triplicate for each sample over a 2 minute period with an injection every 30 seconds.

**3.3.3 AHA Analysis with Gas Chromatography and Mass Spectrometry.** The gas chromatographic analysis used a Thermo instrument (Thermo Fisher Scientific, Waltham, MA). The GC oven was a Trace Ultra series with a TriPlus AS autosampler. The mass spectrometer was a PolarisQ MS. The software used for data analysis was Xcaliber. The injector was held at 240°C with the transfer line at 260°C. Each injection was done with the autosampler and used 1µL injections. The oven was started at 50°C and held for 1 minute, ramped at 8°C per minute to a final temperature of 240°C. The final temperature was held for 4 minutes giving a total run time of 20 minutes. Helium was used at the carrier gas with a flow rate of 1.2mL per minute. Chromatographic data was visualized in an Extracted Ion Chromatogram view. The chromatograms were extracted with the most abundant 3 to 4 ions in each compound specific mass spectrum.

For GC-MS analysis the sample extracts were transferred to 10mL ampoules for derivitization. After the samples from the blood serum and seawater were extracted and transferred to the ampoules they were brought to dryness under a gentle stream of nitrogen while being heated at 30-40°C. The ampoules were then fitted with stir bars and an alcohol, hydrochloric acid solution was added. The alcohol with hydrochloric acid solution was prepared by taking a known mass of alcohol and bubbling hydrochloric acid gas through the solution until enough had been added to produce a mixture that was approximately 1-2M. The molarity of the solution was determined by using a w/w ratio of the components. Immediately after the alcohol with hydrochloric acid solution was added to the reactions the ampoules were sealed using a torch and heated to 70-75°C for

1 hour. After heating the reactions were placed in a freezer to cool for 30 minutes. Once sufficiently cooled the reactions were dried under a gentle stream of nitrogen while heating to 35-40°C. Once dried the reactions were prepared for analysis.

The samples were prepared by reconstituting the dried residues in 1-2mL of isooctane. The reaction mixtures were agitated for 30 seconds using a vortex stirrer. After agitation the solutions were transferred to 2mL autosampler vials for analysis.

### 3.4 HMTBA PURIFICATION

All experiments using 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) took advantage of the purified form of the compound. The Alimet® that was provided was exposed to a purification method that incorporated a bulb to bulb distillation similar to a Kugelrohr distillation apparatus. The purified compound was a yellowish color with a transition temperature close room temperature. The crystalline state of the compound was achieved with refrigeration. Details of the procedure are given below.

**Bulb to Bulb HMTBA Distillation.** The raw Alimet® was transferred to a round bottom flask. The round bottom flask placed inside an oven and was fitted with a double ended collection bulb. Underneath the collection bulb was an ice bath and jack stand. The collection bulb was connected to a vacuum hose which was connected to a rotating motor. The motor was connected inline to a high vacuum pump. The oven was heated to 180-220°C while the vacuum achieved being close to 1mtorr. The HMTBA was heated and distilled to the collection bulb, where it was collected and later transferred to vials for refrigerated storage.

### 3.5 PREPARATION OF STANDARDS

All standards used for calibrating the instrument response were prepared from pure chemicals obtained from commercial vendors. Calibration standards of HMTBA were prepared from the vacuum distilled form.

**3.5.1 Preparation of HMTBA Ester Standards.** The HMTBA esters were all prepared using the same procedure with differing alcohols. In each case a known amount of HMTBA was added to a round bottom flask, fitted with a stir bar and condenser, with enough alcohol (methanol, ethanol, *n*-propanol, and *i*-propanol) to make the reaction mixture approximately 1.0 Molar. The reactions then had HCl gas bubbled through them for 10 minutes. After the addition of HCl the reactions were heated to reflux while stirring until no starting material was observed. Reactions were monitored by Thin Layer Chromatography (TLC) using a 70:30 mixture of Hexane:Ethyl acetate. Once the reaction had gone to completion they were removed from the heat and brought to dryness under vacuum. The product was then reconstituted in Ethyl acetate (100mL), washed with water (2 x 50mL), and brine (2 x 50mL). The Ethyl acetate layer was then dried with anhydrous Sodium sulfate and filtered. It was then brought to dryness under vacuum and solvent removal was completed under high vacuum for 1 hour. Approximately 9 grams of each ester was prepared with each reaction having a yield close to 65%.

**3.5.2 Preparation of Other Alpha Hydroxy Acid Ester Standards.** The other AHAs (Tartaric acid, Malic acid, and Mandelic acid) were prepared in a similar fashion

to the HTMBA ester standards. For the reactions a large batch of *i*-propanol with HCl was produced just prior to the start. The *i*-propanol was produced by bubbling HCl gas through the solution for 20 minutes. Each AHA was added to a round bottom flask that was fitted with a stir bar and condenser. Enough *i*-propanol with HCl was added to bring the reaction to approximately 1.0 Molar. They were then heated to reflux while stirring until no starting material was observed. The reactions were monitored by TLC using a 50:50 mixture of Hexane:Ethyl acetate. Once complete the reactions were removed from the heat and concentrated under vacuum. The product was then reconstituted in Ethyl acetate (50mL), washed with water (2 x 25mL), and brine (2 x 25mL). The Ethyl acetate layers were then dried with anhydrous Sodium sulfate and filtered. It was then brought to dryness under vacuum and solvent removal was completed under high vacuum for 1 hour. Approximately 6 grams of each ester was prepared with each reaction having a yield close to 85%.

**3.5.3 Preparation of S-Methyl HMTBA Standard.** The standard of sulfur methylated salt of HMTBA was prepared in the same manner as the small scale reactions. A known amount of HMTBA (100mg or less) was added to a 20mL ampoule fitted with a stir bar. The methanol was prepared by bubbling HCl gas through the solution until enough HCl had been added to produce a solution of approximately 11 to 12 Molar. The alcohol (no more than 3mL) was then added to the ampoule, which was then sealed. The ampoule was then heated to 70 to 75°C for 4 hours. It was then removed from the heat and allowed to cool in a freezer for 2 hours. The ampoule was then unsealed and the reaction brought to dryness under a stream of nitrogen while heated to 35 to 40°C. The

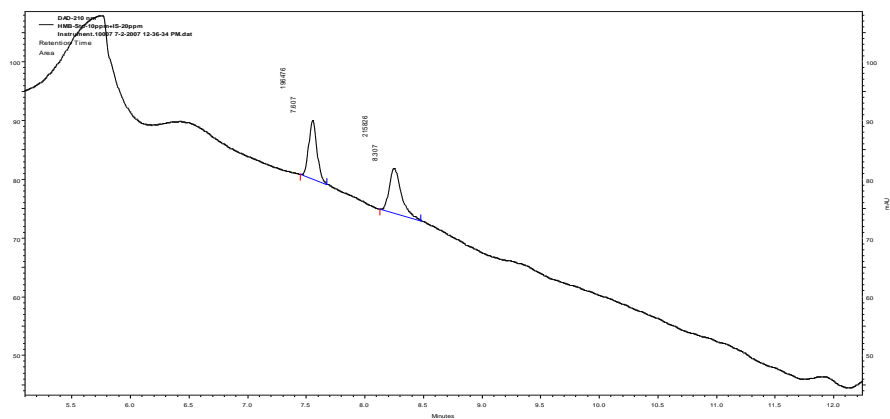
dried residue, which was a brown colored, highly viscous liquid, was then extracted with dried acetone (2 x 5mL) and reconstituted in water (10mL). The water layer was then placed in a -30°C freezer for 6 hours and then freeze dried for 48 hours. The dried residue, which at this point was a slightly brownish crystal, was again extracted with dry acetone (2 x 2mL). The resulting product was a white crystal. The reaction produced approximately 1.2mg of material with a reaction yield close to 90%.



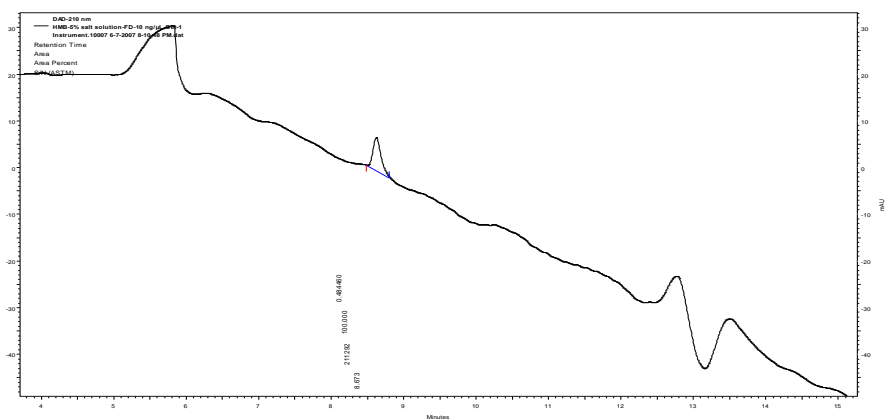
## 4. RESULTS AND DISCUSSION

### 4.1 HPLC DETERMINATION OF HMTBA IN SEAWATER AND BOVINE BLOOD SERUM

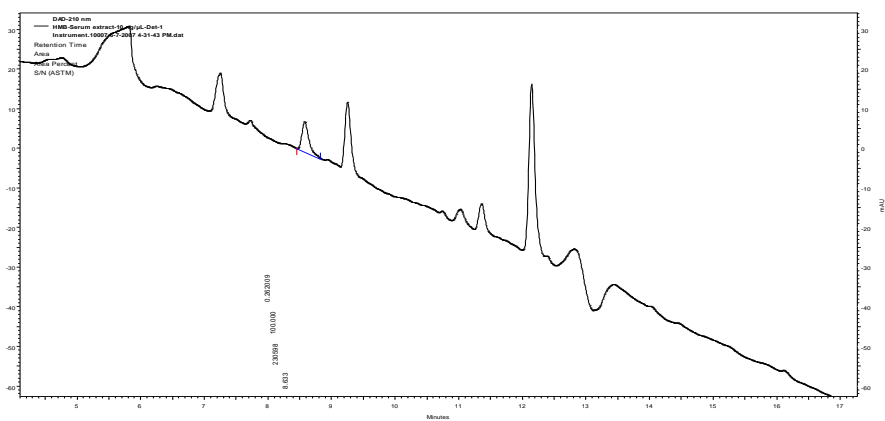
**4.1.1 Method LOD and LOQ Determination.** The 2-Hydroxy-4-(methylthio)butanoic acid (HMTBA) was spiked to a 100% water matrix and instrument sensitivity was determined. The HMTBA was analyzed using diode array detection while monitoring at 210nm. Identity of peaks was established with standards as reference samples. The instrument was found to have a minimum detection limit (MDL or LOD) of approximately 0.2 parts per million with a limit of quantitation (LOQ) of approximately 1.0 parts per million. The instrument performance was also tested with a seawater matrix (5% salts) with similar results. Instrumental performance for HMTBA spiked to blood serum matrix was also tested with a MDL of approximately 0.5 parts per million and a LOQ of approximately 2.0 parts per million. Typical HPLC chromatograms for each method are shown below in Figure 4.1.



(A)



(B)



(C)

Figure 4.1 A typical reversed phase HPLC chromatograms obtained for the standard (A), sea water extract (B) and the bovine blood serum extract (C).

**4.1.2 HPLC Method Performance for Seawater.** The results for the seawater determinations were quite consistent. Our method was validated using samples prepared by ourselves as well as blind analysis samples provided to us. My validation was done over approximately one month testing the reproducibility over many trials and varying HMTBA concentrations. Typical results from my validations are shown below in Table 4.1. After the results from the validation experiments showed good, consistent data, blind analysis samples were done. The samples were prepared and given to us from Novus International. The samples underwent the same extraction procedure and analysis conditions that were used for our validations. The blind analysis samples also showed good, consistent data with typical recoveries falling between 85 and 93%. The samples and data are shown below in Table 4.2.

Table 4.1 Results of Method Validation Simulated Seawater Samples.

<b>Laboratory Simulated Seawater Validation Samples</b>						
<i>Sample</i>	<i>Date Done</i>	<i>Area (HMB)</i>	<i>Amount of HMTBA (<math>\mu</math>g)</i>		<i>% Recovery</i>	<i>Std Dev</i>
			<i>Detected</i>	<i>Spiked</i>		
HMTBA 25 ppm	7-Jun-07	211292	21.0	25	<b>84</b>	4.2%
HMTBA 25 ppm	7-Jun-07	227002	22.4	25	<b>90</b>	
HMTBA 5 ppm	26-Jun-07	86684	4.8	5	<b>96</b>	1.4%
HMTBA 5 ppm	26-Jun-07	88868	4.9	5	<b>98</b>	
HMTBA 10 ppm	2-Jul-07	106440	8.6	10	<b>86</b>	1.4%
HMTBA 10 ppm	2-Jul-07	108451	8.8	10	<b>88</b>	

Table 4.2 HPLC results from blind HMTBA analysis extracted from seawater.

<b>Seawater Samples</b>				
<i>Sample code</i>	<i>Area of HMB</i>	<i>Detected HMB (ppm)</i>	<i>Target HMB (ppm)</i>	<i>% Recovery</i>
NBP-1	1071163	<b>46.0</b>	49.4	<b>93.2</b>
NBP-2	49376	<b>3.7</b>	4.9	<b>75.2</b>
NBP-3	ND	<b>ND</b>	0	<b>NA</b>
NBP-4	496423	<b>22.2</b>	24.6	<b>90.3</b>
NBP-5	477242	<b>21.4</b>	24.6	<b>87.0</b>
NBP-6	476125	<b>21.4</b>	24.6	<b>86.9</b>

**4.1.3 HPLC Method Performance for Blood Serum.** The serum analysis was a problem for us even though it started off working. Our validations were done using bovine blood serum that we acquired from colleagues at the University of Missouri-Columbia. All initial testing and analysis was performed using this serum. The results obtained from this seemed to show good recoveries that were consistent. The blood serum samples also used the same method used for the seawater analysis. The initial validations showed good data with typical recoveries between approximately 90 and 120%. The problem arose when blind analysis samples were provided. The samples showed a marked increase in background interference that could not be removed from the samples. The analysis was repeated many times and no solution could be found. For this

reason, other methods were sought for the analysis of HMTBA in blood serum. The results from the blood serum validation study are shown below in Table 4.3.

Table 4.3 Results of Method Validation Samples for HMTBA in Bovine Serum.

<b>Blood Serum Validation Samples</b>							
<i>Sample code</i>	<i>Date Done</i>	<i>Area (HMB)</i>	<i>Amount of HMB (<math>\mu</math>g)</i>			<i>% Recovery</i>	<i>Std Dev</i>
			<i>Detected</i>	<i>Calculated</i>	<i>Spiked</i>		
HMTBA 10ppm	7-Jun-07	213772	10.6	10.6	10	106	5.9%
HMTBA 10ppm	7-Jun-07	214978	10.7	10.7	10	107	
HMTBA 10ppm	7-Jun-07	243267	11.9	11.9	10	119	
HMTBA 10ppm	7-Jun-07	226994	11.2	11.2	10	112	
HMTBA 25ppm	23-Jun-07	269087	13.1	26.2	25	105	6.6%
HMTBA 25ppm	23-Jun-07	262328	12.8	25.6	25	102	
HMTBA 25ppm	23-Jun-07	230598	11.4	22.8	25	91	
HMTBA 25ppm	23-Jun-07	240017	11.8	23.6	25	94	
HMTBA 10ppm	2-Jul-07	260646	12.1	12.1	10	121	3.5%
HMTBA 10ppm	2-Jul-07	271364	12.6	12.6	10	126	

## 4.2 GC-MS DETERMINATION OF HMTBA IN SEAWATER AND BOVINE BLOOD SERUM

The alpha hydroxy acids (AHAs) were studied with Gas Chromatography using Mass Spectrometric detection because of the instruments capability to achieve relatively high sensitivity. A method was desired to analyze the compounds in a matrix at the sub parts per million levels. This was accomplished using this method for four AHAs, which include HMTBA, Tartaric acid, Malic acid, and Mandelic acid. Tartaric acid and Malic acid each have two carboxylic acid functionalities so as esters they exist as diesters, while mandelic acid only has one carboxylic acid group and is a monoester. Structures of the compounds can be seen in Figure 4.2.

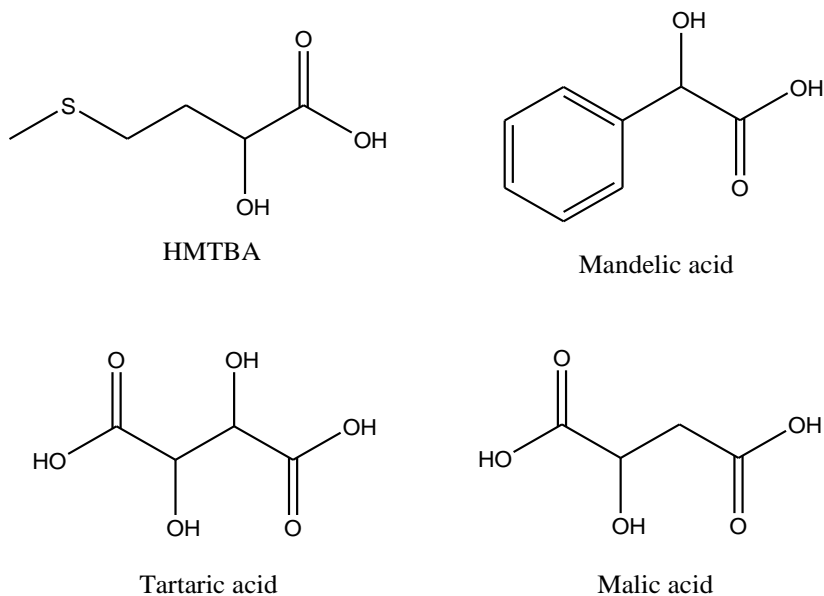


Figure 4.2 Structures of the alpha hydroxy acids studied.

The chromatography for the analytes was visualized as Extracted Ion Chromatograms (EIC) for all analyses performed. This was done to maximize sensitivity of the analysis as well as make the analyte peaks easier to identify. To determine the m/z

ratios used for analysis, standards were used to obtain each compounds mass spectrum, with the standards for each compound being mixed to 20 parts per million. The most abundant ions were then chosen for extraction. Each compounds mass spectrum showing the ions used for the EICs is shown below in Figure 4.3.

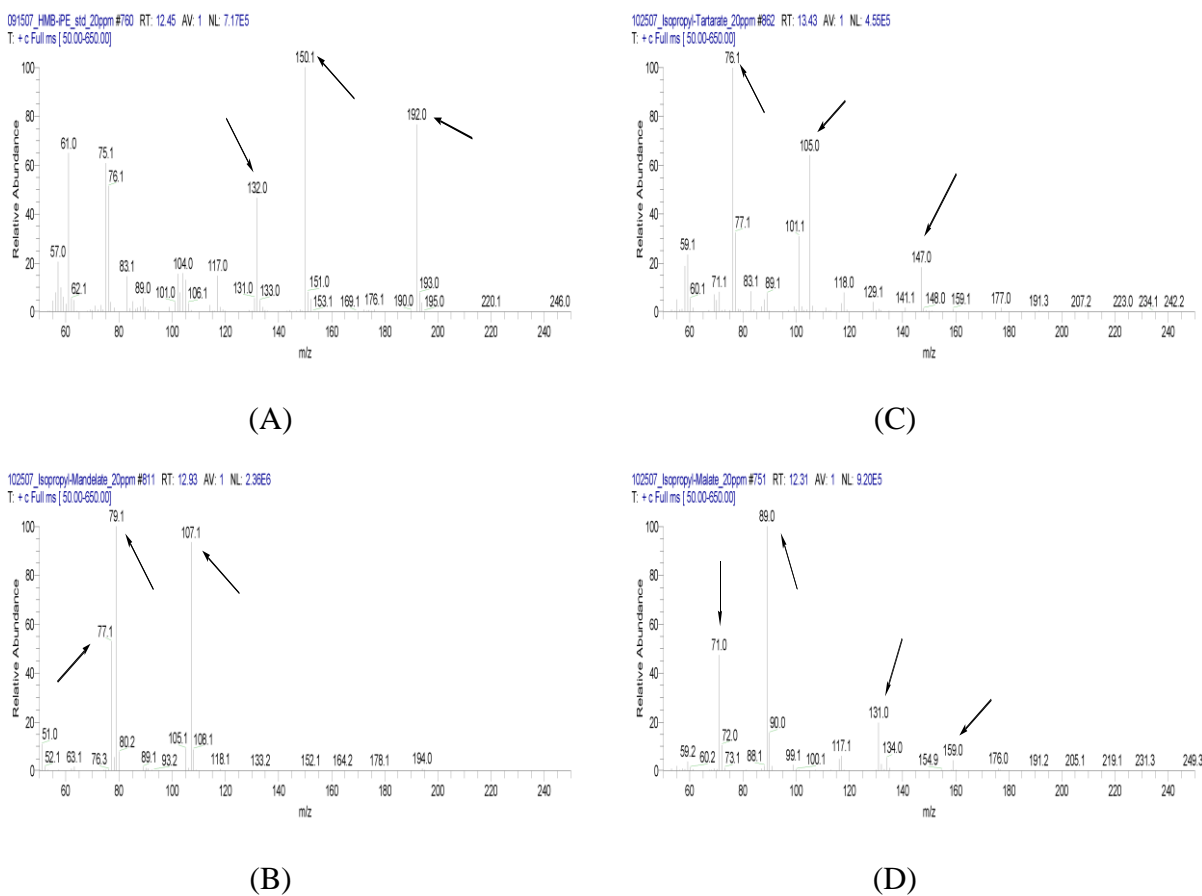


Figure 4.3 EI Mass spectra of HMTBA isopropyl ester (A), Isopropyl Mandelate (B), Diisopropyl Tartrate (C), and Diisopropyl Malate (D).

**4.2.1 Method LOD and LOQ Determination.** The AHAs were tested for instrument sensitivity using ester standards that were prepared. The ester standards were prepared in iso-octane for all trials. The HMTBA was tested as a methyl, ethyl, and



isopropyl ester while the other AHAs were only determined as isopropyl esters. The HMTBA showed almost the same sensitivity for all the esters. Each ester was determined to have a MDL of approximately 5 parts per billion with a LOQ of approximately 25 parts per billion. The MDL and LOQ for Diisopropyl tartrate were found to be approximately 20 and 100 parts per billion. Diisopropyl malate was found to have an MDL and LOQ of approximately 5 and 50 parts per billion while Isopropyl mandelate had an MDL and LOQ of approximately 3 and 10 parts per billion. Instrumental performance was then established for each compound over a range that included the LOD up to 500 parts per billion. The linearity of the instrument over the concentration was very good with each compound having an  $R^2$  value greater than 0.997. This can be seen below in Figure 4.4.

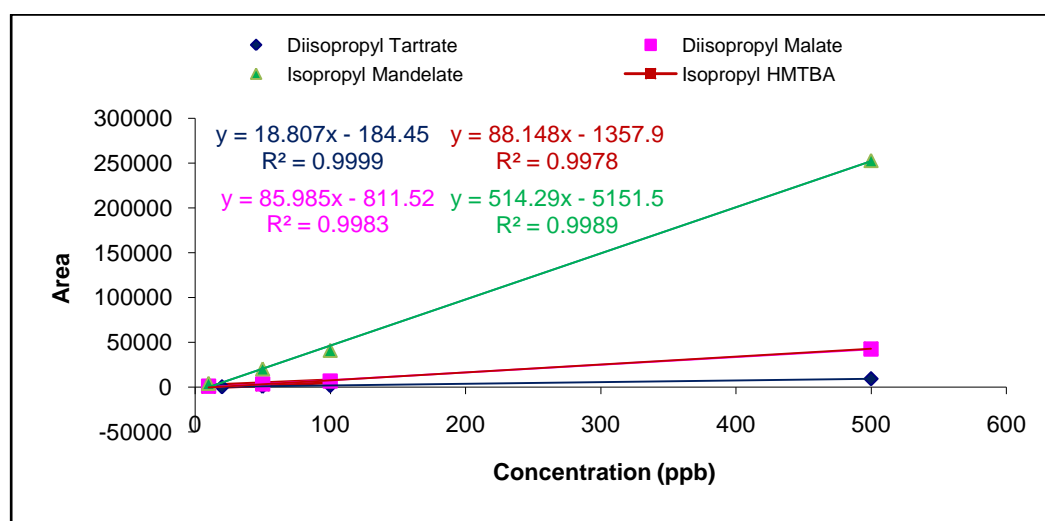
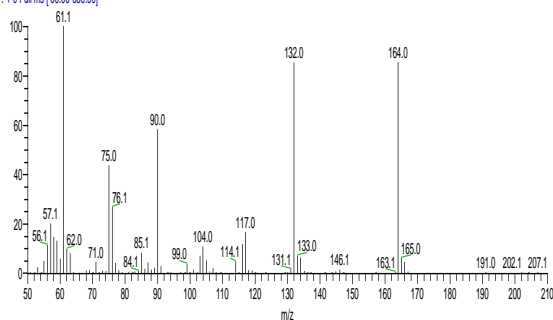


Figure 4.4 Graph showing the linearity of GC-MS response for the AHAs over a the 10 to 500 parts per billion concentration range.

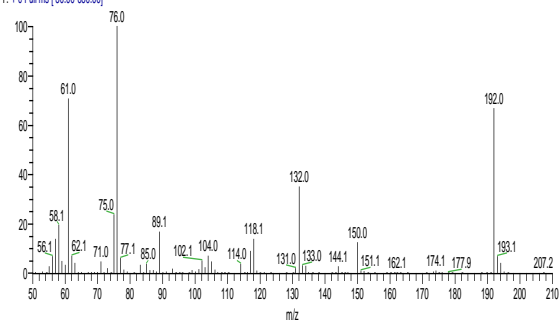
**4.2.2 Esterification of HMTBA.** The esterification reaction efficiency was tested using a matrix free sample of HMTBA. The analyte was spiked to acetone as a standard solution. The solution was then transferred to the reaction ampoule, dried under nitrogen, and the dried residues were reconstituted in the reaction media. The reaction was tested using a variety of alcohols, which include methanol, ethanol, *n*-propanol, and isopropanol. Mass Spectra of the HMTBA ester standards can be seen in Figure 4.5.

082007\_HMB-AE\_std\_20ppm#614 RT: 11.00 AV: 1 NL: 4.07E5  
T: +c Full ms [50.00-650.00]



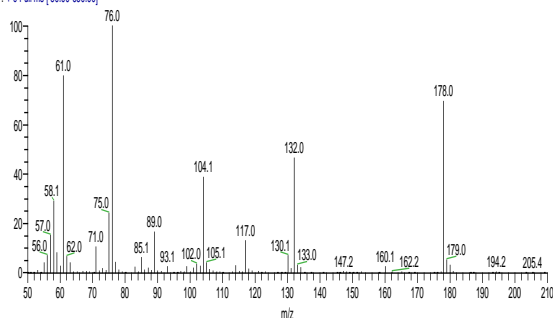
(A)

082007\_HMB-rPE\_nm4\_100ppm#838 RT: 13.34 AV: 1 NL: 5.98E5  
T: +c Full ms [50.00-650.00]



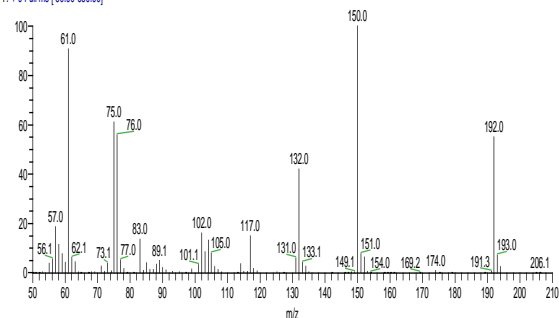
(B)

082007\_HMB-EE\_nm2\_100ppm#708 RT: 12.02 AV: 1 NL: 2.69E5  
T: +c Full ms [50.00-650.00]



(C)

082007\_HMB-rPE\_nm5\_100ppm#749 RT: 12.46 AV: 1 NL: 1.78E6  
T: +c Full ms [50.00-650.00]



(D)

Figure 4.5 Mass spectra of HMTBA esters; (A) methyl ester, (B) ethyl ester, (C) n-propyl ester, (D) isopropyl ester.

Initial experimentation was done using methanol but it was found that methanol lead to inconsistent results with reaction yields varying between 5 and 98%. The cause of this variation was thought to arise from the presence of water in the alcohol as well as the water generated from the reaction. Due to this result a product with greater stability was sought while maintaining the integrity of our esterification method. After testing the previously mentioned alcohols, the most consistent data was achieved with isopropanol. Over many trials a reaction yield between 50 and 75% was achieved, but a yield greater than 70% could not be reached. Reaction results are shown in Table 4.4.

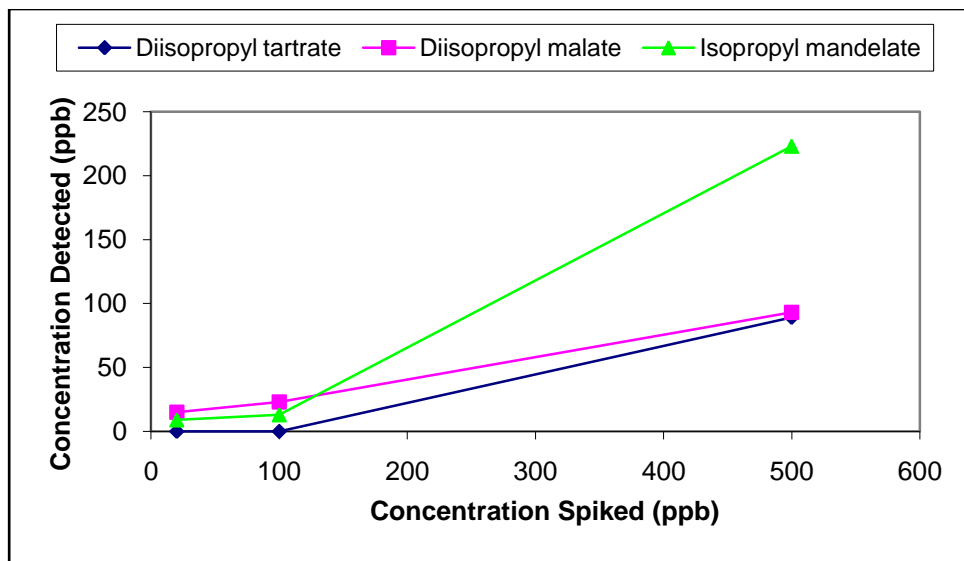
Table 4.4 Esterification efficiency of HMTBA with different alcohols  
(Esterification was done under identical conditions).

<b>HMTBA Esters</b>	<b>Reaction Yield</b>
Methyl ester	22%
Ethyl ester	36%
n-propyl ester	27%
i-propyl ester	70%

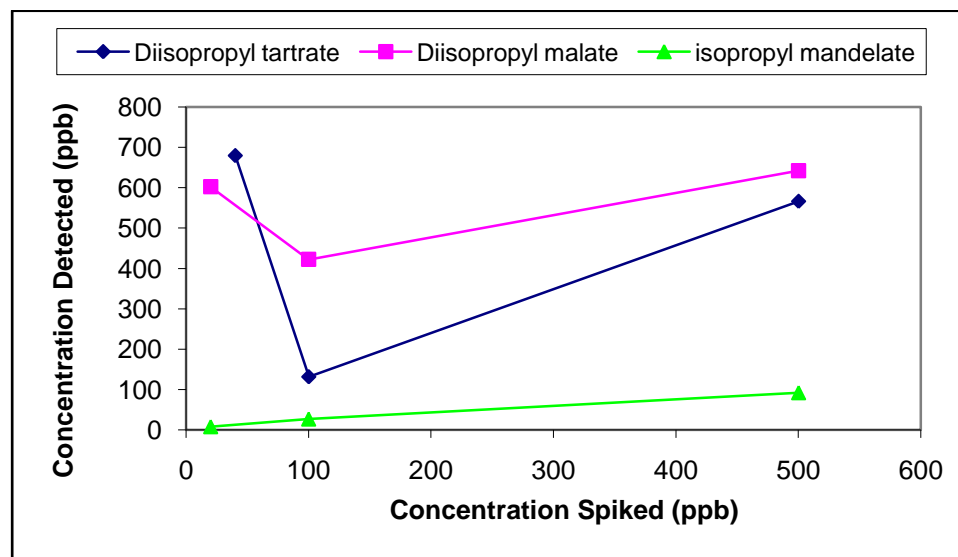
It was found by testing reaction media; the acidic environment produced from the addition of HCl gas in a sealed ampoule was harsh enough to generate the alkyl chloride from the alcohol. The formation of the alkyl chloride promoted the sulfur alkylation, which was the leading cause for a reduced reaction yield. This reaction was observed in both the case for the methanol and isopropanol. Other alcohols were not tested but were

assumed to undergo similar side reactions. It was concluded from these results that the side reaction arising from alkyl chloride formation left the analysis inconsistent. Therefore, another approach was desired.

**4.2.3 Esterification of Other Alpha Hydroxy Acids.** The same procedure was followed in an attempt to perform esterification on the other AHAs that were extracted from seawater and bovine blood serum. In blank trials the AHAs worked relatively well with yields close to 70%. In all cases the yields were found to be very inconsistent when extraction from blood serum and seawater was introduced for all the AHAs studied. The analysis from the seawater showed an increase in the amount of analyte detected as the amount spiked increased but the yields were all relatively low. In the case of the blood serum samples the analysis showed a significant interference for both Tartaric acid and Malic acid but did not affect the Mandelic acid reactions. Even though the Mandelic acid reactions did not show a significant amount of interference, the reactions yields were again found to be low. These trends are shown in Figures 4.6 and 4.7.

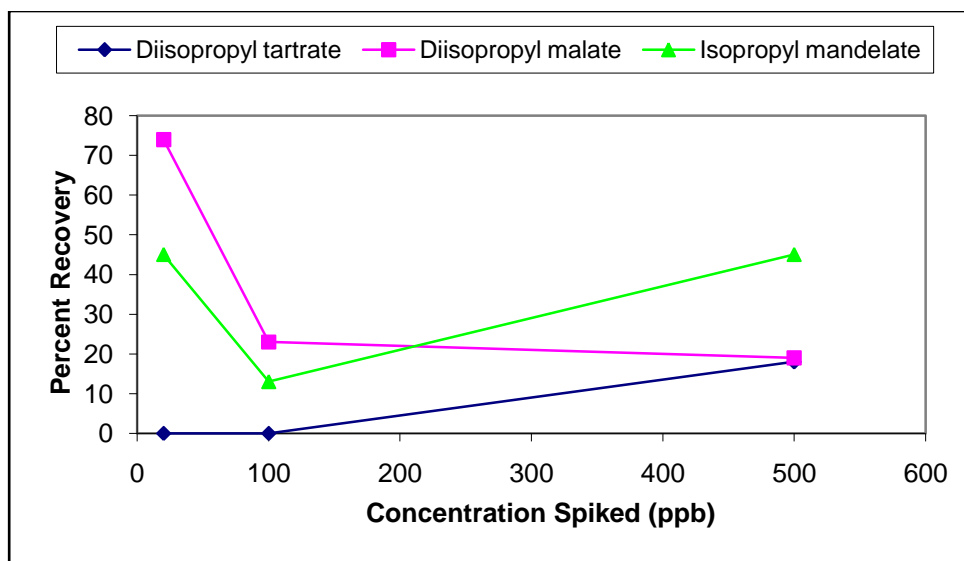


(A)

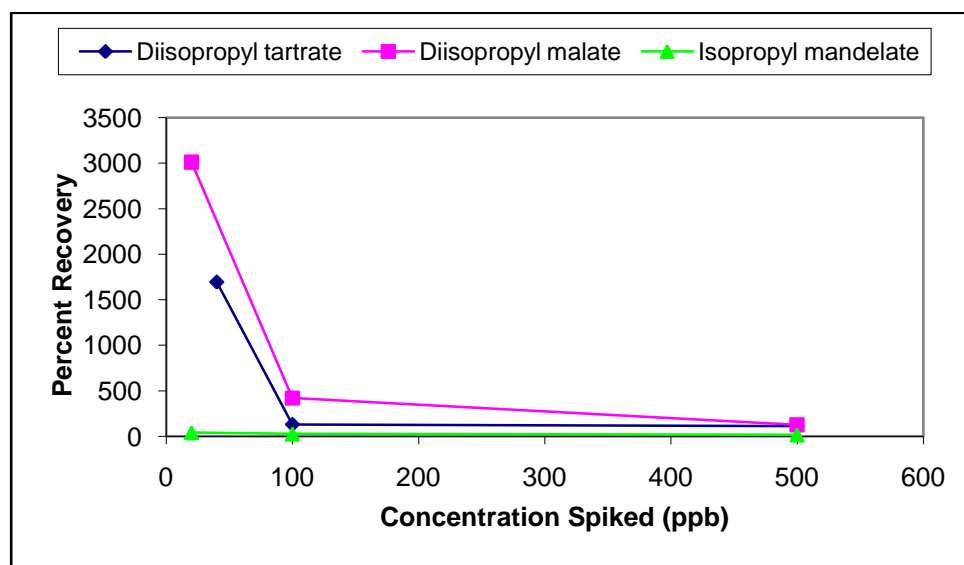


(B)

Figure 4.6 Graphs showing the concentration spiked versus the concentration detected from the esterification reactions from both matrices (A) Seawater, and (B) Bovine blood serum.



(A)



(B)

Figure 4.7 Graphs showing the detected percent recovery versus the concentration spiked from the esterification reactions from both matrices (A) Seawater, and (B) Bovine blood serum.

Findings from the esterification study of Tartaric acid, Malic acid, and Mandelic acid suggested that the method developed provided a reaction environment that was too acidic, leading to the formation of alkyl chlorides that caused side reactions to occur that inhibited the desired esterification.

#### **4.3 ESI-MS/MS DETERMINATION OF HMTBA IN SEAWATER AND BOVINE BLOOD SERUM**

The development of a direct injection with ESI-MS/MS method afforded us many advantages. The most important advantage is that the method allowed us an easy way of derivatizing HMTBA for detection. The sulfur methylation reaction could be done quantitatively using a very similar approach done with the esterification with the only difference in methods being a much higher acidity. The sulfur methylation reactions utilized an HCl concentration close to 12 Molar in methanol. Acidity was kept high to ensure the reaction was completed quantitatively in 1 hour. The only issue that needed to be resolved with this approach was the production of one product.

The MS/MS capabilities allowed us to be more selective. For this reason, it was thought that direct injection with ESI would afford the selectivity needed to eliminate background from the matrix. Also, the MS/MS gave greater sensitivity with a LOD nearing what was achieved using GC-MS, which was 5 parts per billion.

**4.3.1 Sulfur Methylated HMTBA Stability and Ester Conversion.** Due to the high acidity of the reaction two products initially form. The sulfur methylated HMTBA existed both as the free acid and the methyl ester. This problem was resolved easily by

the solvent used to reconstitute the dried samples. The slightly basic solution and a brief resting period was enough to convert the methyl ester product to the free acid. In our case, a solution of 0.01% ammonium hydroxide in water solution with a 1 hour resting period was enough for complete conversion. The sulfur methylated HMTBA also showed good stability in the basic solution. The speed of conversion to the free acid product and its stability is expressed in Table 4.5 and Figure 4.8.

Table 4.5 Stability of the S-Methyl HMTBA in a basic solution.

<b>Hours</b>	<b>Percent HMB</b>	<b>Percent Ester</b>	<b>Percent FA</b>
0.0	3.75	64.8	35.2
1.0	4.20	0.8	99.2
12.0	5.55	0.0	100.0
13.0	5.20	0.0	100.0
36.0	9.35	0.0	100.0
48.0	12.7	0.0	100.0



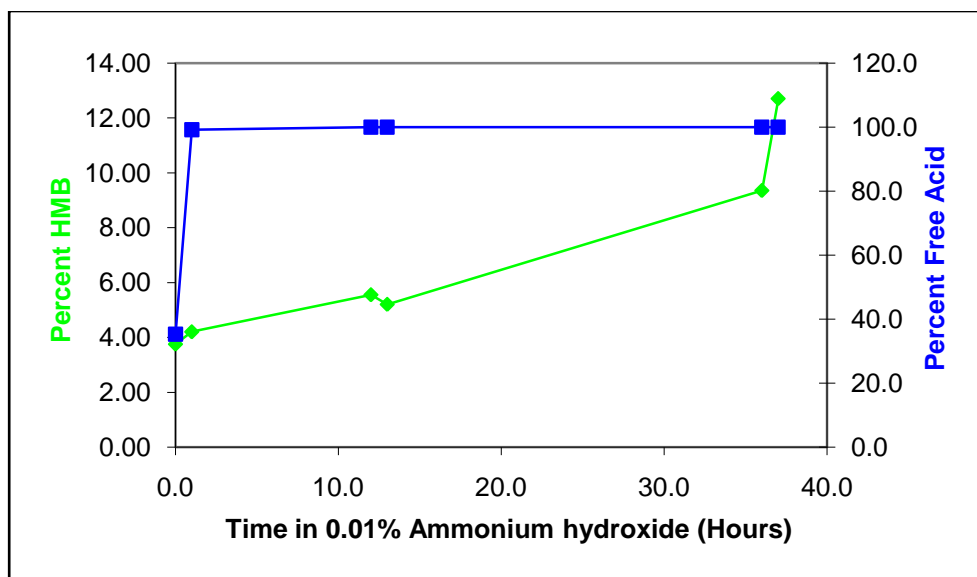


Figure 4.8 Graph showing the data from Table 4.5. S-Methyl HMTBA stability in a basic solution and conversion of the ester to the free acid.

The methyl ester form of the S-Methyl HMTBA salt was converted to the free acid form at 99.2% after only 1 hour. The reaction yield was good over a relatively long period of time. The reaction yield at time zero was close to 96% and only fell to just over 90% yield after 36 hours. The stability of the product assured that the analysis could be done without the worry of product degradation skewing analysis results.

**4.3.2 S-Methyl HMTBA Method Validation.** The validation study utilized the same reaction conditions as the method described previously as well as employing a 1 hour waiting period while the reactions were left to rest in a basic water solution. These reactions were done without extraction from a matrix to ensure the method was consistent. The experiment showed that over the concentration range all the reactions had yields greater than 92%. This data can be seen in Table 4.6. The data from the experiments can be seen below in Table 4.7.

Table 4.6 Reaction yields from validation of conversion of HMTBA to the S-Methyl salt.

Sample	HMB Avg Area	HMB Concentration (ppm)	Percent HMB	Rxn Yield
HMB-S-Me 1ppm T1	61095	0.0446	4.5%	95.5%
HMB-S-Me 1ppm T2	54661	0.0399	4.0%	96.0%
HMB-S-Me 500ppb T1	17281	0.0362	3.6%	96.4%
HMB-S-Me 500ppb T2	30222	0.0632	6.3%	93.7%
HMB-S-Me 100ppb T1	5081	0.0735	7.3%	92.7%
HMB-S-Me 100ppb T2	2893	0.0418	4.2%	95.8%
HMB-S-Me 50ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 50ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 10ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 10ppb T2	0	0.0000	0.0%	100.0%

Table 4.7 Results of the S-Methylation of HMTBA method validation.

Sample	Injection Statistics			Tial Statistics		
	Area Avg	Std Dev	%RSD	Area Avg	Std Dev	%RSD
HMB-S-Me 1ppm T1	1.6E+06	85135	5.3%	1.7E+06	202704	11.7%
HMB-S-Me 1ppm T2	1.9E+06	68712	3.7%			
HMB-S-Me 500ppb T1	9.5E+05	174947	18.4%	9.8E+05	41166	4.2%
HMB-S-Me 500ppb T2	1.0E+06	128097	12.7%			
HMB-S-Me 100ppb T1	2.1E+05	11505	5.5%	2.1E+05	6065	2.8%
HMB-S-Me 100ppb T2	2.2E+05	24217	11.1%			
HMB-S-Me 50ppb T1	3.0E+04	2640	8.9%	3.4E+04	6521	19.1%
HMB-S-Me 50ppb T2	3.9E+04	5422	14.0%			
HMB-S-Me 10ppb T1	1.1E+04	2639	24.7%	1.0E+04	327	3.1%
HMB-S-Me 10ppb T2	1.0E+04	1225	12.0%			

The study also showed a linear reaction response across the concentration range.

The linear response lead us to believe that even at the instruments minimum detection

limit, which is near 10 parts per billion, the reactions were going to near 100% completion. Although no HMTBA starting material was observed below 100 parts per billion because of the instrument detection limits, the reactions were assumed to be quantitative. The linearity response of the Sulfur methylation reactions can be seen below in Figure 4.9.

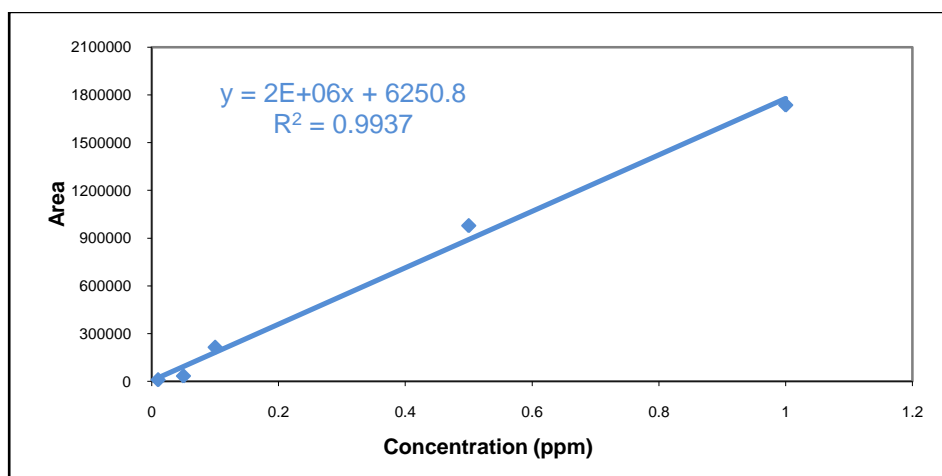


Figure 4.9 Graph showing the linearity of the S-Methylation of HMTBA over a concentration ranging from 10 ppb up to 1ppm.

**4.3.3 Seawater Extracted HMTBA S-Methylation.** At this step in the process the entire method was tested for extraction from a seawater matrix. The HMTBA spiked seawater samples were extracted and brought through the derivatization process. The reactions were done at five different concentrations ranging from 10ppb up to 1ppm. In all cases the reactions were observed to have yields greater than 97%. This can be seen below in Table 4.8. The instrument responses were about 17% of the reactions done without a matrix. The reactions were known to go to completion so this discrepancy was

attributed to the salt effect on the ionization efficiency of the ESI source. The acetone extractions also pulled a small amount of salt through as well, which lead to the decreased ionization efficiency of the samples analyzed. Although the extractions showed a 17% recovery they showed good linearity across the concentration range. The linearity trend can be seen below in Figure 4.11.

Table 4.8 Recovery of HMTBA Extracted from Seawater.

<b>Sample</b>	<b>HMB Avg Area</b>	<b>PAR</b>	<b>Percent HMB</b>	<b>Rxn Yield</b>
HMB-S-Me 1000ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 1000ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 1000ppb T3	0	0.0000	0.0%	100.0%
HMB-S-Me 500ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 500ppb T2	9328	0.0224	2.2%	97.8%
HMB-S-Me 500ppb T3	10033	0.0241	2.4%	97.6%
HMB-S-Me 100ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 100ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 100ppb T3	0	0.0000	0.0%	100.0%
HMB-S-Me 50ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 50ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 50ppb T3	NA	NA	NA	NA
HMB-S-Me 10ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 10ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 10ppb T3	NA	NA	NA	NA

Even though the analysis showed a decrease in response it did show a good response for all samples with no interference from background noise. An example of a

spectrum from the analysis can be seen below in Figure 4.10. The reactions were deemed quantitative and with no interference the analysis would be a viable means for detection. The linearity of the reactions was good with good precision. The analysis statistics are shown below for all samples in Table 4.9.

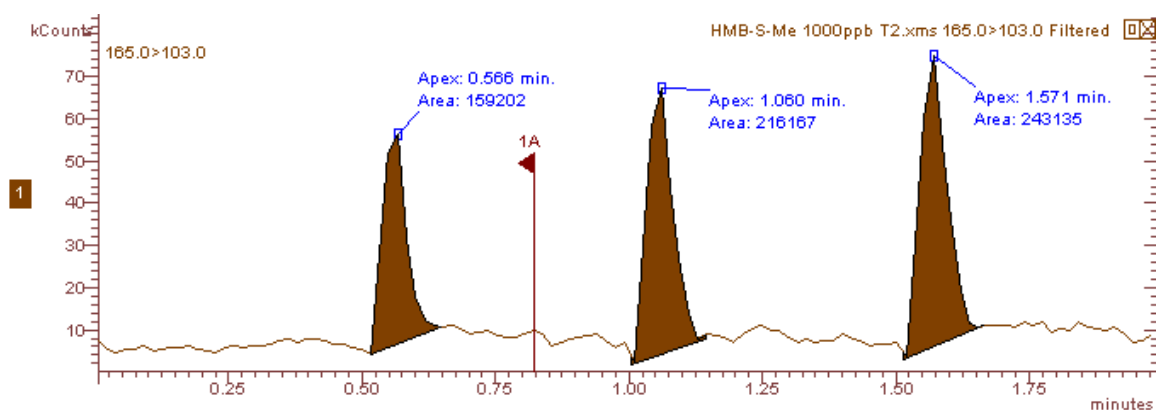


Figure 4.10 Spectrum of S-Methyl HMTBA from seawater extracted sample.

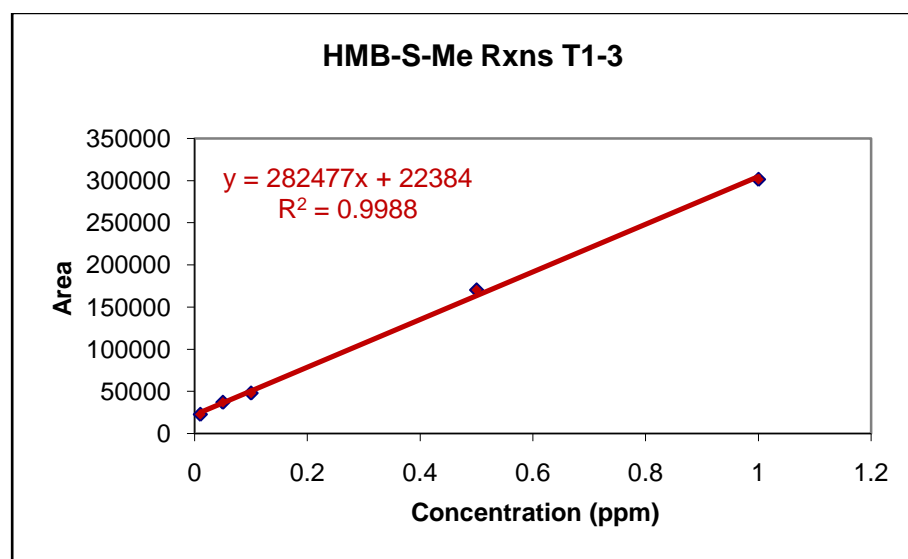


Figure 4.11 Response Linearity for S-Methyl HMTBA extracted from simulated seawater samples.

Table 4.9 Results of S-Methylation of HMTBA from seawater extracted samples.

Sample	Injection Statistics			Tial Statistics		
	Area Avg	Std Dev	%RSD	Area Avg	Std Dev	%RSD
HMB-S-Me 1ppm T1	434040	7264	1.7%	356626	130319	36.5%
HMB-S-Me 1ppm T2	206168	42851	20.8%			
HMB-S-Me 1ppm T3	429670	38791	9.0%			
HMB-S-Me 500ppb T1	324318	28154	8.7%	225528	89014	39.5%
HMB-S-Me 500ppb T2	151558	15818	10.4%			
HMB-S-Me 500ppb T3	200707	25489	12.7%			
HMB-S-Me 100ppb T1	193179	18700	9.7%	103279	77879	75.4%
HMB-S-Me 100ppb T2	56402	12203	21.6%			
HMB-S-Me 100ppb T3	60257	1921	3.2%			
HMB-S-Me 50ppb T1	185967	30145	16.2%	163628	100602	61.5%
HMB-S-Me 50ppb T2	53734	10254	19.1%			
HMB-S-Me 50ppb T3	251182	4319	1.7%			
HMB-S-Me 10ppb T1	180490	5074	2.8%	93407	77831	83.3%
HMB-S-Me 10ppb T2	30627	3397	11.1%			
HMB-S-Me 10ppb T3	69104	10179	14.7%			

Because the analysis showed a 17% recovery from the matrix free standard, the effect of salt on the instrument was tested. This experiment utilized S-methyl HMTBA standard at 500ppb with varying concentrations of salt. Salt solutions of 0.01, 0.05, 0.1, 1, and 5mM were used. Figure 4.12 shows the instrumental response from this analysis with very small amounts of salt having a positive effect on ionization but reaches a tipping point close to 1mM of salt. At a salt concentration of 5mM was shown to decrease the ionization efficiency by close to 50%. A concentration of 5mM of salt in water corresponds to 290ppm and natural seawater has approximately 3.65% salts, which

would correspond roughly to 36,500ppm of salts. The extraction of HMTBA from a seawater matrix could be drastically effected by the extraction of any salt from the matrix. It would take less than 1% of the seawater salt matrix to be extracted to reduce the method efficiency by 50%. For this reason, calibration standards extracted from an identical sample matrix must be used for the analysis.

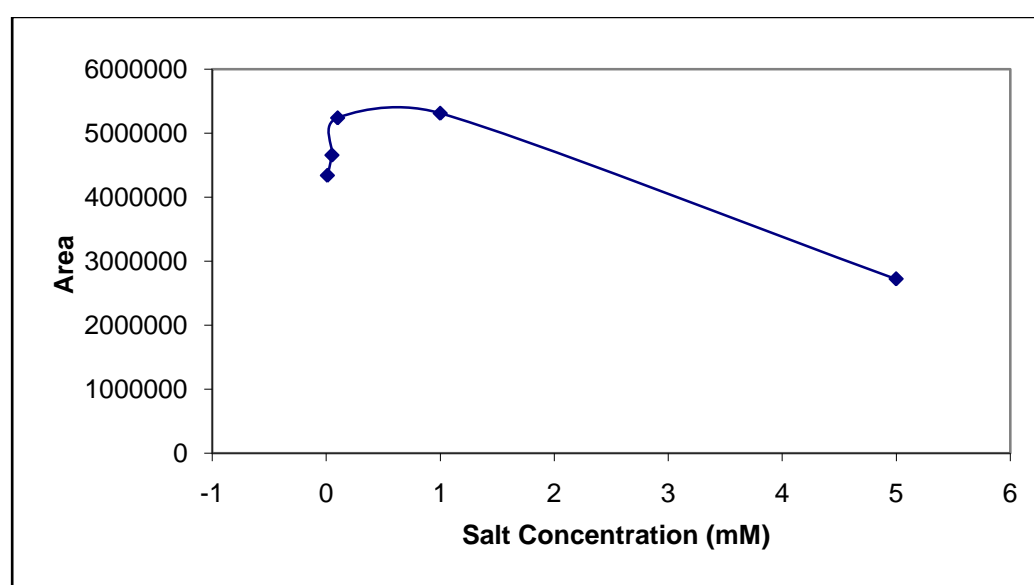


Figure 4.12 Graph showing the effect of salt on the ionization efficiency of an ESI source.

**4.3.4 Bovine Blood Serum Extracted HMTBA S-Methylation.** The process was then tested with extraction from a blood serum matrix. Like the seawater samples, the HMTBA spiked blood serum extracts were tested for derivatization efficiency. These reactions were done over a concentration ranging from 50ppb up to 2ppm. The reaction recovery was found to be good with no samples showing any residual HMTBA. Since no

residual HMTBA was detected the reactions were assumed to be quantitative. These results are shown below in Table 4.10.

Table 4.10 Results from HMTBA extracted from bovine blood serum with derivatization to the S-Methyl salt.

<b>Sample</b>	<b>HMB Avg Area</b>	<b>PAR</b>	<b>Percent HMB</b>	<b>Rxn Yield</b>
HMB-S-Me 2000ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 2000ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 2000ppb T3	0	0.0000	0.0%	100.0%
HMB-S-Me 1000ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 1000ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 1000ppb T3	0	0.0000	0.0%	100.0%
HMB-S-Me 500ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 500ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 500ppb T3	0	0.0000	0.0%	100.0%
HMB-S-Me 100ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 100ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 100ppb T3	0	0.0000	0.0%	100.0%
HMB-S-Me 50ppb T1	0	0.0000	0.0%	100.0%
HMB-S-Me 50ppb T2	0	0.0000	0.0%	100.0%
HMB-S-Me 50ppb T3	0	0.0000	0.0%	100.0%

When comparing the response for the serum extracted samples with the sample done with no matrix, the recovery appears to be 13%. It was thought that the same issue was occurring as seen with the seawater extracted samples. The methanol used for extraction was also extracting a portion of the serum solids. The dissolved serum solids are in turn causing a decreased ionization efficiency that misrepresents the methods true



HMTBA recovery. Even though, it appeared the recoveries were low the reactions showed good linearity over the concentration range tested. The linearity response can be seen in Figure 4.13.

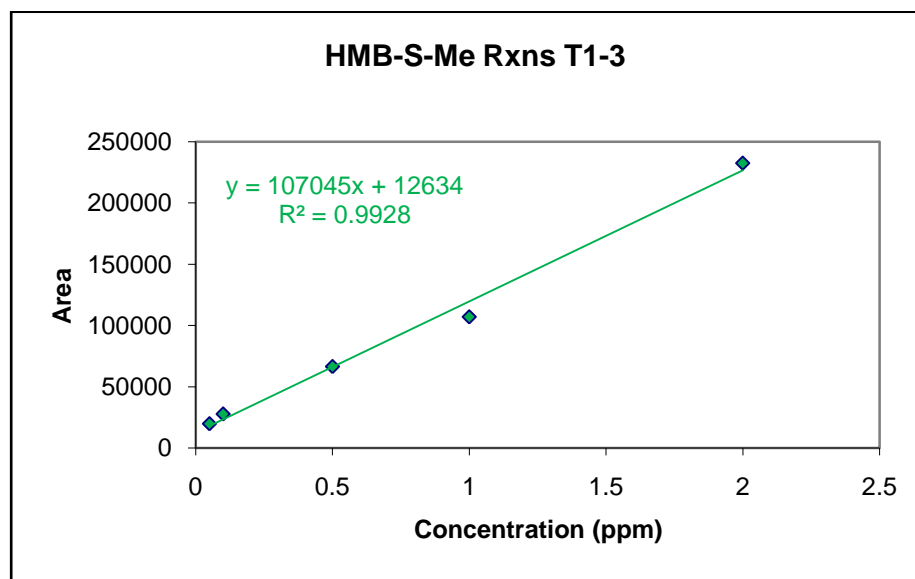


Figure 4.13 Linearity response from the detected S-Methyl HMTBA from bovine blood serum extracted samples.

As seen with the seawater matrix extracted samples the analysis of HMTBA from bovine blood serum sees a dramatic reduction in recovery. It is thought that extracted serum solids have the same effect on ionization efficiency as found with salt and are causing a perceived reduction in extraction recovery. Because of this effect, it was determined that the analysis of a sample in bovine blood serum would require the use of standards extracted from an identical serum matrix for accurate study. A table showing the results of the experiment is shown below in Table 4.11. Although the analysis

showed a decrease in response the background noise was completely eliminated. The S-Methylation of HMTBA was done quantitatively and suffered no interference. An example spectrum is shown if Figure 4.14 below.

Table 4.11 Results of HMTBA S-Methylation reactions from serum extracted samples.

Sample	Injection Statistics			Trial Statistics		
	Area Avg	Std Dev	%RSD	Area Avg	Std Dev	%RSD
HMB-S-Me 2ppm T1	246776	11751	4.8%	232651	17482	7.5%
HMB-S-Me 2ppm T2	213099	24257	11.4%			
HMB-S-Me 2ppm T3	238079	16226	6.8%			
HMB-S-Me 1ppm T1	91226	8820	9.7%	107103	18334	17.1%
HMB-S-Me 1ppm T2	102915	26358	25.6%			
HMB-S-Me 1ppm T3	127169	2981	2.3%			
HMB-S-Me 500ppb T1	54422	6328	11.6%	66529	11694	17.6%
HMB-S-Me 500ppb T2	67402	6457	9.6%			
HMB-S-Me 500ppb T3	77762	15730	20.2%			
HMB-S-Me 100ppb T1	30400	6586	21.7%	27781	7755	27.9%
HMB-S-Me 100ppb T2	19055	1792	9.4%			
HMB-S-Me 100ppb T3	33887	4649	13.7%			
HMB-S-Me 50ppb T1	29833	9699	32.5%	14817	9372	63.3%
HMB-S-Me 50ppb T2	8189	2089	25.5%			
HMB-S-Me 50ppb T3	21444	2591	12.1%			

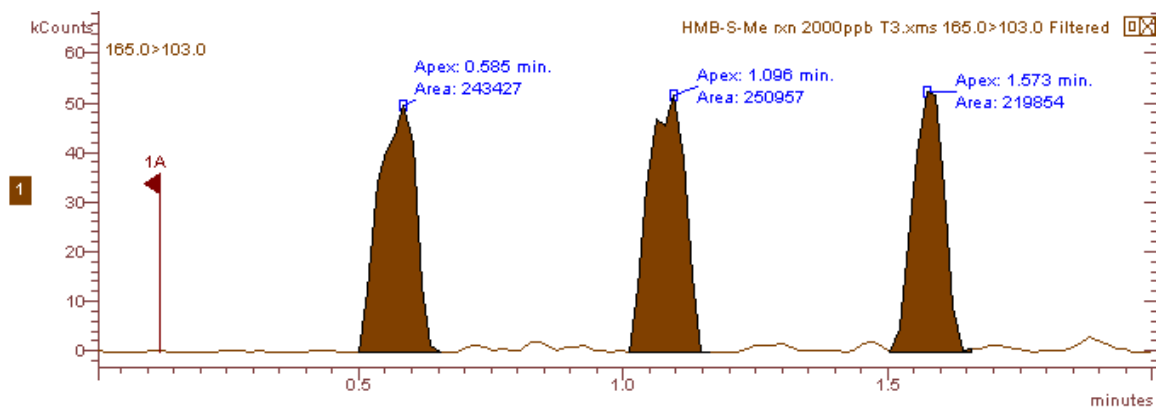


Figure 4.14 Spectrum of S-Methyl HMTBA from serum extracted sample.

## 5. CONCLUSIONS

Animal growth studies have shown that HMTBA is a very efficient methionine supplement in animal nutrition. However, no detailed studies on its uptake and presence in blood have been reported in open literature. Absence of such studies is in part attributed to the lack of a validated analytical methodology for detection of HMTBA in blood. One of the principle goals of the present study was to develop and validate chemical analysis methodology for detection of HMTBA in bovine blood serum.

Three methodologies for the detection of HMTBA in various matrices were developed and validated with varied results. The first methodology was based on HPLC detection. It was shown that HPLC can be successfully employed for detection of HMTBA in relatively simple matrices such as seawater. The methodology yielded an MDL of 0.2ppm and LOQ of 1ppm, however, such methodology could not be used for determination of HMTBA in blood serum because of high interference stemming from this matrix.

Methodologies with better sensitivity and selectivity were therefore investigated for this complex matrix. One such methodology involved esterification of HMTBA and other hydroxy acids followed by the GC-MS determination. HMTBA was esterified with different alcohol including the methyl alcohol, ethyl alcohol, *n*-propyl alcohol, and *i*-propyl alcohol. It was discovered that all esters yielded essentially the same detection and quantification limits 5ppb and 25ppb respectively. However, interference problems were still observed. In addition variation in the yield of ester were observed, these variations in part resulted from methylation (alkylation) of the sulfide moiety. The S-methylation

reaction was therefore examined. It was discovered that under suitable conditions S-methylation can be done with yields  $\geq 90\%$ . The resulting S-methyl HMTBA cation was monitored directly with ESI-(MS) at  $m/z$  165, improved selectivity and sensitivity was obtained with by monitoring fragment ion at  $m/z$  103 that results from CID. This method not only involved minimal sample manipulations but also yielded the best sensitivity and selectivity for HMTBA determination in bovine serum.

## BIBLIOGRAPHY

1. Mish, F.C. et al Eds. Merriam-Webster's Collegiate Dictionary, 10<sup>th</sup> ed., Houghton Mifflin Company: Boston, MA, 1999.
2. Asplund, J.M. Principles of Protein Nutrition of Ruminants; CRC Press: Boca Raton, FL, 1994.
3. Satter, L., Roffler, R.E. Nitrogen requirement and utilization in dairy cattle. *J. of Dairy Sci.* 1975, 58, 1219-1237.
4. Pond, W., Church, D., Pond, K. Concepts of nutrition. In Basic Animal Nutrition and Feeding, 4<sup>th</sup> ed.; Pond, W., Church, D., Pond, K., Eds; John Wiley and Sons, Inc.: N.Y., 1995.
5. Stryler, L. *Biochemistry*; 2<sup>nd</sup> ed.; W.H. Freeman and Company: N.Y., 1981.
6. Lehninger, A.L. *Principles of Biochemistry*; 1<sup>st</sup> ed.; Worth Publications: N.Y, 1982.
7. Cheeke, P.R. In Applied Animal Nutrition: Feeds and Feeding; Cheeke, P.R. Ed., Prentice Hall: Upper Saddle River, NJ, 1999.
8. Schwab, C.G., Bozak, C.K., Whitehouse, N.L., Meshbah, M.M.A. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. *J. Dairy Sci.*, 1992, 75, 3486-3502.
9. Perry, T.W., Cullison, A.E, Lowrey, R.S. *In Feeds and Feeding*. 5<sup>th</sup> Ed; Prentice Hall: Upper Saddle River, NJ, 1999.
10. Baker, D.H. Amino acid nutrition in the chick. *In Advances in Nutrition Research*; Draper, H.H. Ed; Plenum Press: N.Y. 1977; Vol. 1, pp 299-335.
11. Baker, D.H. Utilization of precursors for L-amino acids. *In Amino Acids in Farm Animal Nutrition*; D'Mello, J.P.F. Ed., CAB International: Oxon, U.K., 1994; pp 37-62.
12. Schwab, C.G. Protected proteins and amino acids for ruminants. In Biotechnology in Animal Feeds And Animal Feeding; Wallace, R.J., Chesson, A. Eds.; V.C.H. Press: Weinheim, Germany, 1995; pp 115-141.
13. Bach, A., Stern, M.D. Measuring resistance to ruminal degradation and bioavailability of ruminally protected methionine. *Anim. Feed Sci. Technol.* 2000, 84, 23-32.
14. URL: <http://www.novusint.com>

15. Pas Bernard, J.K. Supplemental animal-marine protein blend and a rumen protected methionine hydroxy analog for lactating dairy cows. *The Professional Animal Scientist*. 1997, 13, 149-154.
16. Stokes, M.R., Clark, J.H., Steinmetz, L.M. Performance of lactating dairy cattle fed methionine or methionine hydroxy analog at two different concentrations of dietary crude protein. *J. Dairy Sci.*, 1981, 64, 1686-1694.
17. Koenig, K.M., Rode, L.M., Knight, C.D., McCullough, P.R. Ruminal escape, gastrointestinal absorption, and response of serum methionine to supplementation of liquid methionine analog in dairy cows. *J. Dairy Sci.* 1999, 82, 355-361.
18. Knight, C.D., Atwell, C.A., Wuelling, C.W., Ivey, F.J., Dibner, J.J. The relative effectiveness of 2-hydroxy-4-(methylthio)butanoic acid and D,L-methionine in young swine. *J. Anim. Sci.* 1998, 76, 781-787.
19. Hosogai, S., Yamanoguchi, K., Iriki, T., Abe, M. Administration of methionine hydroxy analog to ruminants: Effects of lipip metabolism. Azabu Daigaku Juigakubu Kenkyu Hokoku. 1991, 13, 13-17.
20. Belasco, I.J. Fate of carbon-14 labeled methionine hydroxy analog and methionine in the lactating dairy cow. *J. Dairy Sci.*, 1980, 63, 775-784.
21. Patterson, J.A., Kung, Jr. L. Metabolism of D,L-methionine and methionine hydroxy analogs by rumen microorganishs. *J. Dairy Sci.* 1988, 71, 3292-3301.
22. Couch, L.H., Howard, P.C. Quantification of glycolic acid in cosmetic products using reversed phase high performance liquid chromatography. *Inter. J. Cosmetic Sci.* 2002, 24, 89-95.
23. Nicoletti, I., Corradini, C., Cogliandro, E., Cavazza, A. Determination of alpha-hydroxy acids in cosmetic products by high-performance liquid chromatography with a narrow-bore column. *Inter. J. Cosmetic Sci.* 1999, 21, 265-274.
24. Tanabe, S., Toida, T., Kawanishi, T., Togawa, T., Imanari, T. Determination of  $\alpha$ -keto and hydroxy acids by high performance liquid chromatography using ferric perchlorate as a detection reagent. *Anal. Sci.* 1985, 1, 281-284.
25. Dutra, E.A., Santoro, M.I., Micke, G.A., Tavares, M.F., Hackmann, E.R. Determination of  $\alpha$ -hydroxy acids in cosmetic products by capillary electrophoresis. *J. Pharm. and Bio. Anal.* 2006, 40, 242-248.
26. Vorarat, S., Aromdee, C., Podokmai, Y. Determination of alpha hydroxy acids in fruits by capillary electrophoresis. *Anal. Sci.* 2002, 18, 893-896.
27. Yokota, K., Saito, K., Murakami, S., Muromatsu, A., Yamazaki, S. Detection of hydroxy acids with chemiluminescence using ruthenium complex. *Chromatography* 1998, 19(2), 128-129.

28. Matulis, R.M., Guyon, J.C., Spectrophotometric determination of alpha-hydroxy carboxylic acids. *Anal. Chem.* 1964, 36(1), 118-120.
29. Pease, H.L., Prince, J.L., Johnston, E.F. Gas chromatographic method for determining methionine hydroxy analogue residues in cow milk, urine, and tissues using a flame photometric detector. *J. Agric. Food Chem.* 1978, 26(2), 331-334.
30. Ontiveros, R.R., Shermer, W.D., Berner, R.A. An HPLC method for the determination of 2-hydroxy-4-(methylthio)butanoic acid (HMB) in supplemented animal feeds. *J. Agric. Food Chem.* 1987, 35, 692-694.



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