DRUG APPROVAL RESEARCH ON 17α-METHYLTESTOSTERONE

Chairperson: Terence P. Barry, University of Wisconsin-Madison

Industry Advisory Council Liaison: Mark Willows, Binford, North Dakota

Extension Liaison: Laura Tiu, Ohio State University

Funding Request: $223,677

Duration: 18 Months (June 1, 2004-December 31, 2005)

Objectives:

1. Develop a robust and validated high performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS) method to measure 17α-methyltestosterone (MT) in fish feed.
2. Conduct a series of stability studies on MT in fish feed.
3. Gain acceptance from the Center for Veterinary Medicine (CVM) for the series of stability studies.
4. Review and develop a LC-MS method for detecting MT in water.
5. Conduct a biodegradation study of MT in water.
6. Gain acceptance from CVM for the biodegradation study on MT.

Proposed Budgets:

Institution/Unit	Principal Investigator(s)	Objec-tive(s)	Year 1	Year 2	Total
University of Wisconsin-Madison/ Department of Biochemistry	Ashok K. Marwah Padma Marwah	1-6	$114,312	$55,050	$169,362
University of Wisconsin-Madison/ Aquaculture Program	Terence P. Barry	2, 3, 5, & 6	$38,238	$16,077	$54,315
		Totals	**$152,550**	**$71,127**	**$223,677**

Non-funded Collaborators:

Institution/Unit	Collaborator(s)
University of Wisconsin-Madison/ Department of Biochemistry	Henry A. Lardy
University of Wisconsin-Madison/ Aquaculture Program	Jeffrey A. Malison

TABLE OF CONTENTS

JUSTIFICATION

17α-methyltestosterone (MT) is used to manipulate the gender of a variety of fish species cultured in the U.S., including tilapia, hybrid striped bass, yellow perch, sunfish, and esocids. The National Aquaculture New Animal Drug Application (NADA) Coordinator and the Industry Advisory Council of the North Central Regional Aquaculture Center (NCRAC) identified the remaining requirements needed for an original NADA approval for MT in tilapia. These include: (1) develop a validated analytical method to measure MT in fish feed, (2) conduct a series of stability studies for MT in fish feed using protocols accepted by CVM, (3) develop and validate an analytical method for detecting MT in water, and (4) conduct a series of biodegradation studies to elucidate the fate of MT in the aquatic environment.

RELATED CURRENT AND PREVIOUS WORK

Several HPLC and LC-MS methods have been reported in the literature for the analysis of MT in various biological matrices and pharmaceutical preparations (Goudie 1984, Lampert and Stewart 1989, Chiba and Ishii 1991, Cravedi and Delous 1991, Daeseleire et al. 1991, Gleixner et al. 1997, Coddington et al. 2000, Lagana et al. 2001, Cappiello et al. 2003). Only a few, however, deal with analysis of MT in fish feed (Goudie 1984, Coddington et al. 2000), and environmental waters (Lagana et al. 2001, Cappiello et al. 2003). All of these methods suffer with drawbacks such as sketchy details, cumbersome methodology, and lack of validation.

Using a modification of a method developed by Goudie (1984) to analyze MT in fish tissue, Coddington et al. (2000) determined the MT concentration in fish feed using a multi-step extraction technique followed by HPLC analysis. Unfortunately, this method has several major drawbacks including a cumbersome extraction procedure and the absence of a qualifier peak that would allow for the specific quantitation of MT. Moreover, the method was not properly validated, and there were insufficient details on the methodology. Thus, it is uncertain if the method is specific for MT, or would give reproducible results in different laboratories. Finally, the method was never tested for robustness and thus it is uncertain if it would give reliable results under different analytical conditions (i.e., solvent systems, columns, column diameter, etc.).

Lagana et al. (2001) described a multi-residue, atmospheric pressure chemical ionization (APCI), LC-MS/MS method, to measure the concentrations of several natural and synthetic steroids, including MT, in environmental waters. The method was not specifically validated to measure MT and has numerous problems including a non-reproducible solvent gradient, non-specificity, and the use of an external standard method for quantitation. A nano-LC-MS method developed for the analysis of steroid hormones and other organic compounds in water suffers from similar problems.

Preliminary Studies

Preliminary investigations were conducted to develop an understanding of the issues involved in fulfilling the objectives of this study. There were two major areas of concern. First, fish feed is rich in oils and fats that can be detrimental to LC-MS analysis using reverse phase columns. Thus, it will be essential to develop an extraction method that removes all oils and fats from fish feed and still results in high recovery of MT. Second, the concentration of MT in water is expected to be extremely low (<1 ppb). Thus, it will be essential to develop an analytical method that is specific and highly sensitive. To address these issues, preliminary studies were conducted to evaluate liquid-liquid and solid phase extraction of MT from fish feed and water, and the behavior of MT in HPLC and LC-MS analyses were studied. The results shown below are based on a set of single experiments.

Liquid-Liquid Extraction

The partitioning of MT between methanol-water (80:20) and hexane was studied. MT (100 µg) was dissolved in methanol, diluted with water to 80% methanol content, and then extracted twice with hexane. The hexane layer was separated, and the hexane and methanolic layers were evaporated to dryness under nitrogen. The residues were dissolved in mobile phase and subjected to LC-MS analysis. Appropriate controls were run simultaneously.

It was observed that the hexane and methanol-water (80:20) fractions contained 1.6% and 96.6% of the added MT, respectively (Figure 1). The results indicate that it will be possible to remove non-polar constituents from fish feed by partitioning MT between hexane and methanol-water (80:20).

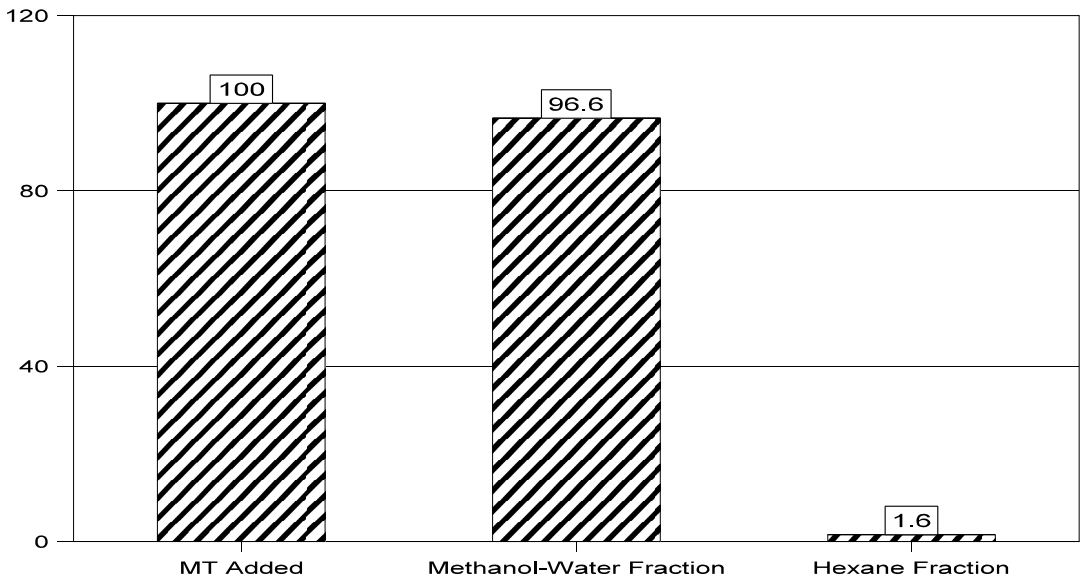


Figure 1. Partitioning of MT between methanol-water (80:20) and hexane. Methanol-water was twice extracted with hexane.

Solid Phase Extraction Studies on MT Standard

The behavior of MT subjected to solid phase extraction was studied next. MT was applied to a polymer-based hydrophilic-lipophilic preconditioned cartridge (Oasis-HLB, Waters). MT was loaded onto the cartridge, washed with 5% methanol, then 50% methanol, and finally eluted with two 1 mL aliquots of methanol. All fractions were collected and subjected to LC-MS analysis. LC-MS analysis revealed that MT was not eluted during loading and washing operations (Figure 2). Over 92.8% of the MT was eluted by the first 1 mL of methanol, and 6.4% was found in the second fraction (Figure 2). It was concluded that MT can be selectively eluted from solid phase extraction cartridges. Extensive study is needed to refine the technique and to establish reproducible procedures.

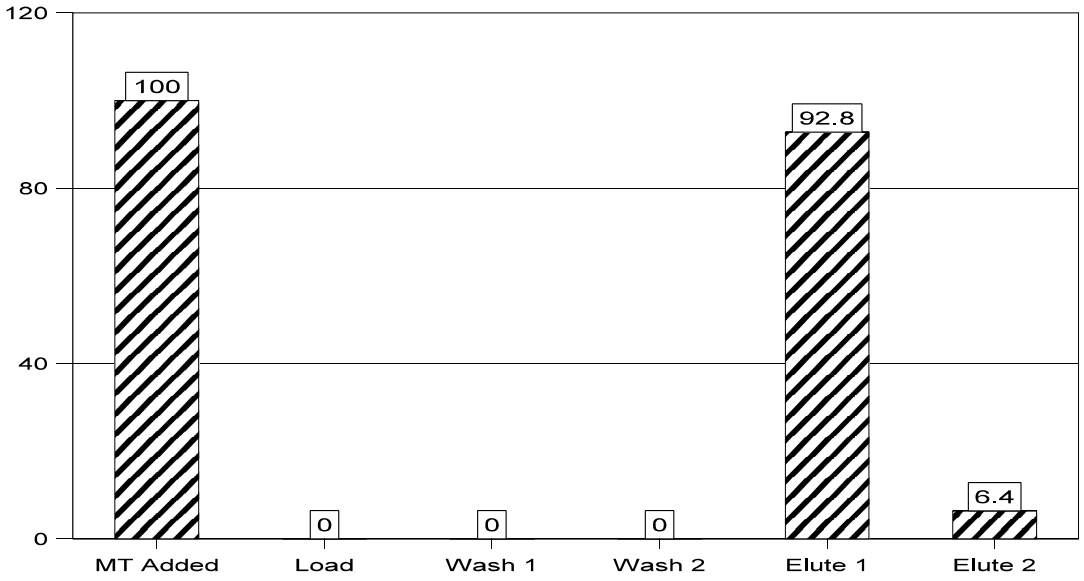


Figure 2. Elution profile of MT using polymer based (Oasis-HLB, Waters) extraction cartridges.

Analysis of Fish Feed for MT

Fish feed (from Rangen, Inc., Idaho; Batch No. R-39D [control] and Batch No. R-39C-163-2003 [containing 60 mg MT/kg fish feed or 0.006%]) was extracted with methanol (3×), diluted with water to 80% methanol content and extracted with hexane. The aqueous methanol was evaporated under nitrogen, and the residue subjected to solid phase extraction on Oasis-HLB (Waters) cartridges as described above. The cartridges were eluted with methanol, and the methanol evaporated to yield an almost colorless residue that was subjected to HPLC and LC-MS analyses. The hexane extract was evaporated to yield a viscous, oily residue that could not be analyzed by HPLC. The hexane residue was re-dissolved in methanol, diluted with water, and washed with hexane. The remaining aqueous-methanol was then analyzed to determine the amount of MT, if any, present in the hexane layer.

HPLC Analysis of Fish Feed for MT

Figures 3.1 and 3.2 shows ultraviolet (UV) chromatograms of fish feed without and with added MT (0.006%), respectively. MT was eluted at 6.23 min.

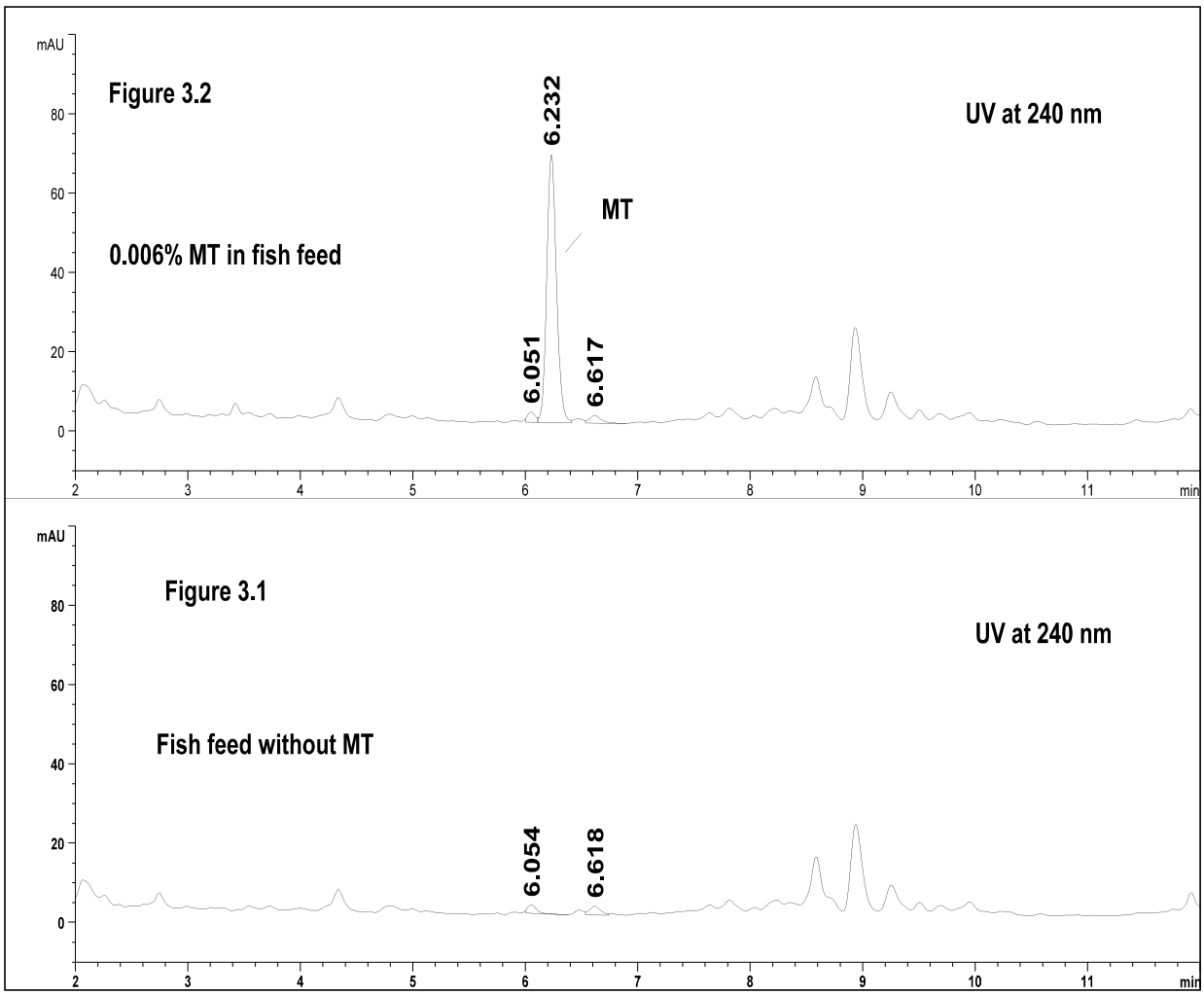


Figure 3. HPLC analysis (UV) of fish feed (Figure 3.1) and fish feed containing 0.006% MT (Figure 3.2).

There was slight interference near the retention time of MT (small peaks at 6.054 and 6.618 min), which will require further refinement of the method. Variables to be tested include: different types of extraction cartridges, HPLC columns, mobile phases, buffers and pH, selection of a qualifier peak, qualifier peak window tolerance, etc. A qualifier peak in quantitative HPLC analysis is used to confirm that the main peak belongs to the expected compound. It is known that the peaks of one compound have a constant response ratio at different wavelengths. The qualifier peak response is a percentage of the main peak response. The qualifier tolerance window specifies the allowed deviation from the expected percentage. The ultraviolet-diode array detector (UV-DAD) spectrum of MT (λ max = 244 nm) is shown in Figure 4.

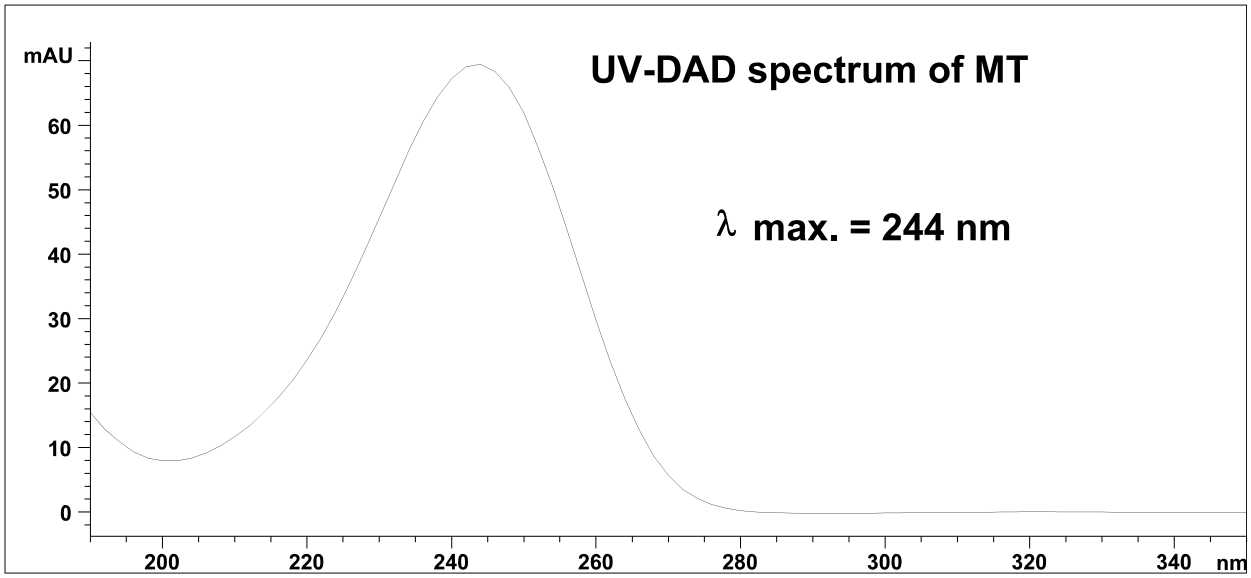


Figure 4. UV-DAD spectrum of MT.

LC-MS Analysis of Fish Feed Extracts for MT

The fish feed samples were also subjected to LC-MS analysis. Mass spectrum of MT showed two major ions at m/z 303 $[M+H]^+$ and 325 $[M+Na]^+$ (Figure 5). Therefore, the total ion current spectra of samples prepared from MT treated fish feed (Figure 6) and fish feed (Figure 7) were subjected to extracted ion analysis at m/z 303 and 325.

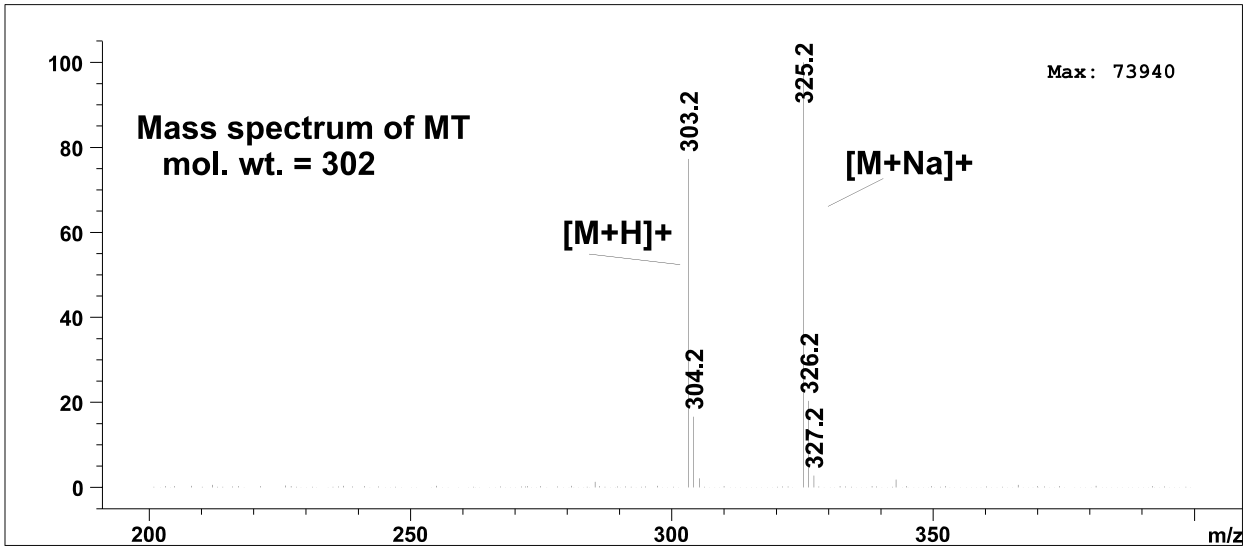


Figure 5. Electro spray ionization mass spectrum of MT.

As seen in Figures 6 and 7, there was no interference at the retention time of MT (6.25 min) for m/z 303 $[M+H]^+$ and 325 $[M+Na]^+$. These results strongly suggest that it will be possible to transform the HPLC method (UV detection only) developed to measure MT in fish feed into an LC-MS method. The advantage of this is that a validated LC-MS method will be much more specific, sensitive, and accurate than a simple UV based method, and will be extremely useful in the detection and identification of MT and its degradation products during stability studies of MT in fish feed.

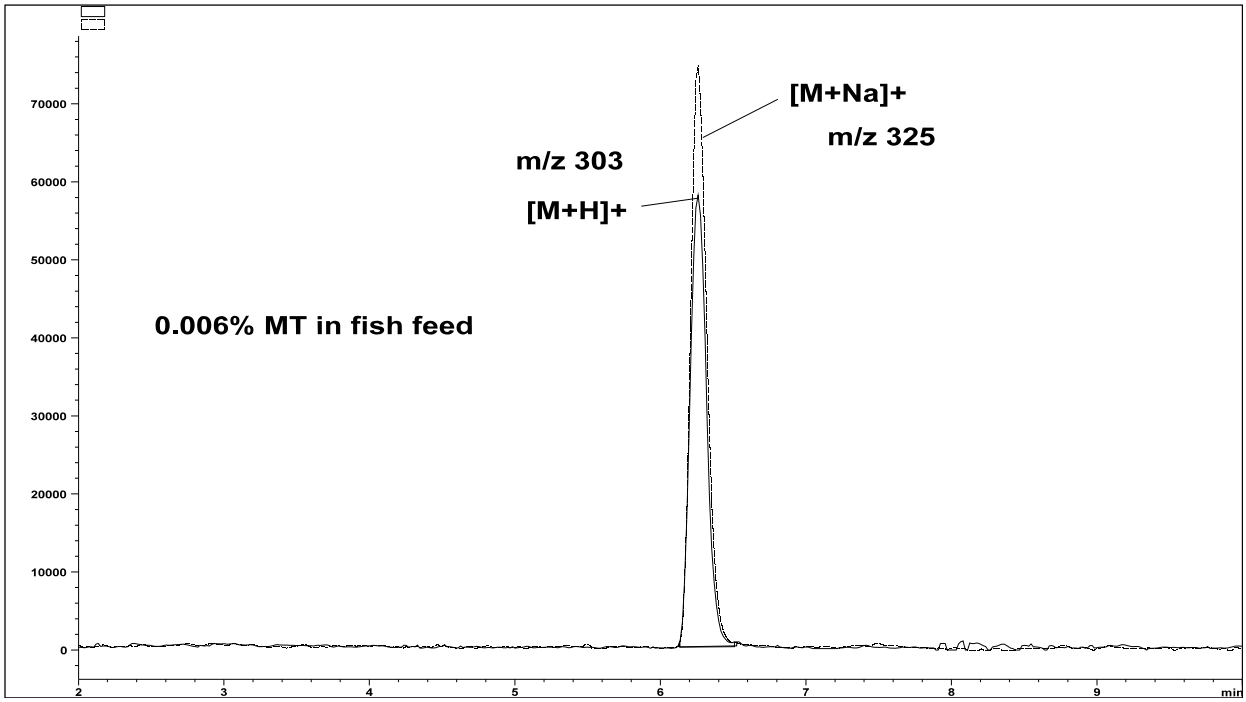


Figure 6. Extracted ion chromatogram of fish feed containing 0.006% MT (60 mg MT/kg fish feed).

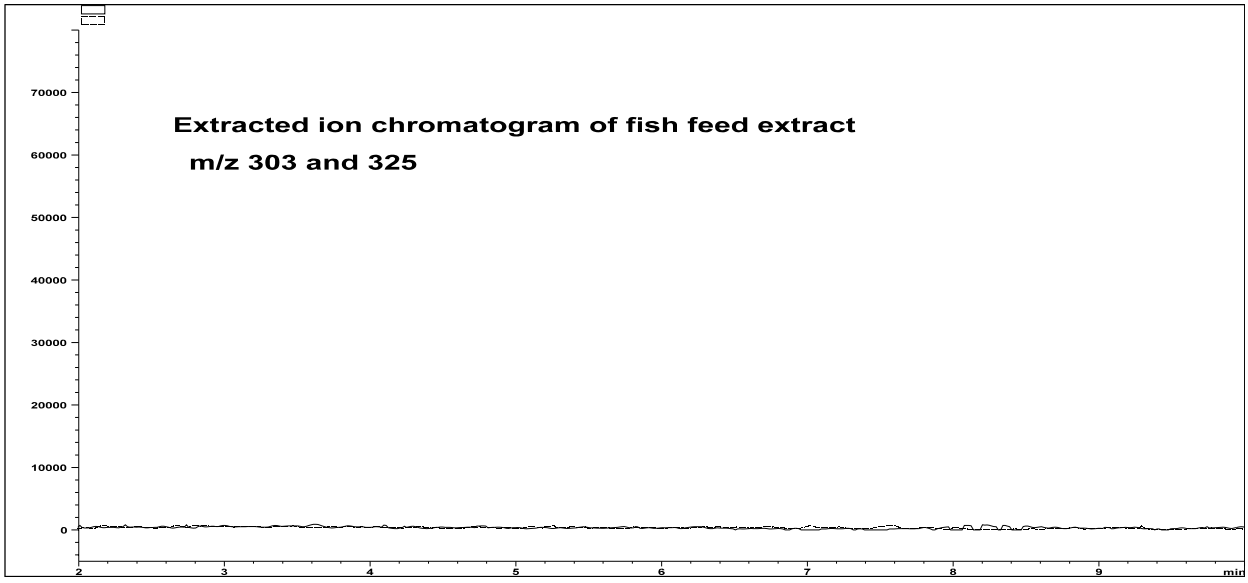


Figure 7. LC-MS analysis (extracted ion chromatogram) of fish feed extract. No interference is seen.

LC-MS Analysis of Hexane Wash for MT

MT was re-extracted from the hexane extracts (as mentioned above) and subjected to HPLC and LC-MS analysis. The results are shown in Figure 8 (UV-DAD) and Figure 9 (LC-MS). Only about 1.5% of the MT was present in the hexane extracts. This is consistent with the data obtained in the liquid-liquid extraction study of MT standard (Figure 1). Of great significance is the finding that the presence of other constituents in fish feed has no significant effect on the partitioning of MT.

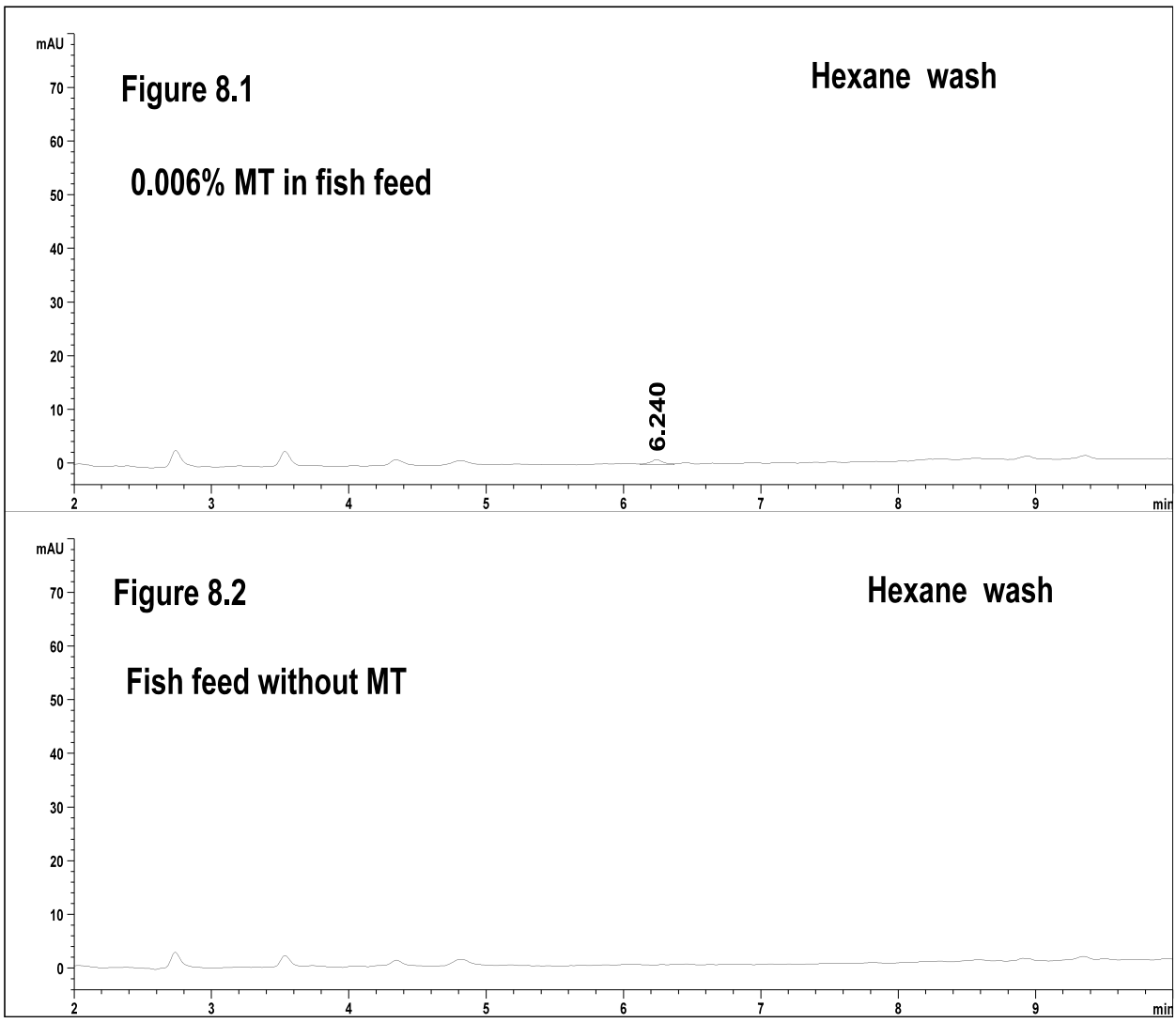
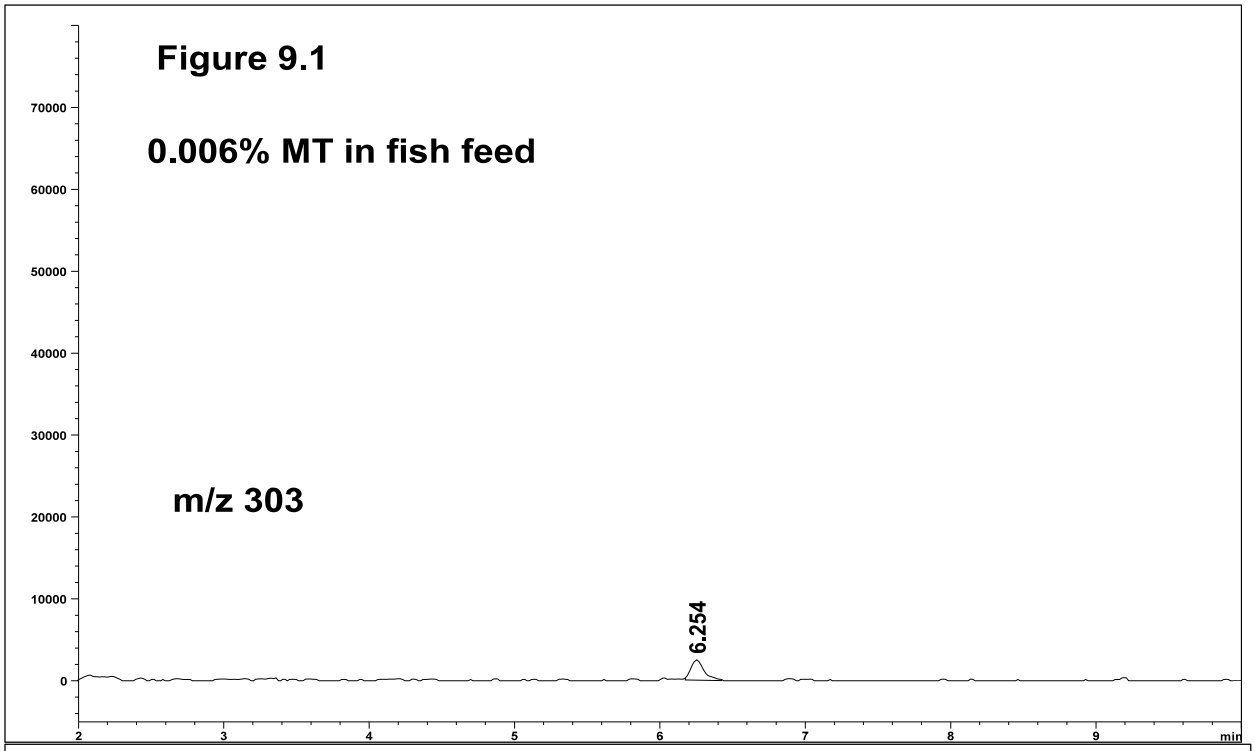


Figure 8. UV analysis of hexane wash of fish feed containing 0.006% MT (Figure 8.1) and fish feed without MT (Figure 8.2). Trace amount of MT is seen at 6.24 min in Figure 8.1.



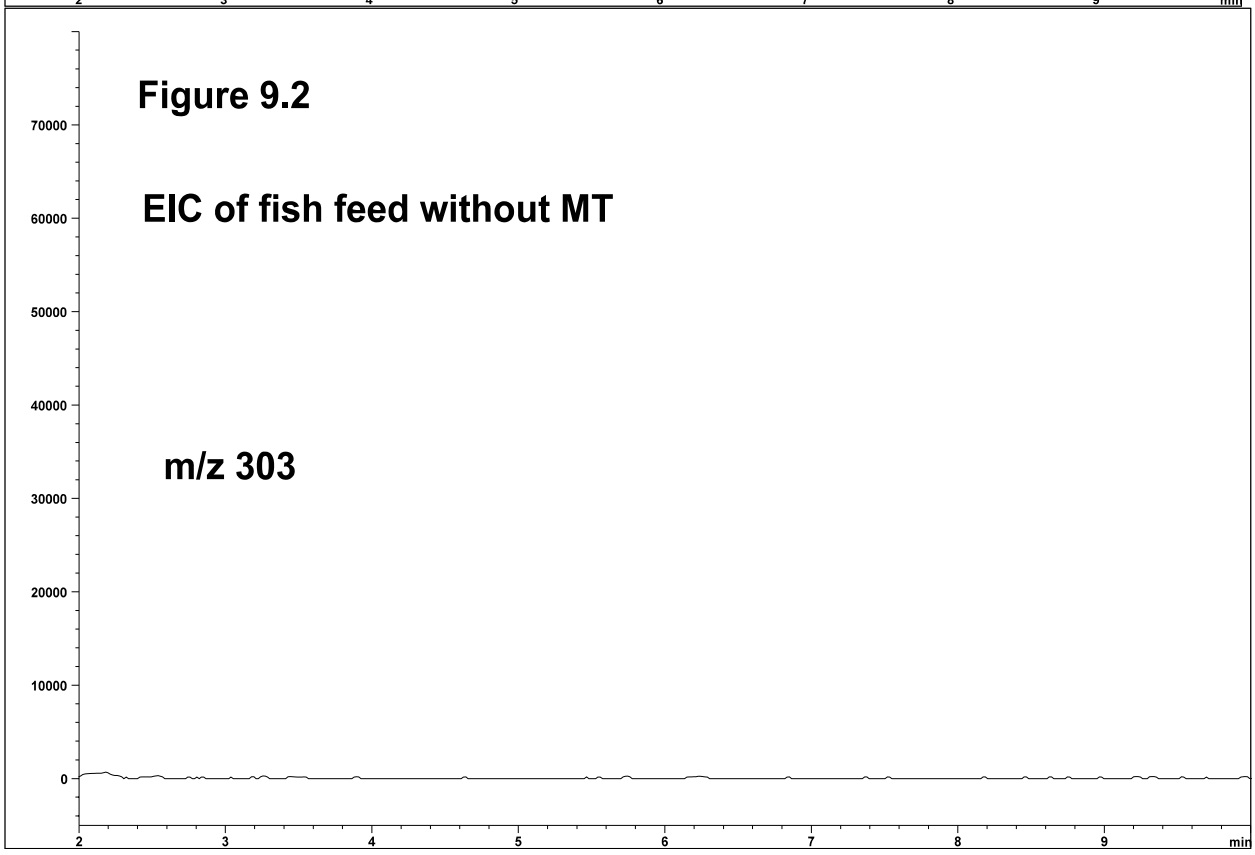


Figure 9. LC-MS analysis (extracted ion analysis [EIC]) of hexane wash for the presence of MT. Only a trace amount of MT was found to be present.

Water Analysis for Trace Amounts of MT

MT (2 ng) was added to 100 mL water to give an effective concentration of 20 ng/L. The water was passed through a solid phase extraction cartridge at a rate of about 10 mL/min. The cartridge was washed with 5% methanol, then 50% methanol, and the MT was eluted with 5 mL methanol. The methanol was evaporated, the residue was reconstituted in LC-MS mobile phase (200 µL), and 10 µL (equivalent to 100 pg of MT) was subjected to electro spray ionization LC-MS analysis in selected ion monitoring mode (SIM) for m/z 303 $[M+H]^+$. Even at the low level of 100 pg, there was a very well defined MT peak (Figure 10.1). Further LC-MS studies are needed, however, to improve the resolution of MT from other peaks present in the chromatogram. The LC-MS analysis of 100 pg MT that was not first extracted (i.e., the MT that was added directly) under identical conditions is shown in Figure 10.2. Comparison of peak areas and heights indicated a near quantitative (>99%) recovery of MT from water.

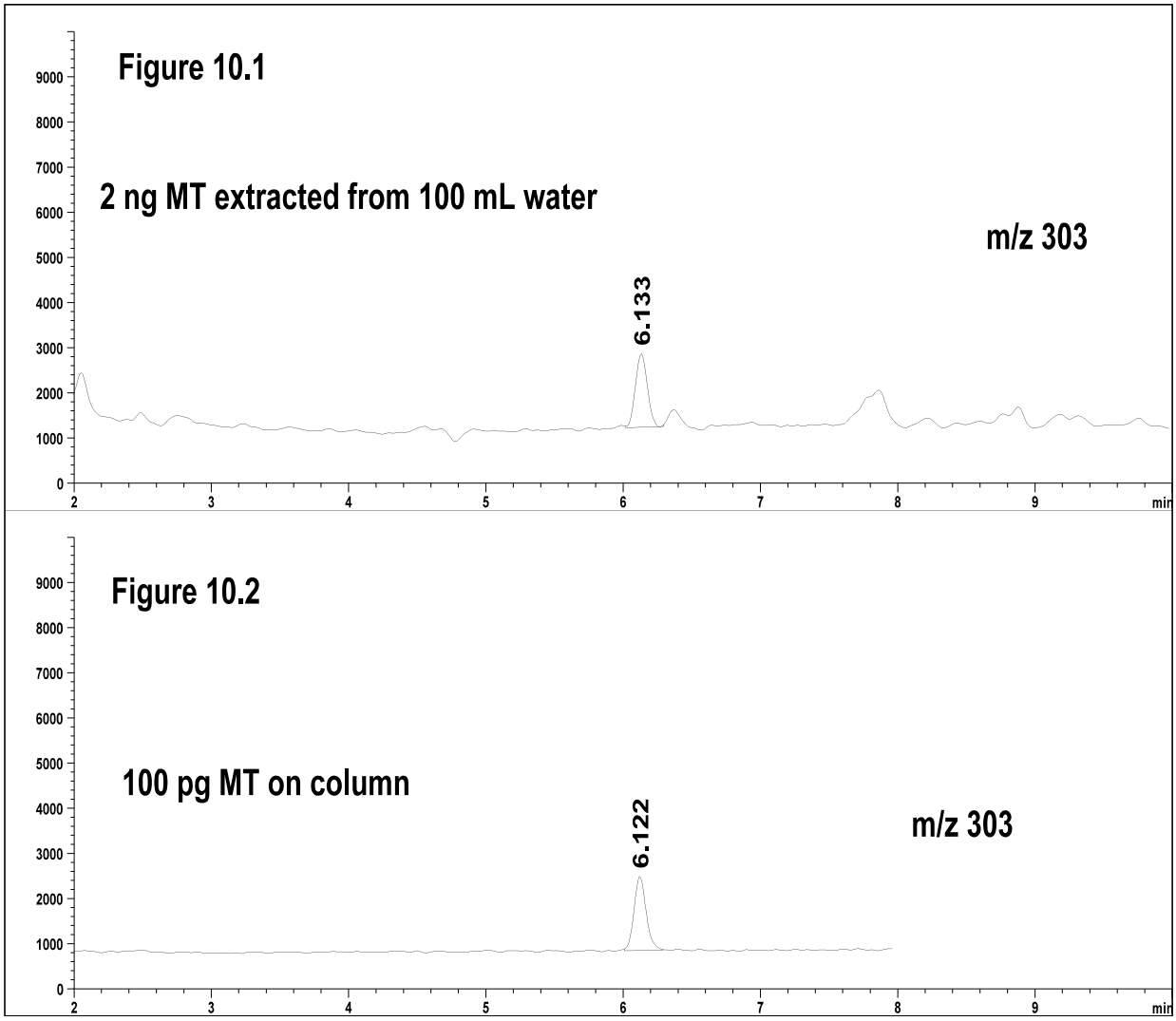


Figure 10. 2 ng MT extracted from 100 mL water. Equivalent of 100 pg of MT was injected onto the column (Figure 10.1). Figure 10.2 shows LC-MS of 100 pg of chemical standard MT (sample was directly injected, there was no extraction step). Comparison of Figures. 10.1 and 10.2 illustrates that the recovery of MT from water exceeded 99%.

Sensitivity and Microbore HPLC and Electrospray Ionization (ESI-MS)

The above-mentioned work was done using normal flow LC-MS analysis on a 4.6 mm internal diameter (I.D.) column. Conventional LC-MS is generally suited using columns with an i.d. of 2.1– 4.6 mm. When the same volume and concentration of a sample is injected onto a smaller diameter column, however, the peak height increases because there is less column dispersion. This phenomenon will occur when the detector is concentration sensitive as in electrospray ionization mode (ESI). Because the HPLC system is equipped with a capillary pump and auto sampler, the system delay volume can be minimized which permits the use of smaller diameter columns (1.0 mm and 0.32 mm I.D.), which markedly improves the sensitivity of the analytical procedure. To test the validity of this concept, equal amounts of MT (100 pg) were injected on 4.6 mm and 1.0 mm I.D. columns. A more than five-fold increase in the sensitivity of MT was observed when a 1.0 mm I.D. column was used (Figures 11.1 and 11.2). It should be possible to even further improve this sensitivity by reducing the column I.D. further, although there will be a limit to this effect determined by parameters including injection volume, dwell volume, and back pressure that must be evaluated empirically.

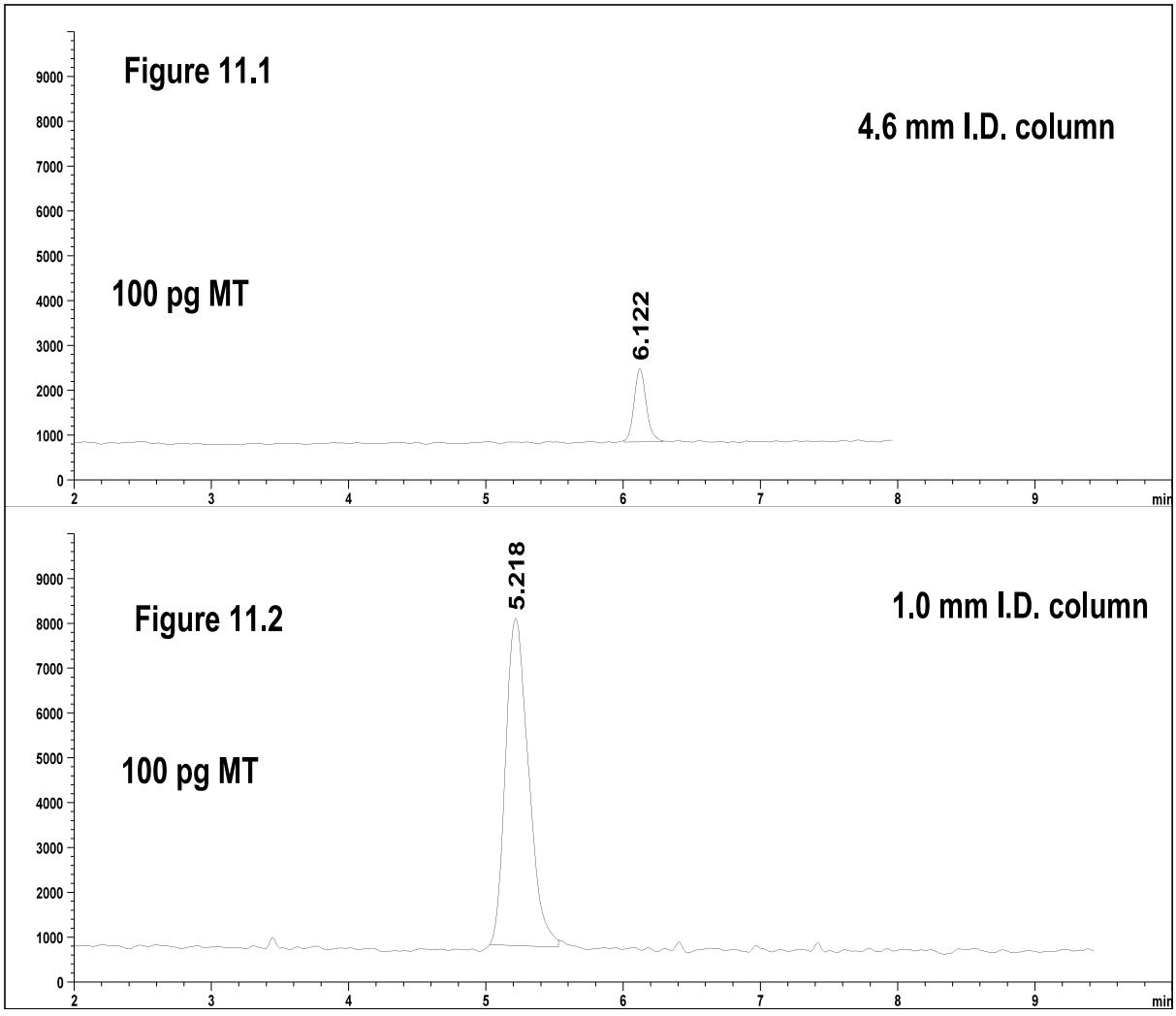


Figure 11. Narrow bore columns greatly improve sensitivity. 100 pg MT was injected on a 4.6 mm column (Figure 11.1) and a 1.0 mm I.D. column (Figure 11.2). Difference in retention times is due to difference in column lengths.

ANTICIPATED BENEFITS

The University of Wisconsin-Madison (UW-Madison) team of steroid biochemists, analytical chemists, and aquaculturists will complete all of the proposed objectives, and communicate the findings via written reports and publications to Auburn University, Rangen Feeds, and CVM. This work will ultimately lead to the approval of MT for use by the U.S. aquaculture industry.

OBJECTIVES

1. Develop a robust and validated high performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS) method to measure 17α-methyltestosterone (MT) in fish feed.

2. Conduct a series of stability studies on MT in fish feed.

3. Gain acceptance from the Center for Veterinary Medicine (CVM) for the series of stability studies.

4. Review and develop a LC-MS method for detecting MT in water.

5. Conduct a biodegradation study of MT in water.

6. Gain acceptance from CVM for the biodegradation study on MT.

PROCEDURES

Good Laboratory Practice

All of the proposed research will adhere to strict elements of Good Laboratory Practice (GLP) to insure that the chemical and physical measurements are made properly, interpreted correctly, and reported with appropriate estimates of error and confidence levels. In brief, standard operating procedures (SOPs) will be written out in detail and submitted to CVM for approval prior to the start of the experiments. Statistical procedures will adhere to CVM requirements as specified in the SOPs. The validity of instrumental data will be tested on a regular basis to establish the continuing acceptable operation of laboratory instruments within prescribed specifications. Only the highest-grade analytical reagents will be used, and all reagents/materials will be labeled with information related to its certification value, date, and expiration time. Ph.D. scientists will conduct all of the proposed experiments. A rigorous specimen/sample-tracking system will be used to maintain the unmistakable connection between a set of analytical data and the specimen and/or samples from which they were obtained. Finally, meticulous records will be kept as per accepted GLP guidelines.

Develop Method to Measure MT in Fish Feed (Objective 1)

Existing HPLC methods to measure MT in fish feed lack proper validation, specificity, sensitivity, and robustness. UW-Madison researchers propose to develop a robust, validated method to measure MT in fish feed. First, the focus will be on developing an efficient, and reproducible procedure for extracting MT from fish feed. The effect of feed particle size (grinding), various solvents, sonication, etc. on the extraction of MT from the feed, and the use of liquid/liquid and solid phase extraction procedures for sample clean up will be evaluated. Optimal conditions will be those giving maximum recovery and minimum baseline noise. Every stage of the extraction procedure will be monitored using a mass detector in series with a UV-DAD. LC-MS is the preeminent analytical method for the analysis of steroids and will be used to substantiate all data generated using HPLC. Using the LC-MS instrument in this way, UW-Madison researchers are confident that they will develop an efficient, practical, robust, and validated HPLC method to detect and measure MT in fish feed. The method will be written in a detailed SOP format, and detailed instructions will be included for every stage of the method.

The HPLC method to be developed and validated will use narrow bore columns to enhance resolution and increase sensitivity, and a diode array detector which will enable inclusion of a qualifying wavelength for MT analysis, thus making the method highly specific to MT. UW-Madison scientists will rigorously establish the limits of detection (LOD), limits of quantitation (LOQ), accuracy, precision, linearity, and range of the assay. Calibration curves will be plotted for the range studied and data subjected to statistical evaluation (linearity, correlation coefficients, etc.). The robustness of the method will be tested by creating deliberate variations in parameters such as mobile phase composition, flow rate, column temperature, pH, buffer concentrations, and columns from different lots and different manufacturers. The complete method, including extraction procedure, HPLC details, equipment and materials used, etc., will be thoroughly documented and submitted along with chromatograms and data generated for method validation. The validated procedure will also be used with equal precision and accuracy to detect/quantify MT in the stability studies described below under Objectives 2 and 3. The proposed method will be developed using CVM Guidelines for Industry Nos. 63 and 64, CVM letter of May 25 2001, and in regular consultation with CVM. The salient features of the method development and validation are discussed below.

Equipment

A state-of-the-art HPLC-MS system is available for the exclusive use of this group. Internal diameter, length, and internal volume of the HPLC tubing used to make connections will be specified, and the volume of diode array ultra violet flow cell will be recorded to facilitate method transfer. HPLC equipment will be subjected to operational qualification and performance qualification to ensure that specific modules of the system are operating according to specifications.

System Suitability

System suitability tests will be performed before, during, and after the analysis to ensure system performance. Plate counts, tailing factor, resolution, and reproducibility will be determined from replicate injections of standard MT and the internal standard and data documented. MT will also be analyzed by U.S. pharmacopoeial method to ensure system performance as per CVM Guidance Document for Analytical procedures (Guideline No. 64).

Reagents and Chemicals

Source and quality of all reagents and chemicals used in this study will be documented. All solvents used will be of HPLC grade. Distilled water, deionized and purified by Nanopure Ultra Pure Water System from Barnstead (Dubuque, Iowa) will be used (18.25 ± 0.05 MΩ-cm).

Preparation and Storage-Stability of Standard Solutions

Standard stock solutions will be prepared by dissolving separately 25 mg of MT and an internal standard steroid (steroid not yet determined) in 25 mL of methanol. Working solutions will be prepared from these stock solutions by serial dilution. All solutions will be stored at -20°C and checked by LC-MS monthly for homogeneity and possible deterioration to determine storage life.

Preparation of Mobile Phase

The mode of preparation of the mobile phase will be documented and rigorously followed during the course of the study. Buffer concentration and pH will be clearly specified and the method of buffer preparation will be recorded.

Extraction Procedure

A procedure will be developed to extract MT from fish feed. The efficiency of liquid-liquid and solid phase extractions will be evaluated using various solvents and a variety of extraction cartridges. Values giving reproducible data will be included in the method. The effects of other variables including grinding, sonication, temperature etc. on the extraction of MT from fish feed will be studied to increase extraction efficiency, if warranted.

Effect of Injection Volume

Injection volume can have a pronounced effect on the shape of early eluting peaks and may result in compromised column performance. Therefore, the effect of injection volume on peak shape of MT will be studied to find out effect of column length on injection volume. Samples containing known amounts of MT in different volumes of mobile phase of initial concentration will be injected onto the chromatograph and peak width and peak height will be recorded.

Dwell Volume

The dwell volume of the system (the volume of the mobile phase that passes through the head of the column before the actual gradient starts) attains significance for HPLC analysis carried out using mobile phase gradients. It is particularly important for early eluting peaks, which may elute in the dwell volume, thus essentially eluting isocratically. Dwell volume varies from instrument to instrument, and if known, helps in reproducing gradient analysis on different instruments. The dwell volume of the system will be determined graphically by replacing the column with a short piece (50 mm) of HPLC tubing and by running a gradient (0–100% in 10 min) of water versus UV absorbing water-miscible mobile phase such as acetone and by recording the response at 254 nm.

Standard Curve

Calibration standards will be prepared using at least five different concentrations of MT in fish feed. The standard samples will be prepared by adding appropriate amounts of MT.

Calibration

The ratios of peak response (areas/height) of MT to that of internal standard will be correlated with the standard concentration over the specified range. After analysis, a standard curve for MT will be constructed by regression analysis of peak response ratio (y-axis) and MT concentration (x-axis) using Agilent Chemstation software. The data will be analyzed using both peak area and peak height. The analysis giving the most accurate results will be used in the final method.

Recovery

The recovery of MT will be determined by comparing peak area ratios from fish feed spiked with known amounts of MT processed as described earlier versus peak area ratios of the same concentrations obtained by spiking the extracted matrix with known concentrations. Each concentration will be analyzed five times.

Accuracy and Precision

The accuracy and precision of the method used will be established across the specified range of the analytical procedure (e.g., 30–120 mg MT/kg fish feed). The intra-assay precision and accuracy of the method will be evaluated by analyzing, during a single run, five replicates of spiked samples at five different concentrations selected from the specified range against a separate calibration curve. The inter-assay precision and accuracy will be assessed by analyzing spiked quality control samples at five different concentrations during different runs (N = 5) against an independent calibration curve. Accuracy will be evaluated as percentage error. Results will be compared with U.S. pharmacopoeial method for MT accuracy = [(mean of measured - mean of added)/mean of added]×100. The precision will be given by inter-assay and intra-assay coefficient of variation. Confidence intervals will be reported.

Specificity

During method development care will be taken to ensure the specificity of the method. Different columns (C18 versus C8 versus CN, etc.), and mobile phase conditions (e.g., isocratic versus gradient, different organic modifiers, pH, etc.) will be studied to ensure proper resolution of MT from impurities and degradation products. To ensure specificity, MT will be analyzed using a qualifying wavelength in combination with the wavelength used for quantitation. A qualifier peak is used to confirm that the main peak belongs to the expected

compound. It is known that the peaks of one compound have a constant response ratio at different wavelengths or ions. The qualifier peak response is a percentage of the main peak response. The qualifier tolerance window specifies the allowed deviation from the expected percentage. This required range of the ratio between the wavelength used for estimation, and the qualifying wavelength, will be derived by analyzing the UV spectrum of the pure MT standard.

To evaluate the specificity of the method, fish feed (without added MT) will be subjected to the assay procedure and the retention times of endogenous substances in fish feed will be compared with those of compounds of interest (MT and the internal standard). Interference from the internal standard on the retention time of MT and vice versa will be checked to rule out the presence of any interfering impurities.

Likely impurities such as 17β-hydroxy MT, if available, will be subjected to co-chromatography with MT to further study the specificity of the method. Samples of MT will be degraded high heat, acid, alkali, and peroxide oxidation. The products will then be analyzed by the developed method and by U.S. pharmacopoeial method. The UV spectrum of MT will be recorded and monitored using an online diode array detector during all these studies and data will be subjected to peak purity analysis to determine its homogeneity.

The complete study will be monitored using an online mass detector. The mass spectral data will provide an additional proof of specificity of the method. The specificity of the method will be further assessed by injecting different steroids (corticoids, androgens, estrogens, etc.) and related compounds and by evaluating the chromatogram obtained using the diode array ultra violet detector and mass detector mode.

Internal Standard

The analytical method for MT in fish feed will be developed using an appropriate internal standard. An internal standard is a compound of known structure and purity which has chromatographic behavior similar to that of the analyte and is stable under the conditions of extraction and analysis. A known concentration of internal standard is added to the matrix prior to extraction and analysis. Typically, internal standard can correct for matrix effects (differences between standard and samples) and instrumental drift, injection errors, dilutions, evaporations, etc. Quantitation results are computed using the response ratio of the sample analyte to that of internal standard (areas as well as concentrations).

Linearity

A linear relationship will be evaluated for UV-DAD data across the range of the analytical procedure using at least five data points, and the data processed statistically. ESI-MS has a relatively narrow linear dynamic range because the ESI response may level out even at sub-nanogram concentrations leading to calibration plots (straight lines to markedly curved plots) which vary from compound to compound and even for the same compound depending upon the LC-MS conditions (Law and Temesi 2000). Therefore, a range of non-linear curve fitting routines will be investigated for LC-MS data to ensure that response curves are accurately defined.

Range

The intended application of the present procedure is to develop a validated method for the analysis of 60 mg MT/kg fish feed. UW-Madison scientists intend to study 10–200% of this concentration. The linearity, accuracy, and precision will be studied in samples containing 12–120 mg MT/kg fish feed. All specified parameters will be studied within and at the extremes of this range. Although not strictly required for the purposes of the present investigation, the limits of detection and the limits of quantitation of the method will also be determined.

Limit of Detection (LOD)

The limit of detection will be defined as the sample concentration of MT resulting in a peak height of 3× the signal to noise ratio (S/N).

Limit of Quantitation (LOQ)

The limit of quantitation will be defined as the sample concentration of MT resulting in a peak height of 10× S/N.

Robustness

The robustness of the analytical method will be evaluated by creating deliberate variations in the method parameters, and evaluating the effects of these variations on the resolution of MT. Parameters to be tested in the HPLC system include: mobile phase composition, buffer (if used) strength and pH, mobile phase flow rate (± 10% variations), column temperature, and the volume of flow through the UV detector cell on peak width and sensitivity. Different columns from the same lot and similar columns from different manufacturers will also be studied. Parameters to be tested in the mass spectrometer include drying gas flow, drying gas temperature, and capillary voltage. Replicate analysis ($N > 3$) will be carried out to obtain reproducible data for robustness studies.

Conduct Stability Studies on MT, and Gain Acceptance from CVM for the Stability Studies (Objectives 2 and 3)

Stability studies for MT in fish feed using the validated HPLC and LC-MS methods developed under Objective 1 are proposed. The stability studies will be conducted following CVM guidelines as mentioned in the Final Guidance section of "Guidance for Industry: Stability Testing for Medicated Premixes; VICH GL8" (CVM Guidelines, Nos. 5 and 91). Conditions to be evaluated include storage temperature, humidity, and length of storage. The manufacturer (Rangen) currently produces MT-treated feed every six months, which will be the maximum length of the stability studies. All tests will be conducted in triplicate using three separate batches of MT-treated feed. Thus, it will take 18 months to complete the proposed studies. Degradation curves will be plotted for various parameters studied (different temperatures, humidities, storage time etc.) to better understand the data.

Long-term Storage Studies

Moderate environmental conditions (25 ± 2°C and 60 ± 5% relative humidity [RH]) will be used for long-term storage conditions. Samples will be analyzed on a monthly basis for six months using the HPLC and LC-MS methods developed under Objective 1.

Short-term Storage Studies

More severe environmental conditions (40 ± 2°C and 75 ± 5% RH) will be used for the short-term storage, also called accelerated testing. Samples will be analyzed at regular intervals (at week 0,1 2, 3, and 4) using developed validated HPLC and LC-MS methods.

Additional Testing

In addition to the highly controlled storage conditions described above, the stability of MT in fish feed stored under less rigorously controlled conditions designed to mimic the most likely storage conditions used by fish farmers will also be tested. There will be three of these conditions: (1) stored in a sealed plastic container in a typical household freezer (-15°C); (2) stored in a sealed plastic container in a typical household refrigerator (5°C); and (3) stored in the original bag at room temperature (17–22°C) and ambient changes in humidity. Samples of feed from each of these conditions will be sampled on a monthly basis for six months. In all of these trial, the feed with will be thoroughly mixed prior to sampling in ensure that oil migration, the settling of fines, or other physical factors do not interfere with the precise measurement of MT stability in fish feed. Additionally, MT will be stored in an original bag and samples will be withdrawn monthly for six months from the top, middle, and bottom of the bag without moving the bag, and analyzed for MT content to evaluate the possible migration of MT in oil.

After the results of the first six-month stability trial have been obtained, additional stability testing may be conducted to evaluate other storage conditions of interest. For example, an intermediate storage condition

(e.g., 30 ± 2°C and 60 ± 5% RH) will be evaluated if significant changes in MT are observed under the accelerated testing conditions. Studies will also be conducted to determine if the MT in fish feed becomes partitioned between the oil and solid fractions of the feed matrix. Experiments will also be conducted to find out how much of MT is lost at the time of feeding when carried off in the surface oil film. CVM will be consulted before undertaking any additional studies, and if warranted or required, detailed protocols will be prepared and approved by the CVM before such studies are conducted.

Review and Develop a LC-MS Method for Detecting MT in Water (Objective 4)

The LC-MS/MS method reported by Lagana et al. (2001) for the detection of MT in water has been critically reviewed. As discussed above, this method has numerous problems and cannot be used in the proposed studies. The method uses APCI mode that does not work well on polar, non-volatile compounds such as MT. ESI is the method of choice for such compounds. Other factors being equal, ESI is more sensitive and gives less background noise than APCI. The sensitivity of a variety of steroids is enhanced several fold in ESI mode by addition of appropriate ions such as Na^+, Li^+, Ag^+, H^+, etc. to the mobile phase has recently been demonstrated (Marwah et al. 2002). Because the ESI technique is concentration dependent, a capillary LC-MS-ESI method using microbore columns will result in a highly sensitive method for the detection and quantification of MT in water. Without doubt, it will be far better and more economical to develop a simple, sensitive LC-MS-ESI method for the detection of MT in water, rather than using a LC-MS/MS-APCI method that is not specifically developed for MT.

UW-Madison scientists propose to develop a highly sensitive capillary LC-MS-ESI method in SIM mode using an appropriate internal standard. The method will be optimized and validated for determination of very low concentrations of MT in water. The effects of mobile phase additives (pre-column and post-column) will be studied, and the method will be subjected to rigorous method validation, as described above under Objective 1. The LOD and LOQ will also be determined. The CVM Guidelines will be strictly adhered to, and method validation and robustness studies will be carried out as described under Objective 1. As discussed above, preliminary studies using water spiked with MT at 2 ppb (20 ng/L) indicate that MT concentrations of < 0.1 ppb can be measured using a conventional 4.6 mm I.D. column. An approximately ten-fold increase in sensitivity using microbore columns (<0.01 ppb) is expected.

Conduct a Biodegradation Study on MT, and Gain Acceptance from CVM for the Biodegradation Studies (Objectives 5 and 6)

The LC-MS method developed under Objective 4 will be used to conduct biodegradation studies on MT in water. Guidelines for measuring the transformation rate of MT in a water-sediment system under both aerobic and anaerobic transformations in aquatic sediment systems as specified by OECD guidelines will be strictly followed (OECD 2002).

In brief, [^{14}C]-labelled MT will be used. At least one million dpms [^{14}C]-MT will be added to each incubation at a final MT concentration of 1 ppb. Two sediments will be tested under both aerobic and anaerobic conditions. Each experiment will be conducted for approximately three months (as per OECD guidelines), and there will be a total of six sampling points (time 0, and every two weeks thereafter for three months). Thus, there will be a total of 24 treatment groups (2 conditions × 2 sediments × 6 times). Each treatment group will be replicated three times for a total of 72 samples.

Preliminary experiments will be conducted to determine if MT is degraded in water-sediment systems to volatile metabolites. This will be accomplished by conducting a set of studies using a gas flow-through apparatus (OECD 2002). If no volatile MT metabolites are produced, all subsequent experiments will be conducted in Biometers (OECD 2002).

Sediments to be used in the experiments will be obtained locally and fully characterized according to OECD guidelines. All sediment analyses will be conducted at the Wisconsin State Laboratory of Hygiene, Madison, Wisconsin. All samples will be extracted and analyzed by HPLC according to the methods developed in Objective 5 using an on line flow scintillation analyzer (radioactivity detector). This technique will provide an accurate degradation profile of MT, because the matrix components, being devoid of radioactivity, cannot interfere in the detection procedure.

FACILITIES

The laboratory at the Department of Biochemistry-Enzyme Institute is fully equipped to conduct the proposed analytical research. A state-of-the-art HPLC-MS system is available for use in this project. The LC-MS system (Agilent 1100 series) consists of a capillary pump with microdegasser, a quaternary pump with normal degasser, a capillary autosampler, a normal autosampler, a Rheodyne injector, a thermostated column oven, a diode array UV detector, a Gilson fraction collector, and a single quadruple mass detector. In addition, the UW-Madison Department of Pharmacy and the UW-Madison Biotechnology Center have a state-of-the-art ion-trap mass spectrometer and a Fourier transform mass spectrometer available on a fee-for-use basis. These instruments can be used for identifying MT breakdown products, if necessary. Stability studies will be conducted in the analytical laboratory of the UW Aquaculture Program, and at the UW Biotron—a state-of-the-art facility on the UW-Madison campus that permits investigators to conduct studies under strictly regulated environmental conditions, including the combinations of light, temperature, and humidity to be tested in this investigation.

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CVM (Center for Veterinary Medicine) Guidance Document On Drug Stability Studies. CVM Guidance for Industry: International Cooperation on Harmonisation of Technical Requirements for Approval of Veterinary Medicinal products (VICH); Final Stability Testing for Medicated Premixes (VICH GL8) Availability. Guideline No. 91. http://www.fda.gov/cvm/guidance/fguide91.PDF

CVM (Center for Veterinary Medicine) Guidance Document On Drug Stability Studies. CVM Guidance for Industry: Validation of Analytical Procedures: Definition and Terminology. Guideline No. 63. http://www.fda.gov/cvm/guidance/guida63.pdf

CVM (Center for Veterinary Medicine) Guidance Document For Analytical Procedures. CVM Guidance for Industry: Validation of Analytical Procedures: Methodology: Final Guidance. Guideline No. 64. http://www.fda.gov/cvm/guidance/guida64.pdf

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Gleixner, A., H. Sauerwein, and H.H.D. Meyer. 1997. Detection of anabolic steroid methyltestosterone in hair by HPLC-EIA. Chromatographia 45:49-51.

Goudie, C. A. 1984. Extraction of synthetic androgens from fish muscle and quantitation by HPLC. Steroids 44:241-252.

Lagana, A., G. Fago, A,. Marino, and D. Santaarelli. 2001. Liquid chromatography tandem mass spectrometry applied to analysis of natural and synthetic steroids in environmental waters. Analytical Letters 34:913-926.

Lampert, B. L., and J.T. Stewart. 1989. Determination of anabolic steroids and zeranol in human serum by isocratic reversed-phase HPLC on silica. Journal of Chromatography 12:3231-49.

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Marwah, A., P. Marwah, and H.A. Lardy. 2001. Liquid chromatography-electrospray ionization mass spectrometric analysis of corticosterone in rat plasma using selected ion monitoring (SIM). Journal of Chromatography B 757:333-42.

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OECD (Organisation for Economic Co-operation and Development) Guideline for Biodegradation Study. OECD Guidelines for the Testing of Chemicals: Aerobic and Anaerobic Transformation in Aquatic Sediment Systems. OECD Guideline No. 308. 19 pages. http://www.oecd.org/dataoecd/44/10/2741307.pdf

PROJECT LEADERS

State	Name/Institution	Specialization
Wisconsin	Terence P. Barry University of Wisconsin-Madison	Endocrinology, Aquaculture
	Ashok Marwah University of Wisconsin-Madison	Bio-analytical Chemistry, Organic Chemistry
	Padma Marwah University of Wisconsin-Madison	Bio-analytical Chemistry, Organic Chemistry

PARTICIPATING INSTITUTION AND PRINCIPAL INVESTIGATORS

University of Wisconsin-Madison

Terence P. Barry
Ashok K. Marwah
Padma Marwah

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0022
Expires 5/31/98

BUDGET

ORGANIZATION AND ADDRESS Board of Regents of the University of Wisconsin System Madison, WI				**USDA AWARD NO.** Year 1: Objectives 1-6	
PRINCIPAL INVESTIGATOR(S)/PROJECT DIRECTOR(S) Terence P. Barry, Ashok Marwah, and Padma Marwah				Duration Proposed Months: 12 **FUNDS REQUESTED BY PROPOSER**	Duration Awarded Months: _____ **FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages 1. No. of Senior Personnel	**CSREES FUNDED WORK MONTHS**				$
	Calendar	Academic	Summer		
a. 3 (Co)-PI(s)/PD(s)	16.8			$76,082	
b. ___ Senior Associates					
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates-Postdoctorates					
b. ___ Other Professional					
c. ___ Graduate Students					
d. ___ Prebaccalaureate Students					
e. ___ Secretarial-Clerical					
f. ___ Technical, Shop and Other					
Total Salaries and Wages . . . . . →				$76,082	
B. Fringe Benefits (If charged as Direct Costs)				$25,868	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) . . . . . →				$101,950	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for **each** item.)					
E. Materials and Supplies				$39,000	
F. Travel 1. Domestic (Including Canada)				$1,000	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges					
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				$10,600	
J. Total Direct Costs (C through I) . . . . . →				$152,550	
K. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)					
L. Total Direct and Indirect Costs (J plus K) . . . . . →				$152,550	
M. Other . . . . . →					
N. Total Amount of This Request . . . . . →				$152,550	$
O. Cost Sharing (If Required Provide Details)	$				

NOTE: Signatures required only for Revised Budget — This is Revision No. →

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director		
Authorized Organizational Representative		

Form CSREES-55 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0022
Expires 5/31/98

BUDGET

ORGANIZATION AND ADDRESS Board of Regents of the University of Wisconsin System Madison, Wisconsin				**USDA AWARD NO.** Year 2: Objectives 1-6	
				Duration Proposed Months: 6	Duration Awarded Months: _____
PRINCIPAL INVESTIGATOR(S)/PROJECT DIRECTOR(S) Terence P. Barry, Ashok Marwah, and Padma Marwah				**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				$
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. 3 (Co)-PI(s)/PD(s)	8.4			$39,796	
b. ___ Senior Associates					
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates-Postdoctorates					
b. ___ Other Professional					
c. ___ Graduate Students					
d. ___ Prebaccalaureate Students					
e. ___ Secretarial-Clerical					
f. ___ Technical, Shop and Other					
Total Salaries and Wages . . . . . →				$39,796	
B. Fringe Benefits (If charged as Direct Costs)				$13,531	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) . . . . . →				$53,327	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for **each** item.)					
E. Materials and Supplies				$11,000	
F. Travel				$1,000	
1. Domestic (Including Canada)					
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges					
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				$5,800	
J. Total Direct Costs (C through I) . . . . . →				$71,127	
K. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)					
L. Total Direct and Indirect Costs (J plus K) . . . . . →				$71,127	
M. Other . . . . . →					
N. Total Amount of This Request . . . . . →				$71,127	$
O. Cost Sharing (If Required Provide Details)	$				

NOTE: Signatures required only for Revised Budget — This is Revision No. →

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director		
Authorized Organizational Representative		

Form CSREES-55 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0022
Expires 5/31/98

BUDGET

ORGANIZATION AND ADDRESS Board of Regents of the University of Wisconsin System Madison, WI				**USDA AWARD NO.** Years 1 & 2: Objectives 1-6	
				Duration Proposed Months: 18	Duration Awarded Months: _____
PRINCIPAL INVESTIGATOR(S)/PROJECT DIRECTOR(S) Terence P. Barry, Ashok Marwah, and Padma Marwah				**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				$
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. 3 (Co)-PI(s)/PD(s)	25.2			$115,879	
b. ___ Senior Associates					
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates-Postdoctorates					
b. ___ Other Professional					
c. ___ Graduate Students					
d. ___ Prebaccalaureate Students					
e. ___ Secretarial-Clerical					
f. ___ Technical, Shop and Other					
Total Salaries and Wages . . . . . →				$115,879	
B. Fringe Benefits (If charged as Direct Costs)				$39,399	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) . . . . . →				$155,278	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for **each** item.)					
E. Materials and Supplies				$50,000	
F. Travel				$2,000	
1. Domestic (Including Canada)					
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges					
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				$16,400	
J. Total Direct Costs (C through I) . . . . . →				$223,678	
K. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)					
L. Total Direct and Indirect Costs (J plus K) . . . . . →				$223,678	
M. Other . . . . . →					
N. Total Amount of This Request . . . . . →				$223,678	$
O. Cost Sharing (If Required Provide Details)	$				

NOTE: Signatures required only for Revised Budget — This is Revision No. →

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director		
Authorized Organizational Representative		

Form CSREES-55 (6/95)

BUDGET EXPLANATION FOR THE UNIVERSITY OF WISCONSIN-MADISON

Objectives 1-6

A. Salaries and Wages. Salaries and fringe benefits are requested for Ashok Marwah and Padma Marwah at 55% time each, and for Terence Barry at 30% time.

B. Fringe Benefits. The UW-Madison fringe benefit rate for faculty and academic staff is 34%.

E. Materials and Supplies. Total costs: chemicals ($4,000); reagents and buffers ($5,000); steroid standards ($3,000); liquid nitrogen and nitrogen gas ($4,000); solvent and sample filters ($3,000); HPLC columns and guard columns ($7,000); solid phase extraction cartridges ($4,000); consumables, including vials, vial inserts, caps, peek tubing, peek fittings, gloves, wipes, pipette tips, scintillation vials, scintillation fluid, and replacement UV lamps for the UV-DAD detector ($7,000); glassware, plastic tubes, and other miscellaneous supplies ($5,000); biometers ($2,000), ultrasonic bath ($1,000); and custom synthesized ^{14}C-labelled MT ($5,000).

F. Travel. Annual costs: transportation, lodging, and meal expenses for two senior personnel to present results at a national conference at a location to be determined; chairman to attend NCRAC meetings yearly at a location to be determined to present results.

I. All Other Direct Costs. Total costs: service contracts on the HPLC ($4,000) and LC-MS ($8,000), ion trap and FT-MS services ($2,400), and Biotron rental ($2,000).

BUDGET SUMMARY

Year 1

	Ashok Marwah	Padma Marwah	Terence P. Barry	TOTALS
Salaries and Wages	$30,965	$30,387	$14,730	$76,082
Fringe Benefits	$10,528	$10,332	$5,008	$25,868
Total Salaries, Wages, and Fringe Benefits	$41,493	$40,719	$19,738	$101,950
Nonexpendable Equipment				$0
Materials and Supplies	$21,000		$18,000	$39,000
Travel	$500		$500	$1,000
All Other Direct Costs	$10,600			$10,600
TOTAL PROJECT COSTS	**$73,593**	**$40,719**	**$38,238**	**$152,550**

Year 2

	Ashok Marwah	Padma Marwah	Terence P. Barry	TOTALS
Salaries and Wages	$16,102	$15,801	$7,893	$39,796
Fringe Benefits	$5,475	$5,372	$2,684	$13,531
Total Salaries, Wages, and Fringe Benefits	$21,577	$21,173	$10,577	$53,327
Nonexpendable Equipment				$0
Materials and Supplies	$6,000		$5,000	$11,000
Travel	$500		$500	$1,000
All Other Direct Costs	$5,800			$5,800
TOTAL PROJECT COSTS	**$33,877**	**$21,173**	**$16,077**	**$71,127**

SCHEDULE FOR COMPLETION OF OBJECTIVES

Objective 1: Initiated and completed during the first four months of the project.

Objectives 2 and 3: Initiated and completed during months 4–10 of the project.

Objective 4: Initiated and completed during months 8–12 of the project.

Objectives 5 and 6: Initiated and completed during months 13–18 of the project.

LIST OF PRINCIPAL INVESTIGATORS

Terence P. Barry, University of Wisconsin-Madison

Ashok K. Marwah, University of Wisconsin-Madison

Padma Marwah, University of Wisconsin-Madison

VITA

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EDUCATION

B.S. University of Wisconsin-Madison, 1977, Zoology
M.S. University of Hawaii and Hawaii Institute of Marine Biology, 1989, Zoology
Ph.D. University of Wisconsin-Madison, 1992, Endocrinology-Reproductive Physiology

POSITIONS

Associate Scientist (2003-present), Assistant Scientist (1997-2003), Associate Researcher (1994-1997), Assistant Researcher (1990-1994), University of Wisconsin Aquaculture Program
Research Associate (1986-88), USAID:PSTC, Iloilo, Philippines

SCIENTIFIC AND PROFESSIONAL ORGANIZATIONS

American Fisheries Society, Physiology Section

SELECTED PUBLICATIONS

Roberts, S., T.P. Barry, J.A. Malison, and F. Goetz. In press. Production of a recombinantly-derived growth hormone antibody and the characterization of growth hormone levels in yellow perch. Aquaculture.

Barry, T.P., M.J. Unwin, J.A. Malison, and T. P. Quinn. 2001. Free and total cortisol levels in semelparous and iteroparous chinook salmon (*Oncorhynchus tshawytscha*). Journal of Fish Biology 59:1673-1676.

Barry, T.P., J. Riebe, J.J. Parrish, J.A., and Malison. 1997. Effects of 17α,20β-dihydroxy-4-pregnen-3-one on cortisol production by rainbow trout interrenal tissue in vitro. General and Comparative Endocrinology 107:172-181.

Barry, T.P., J. Malison, J. Held, and J.J. Parrish. 1995. Ontogeny of the cortisol stress response in rainbow trout. General and Comparative Endocrinology 97:57-65.

Barry, T.P., A.F. Lapp, T.B. Kayes, and J.A. Malison. 1993. Validation of a microtitre plate ELISA for measuring cortisol in fish and comparison of stress responses of rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*). Aquaculture117:315-363.

Barry, T.P., P. Thomas, and G.V. Callard. 1993. Stage-related production of 21-hydroxylated progestins by the dogfish (*Squalus acanthias*) testis. Journal of Experimental Zoology 265:522-532.

Barry, T.P., K. Aida, T. Okumura, and I. Hanyu. 1990. The shift from C-19 to C-21 steroid synthesis in spawning male common carp, Cyprinus carpio, is regulated by the inhibition of androgen production by progestogens produced by spermatozoa. Biology of Reproduction 43:105-112.

Barry, T.P. and E.G. Grau. 1986. Estradiol-17β and thyrotropin-releasing hormone stimulate prolactin release from the pituitary gland of a teleost fish in vitro. General and Comparative Endocrinology 62:306-314.

VITA

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EDUCATION

B.S. Lucknow University, Lucknow, India, 1973, Chemistry, Physics and Mathematics
M.S. Lucknow University, Lucknow, India, 1975, Chemistry
Ph.D. JNT University, Hyderabad, India, 1995, Chemistry/Synthetic and Analytical Studies

POSITIONS

Senior Scientist (2000-present) and Associate Researcher (1996-2000), Institute for Enzyme Research, University Wisconsin-Madison
Consultant (2003) for LC-MS (single quad. and ion trap), Agilent Technologies
Research Scholar (1992), Center for Neurochemistry (CNRS), University Louis Pasteur, Strasbourg, France
Senior Research Executive (1993-1995), Research Executive (1985-1993), and Research Chemist (1979-1985), Research Center, Indian Drugs & Pharmaceuticals Ltd., India.
Junior Research Fellow (1976-79), Council for Scientific and Industrial Research, New Delhi, India.

SCIENTIFIC AND PROFESSIONAL ORGANIZATIONS

American Chemical Society
American Society for Mass Spectrometry

SELECTED PUBLICATIONS

Marwah, P., A. Marwah, and H. A. Lardy. 2003. Microwave assisted selective enolization of steroidal ketones and efficient acetylation of sterols in semisolid state. Tetrahedron 59(13): 2273-87.

Lardy, H.A., A. Marwah, and P. Marwah. 2002. Transformations of DHEA and its metabolites by rat liver, Lipids 37:1187-91.

Marwah, A., P. Marwah, and H. A. Lardy. 2002. Analysis of ergosteroids VIII: Enhancement of signal response of natural steroidal compounds in liquid chromatographic-electrospray ionization mass spectrometric analysis by mobile phase additives. Journal of Chromatography A 964:137-51.

Marwah, A., P. Marwah and H. A. Lardy. 2002. Ergosteroids VII. Perchloric acid induced transformations of 7-oxygenated steroids and their bio-analytical applications: A liquid chromatographic-mass spectrometry study. Bioorganic Chemistry 30:233-48.

Marwah, A., P. Marwah, and H. A. Lardy. 2002. Ergosteroids VI. Metabolism of DHEA by rat liver in vitro: A liquid chromatography-mass spectrometric study. Journal of Chromatography B 767:285-299.

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POSITIONS

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Research Associate (1993), Department of Chemistry, University of Illinois-Chicago
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SELECTED PUBLICATIONS

Marwah, P., A. Marwah, and H. A. Lardy. 2003. Microwave assisted selective enolization of steroidal ketones and efficient acetylation of sterols in semisolid state. Tetrahedron 59(13): 2273-87.

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