出國報告（出國類別：開會）

參加
「國際 AOAC 動物用藥檢驗方法
專家評審會議」
報告

服務機關：行政院衛生署藥物食品檢驗局

姓名職稱：曾素香 副研究員

出國地區：美國

出國期間：98 年 6 月 14 日至 6 月 19 日

報告日期：98 年 9 月 7 日

摘要

國際公定分析化學家協會（AOAC, international）於 6 月 15 日至 17 日於美國總部舉辦動物用藥檢驗方法專家評審會議，本人有幸獲台灣 AOAC 及本局推薦，受邀擔任該會議委員，其他與會委員則來自美國食品藥物管理局（US FDA）、美國農業部（USDA）、加拿大食品檢驗局（CFIA）、法國食品安全局（AFSSA）、荷蘭國家公共衛生及環境研究所（RIVM）、 美國 Covance 實驗室及美國印地安納波里大學等單位，計 10 人。3 天討論之動物用藥子題分別爲孟寧素/那寧素（Monesin/Narasin）、泰黴素（Tylosin）及培林（Ractopamine），委員們就已收集之文獻進行意見發表、溝通交流及投票，目的爲選出合適的檢驗方法進行後續確效實驗，以未來作爲 AOAC 公定方法。此次爲國際 AOAC 首次邀請亞洲國家參與，本人有幸代表藥物食品檢驗局實際參與開會，收獲良多。國際 AOAC 研擬公定方法之過程，十分值得未來食品藥物管理局借鏡。建議食品藥物管理局應研擬長期計畫，進行公告方法之研擬、審查及指定等工作，邀集學者、專家、相關實驗室參與，進行方法回顧、評估、審查、方法確效及實驗室間比對等工作。未來應持續積極參與國際 AOAC 相關會議，提升研究檢驗水平及視野，並促進專家交流，以因應日益複雜之國際食品安全檢驗問題。

目次

壹、目的

此次國際 AOAC 舉辦之專家評審會議（Expert Review Panel, ERP），經費由美國 ELANCO 公司提供，其爲生產動物用藥之公司。此會議之目的爲選出合適的培林等動物用藥檢驗方法進行後續確效實驗，以作爲 AOAC 公定方法。國際 AOAC Dr. Al Pohand (Chief Scientific Officer)與台灣關係良好，請台灣 AOAC 分會提供參加專家人選，本人有幸獲台灣 AOAC 孫璐西理事長及本局推薦，受邀擔任該會議委員，得有機會瞭解國際 AOAC 研擬公定方法之運作模式及程序，並提供專業上的協助。雖然參與該專家會議之壓力不小，但基於機會得來不易且與本局業務密切相關，故積極投入此項國際事務工作，期待此行獲得之資訊及心得可提供明年正式成立之衛生署食品藥物管理局相關業務運作時之參考。

貳、過程

國際公定分析化學家協會（AOAC, international）於 6 月 15 日至 17 日於美國總部（位於馬里蘭州蓋茲堡市）舉辦培林等動物用藥檢驗方法專家評審會議，除了本人外，其他與會委員則包括美國食品藥物管理局（US FDA）1 人、美國農業部（USDA）3 人、加拿大食品檢驗局（CFIA）法國食品安全局（AFSSA）1 人、荷蘭國家公共衛生及環境研究所（RIVM）1 人、 美國 Covance 實驗室 1 人及美國印地安納波里大學 1 人，計 10 人，其姓名及單位如下：

1. 美國食品藥物管理局（US FDA）：1 人
   Dr. Valerie Reeves
   Center for Veterinary Medicine, FDA (USA)
2. 美國農業部（USDA）：3 人
   (1) Dr. In Suk Kim
   Lead Chemist
   Office of Public Health Science, Western laboratory, FSIS (Food Safety and Inspection Service), USDA, (USA)
   (2) Dr. Katerina mastovska
   Research Chemist
   Agricultural Reserach Service, Eastern Regional Research Center, USDA (USA)
   (3) Dr. Weillin Shelver
   Agricultural Research Service, Biosciences Research Laboratory, USDA (USA)
3. Dr. Matt Rodewald
   Covance laboratories (USA)
4. Dr. Lindell Ward
   Assistant Professor of Chemistry
   Department of Chemistry
   University of Indianapolis
5. 加拿大食品檢驗局（CFIA）： 1 人
   Dr. Joe Boson
   Centre for Veterinary Drug Residues, CFIA Saskatoon Laboratory
   Canadian Food Inspection Agency (Canada)
6. 法國食品安全局（AFSSA）： 1 人
   Dr. Erik Verdon
   Head of unit, veterinary drug residue unit

Deputy head of EU community reference laboratory in charge of antibiotic residues in food from animal origin
AFSSA (French Food Safety Agency)

7. 荷蘭國家公共衛生及環境研究所（RIVM）：1 人
Dr. Leendert van Ginkel
Head, Laboratory for Food and Residue Analysis
Nutrition, Medicines and Consumer Safety Division,
National Insitute for Public Health and the Environment (RIVM-CRL, the Netherland)
8. 藥物食品檢驗局(BFDA)：1 人
曾素香副研究員

此會議討論之動物用藥子題為 6 月 15 日孟寧素/那寧素（Monesin/Narasin）、6 月 16 日泰黴素（Tylosin）、6 月 17 日培林（Ractopamine）。於會議進行前委員們即被分配就國際 AOAC 收集之文獻進行回顧（review），並需於開會前繳交。每篇文獻皆有主要回顧人（primary reviewer）及次要回顧人（secondary reviewer）。每位委員交出之回顧報告，其書寫內容需包括方法摘要（summary of method）、方法之優缺點、方法清楚度（method clarity）、方法應用範圍（method scope）、方法確效報告及建議等，方法確效部分，需評估方法最佳化、鑑別性及方法表現（分析濃度範圍、線性關係、精確性/回收率及精密度）等。國際 AOAC 於開會前即將各委員之回顧報告進行彙整後傳送給所有委員參考，此部分資料為 AOAC 內部審查資料不可外流，故不附在本報告中。此次本人計被分配 10 篇文獻，填報的回顧報告見附件一。

會議第一天即由國際AOAC Dr. Al Pohland針對專家評審會議(Expert Review Panel, ERP）施行策略及流程進行介紹，整理如下。

<u>專家評審會議之啓動</u>：由專案利害關係人（Stakeholder）或組織（Stakeholder body）向 AOAC 申請創立 ERP，並需提出討論主題、預期產出及建議專家名單。申請資料送 AOAC Chief Science Officer (CSO)，由 CSO 研擬 ERP 成員名單，由公定方法委員會（Official Methods Board, OMB）審核決定，並指定 ERP 會議主席。ERP 會議主席將負責規劃會議進行模式，以順利討論並作出建議，決定可進行進一步評估之方法及試驗材料等，會議後將所得結論及建議送交 OMB 及申請人（單位）。ERP 會議參與成員可包括具投票權的成員（專家委員）及不具投票權的成員（AOAC 職員、申請單位人員及觀查員）。

選擇方法過程：ERP 將就收集到的方法進行仔細評估，並選出適當的方法進行後續確效試驗，方法之基本要求爲：符合目的、可應用於所需檢體範圍、方法描述清楚、方法表現滿意、具有單一實驗室評估數據（single laboratory validation data）。ERP 委員將選出最好的方法（一個或數個）進行進一步確效及流程修正，並指定實驗測試之基質種類。

總結報告（Summary Report）：ERP 主席於會議結束後完成之總結報告，必需包括委員建議、選出方法之修飾部分、確效實驗採用基質等內容，並及時送交 ERP 委員確認，ERP 委員必須於期限內回覆意見。最終總結報告將送申請者，副本送 OMB 主席。

ERP 會議後事宜（Post-ERP Activities）：EPR 會議後，AOAC 仍可請委員會或廠商提供協助，協助事項包括：(1)取得測試用檢體或參與後續確效實驗(2)研擬及評估確效試驗計畫(3)評估確效試驗結果及檢驗方法草案。

三天的會議皆進行的十分順利，第一天由加拿大食品檢驗局 Dr. Joe Boison 擔任主席，第二天主席爲美國印地安納波里大學 Dr. Lindell Ward，第三天主席爲荷蘭國家公共衛生及環境研究所 Dr. Leendert van Ginkel。主席們皆準備相當充分且主持得相當順暢，參與委員們得以進行充分溝通、討論及投票，順利選出合適的檢驗方法進行後續確效實驗，以作爲未來 AOAC 公定方法研擬之參考。三天會議的總結報告見附件二，受邀的中國代表委員 Dr. Guo-Fang Pang 無法參加會議，雖未交分配到的回顧報告，卻參考了三天會議主題之文獻方法研擬出流程草案並實驗進行確效，將實驗結果整理成文件提供與會委員參考。茲將討論主題動物用藥之重點及決議內容整理如下：

一、孟寧素（Monesin）/那寧素（Narasin）（6 月 15 日）

會議開始，主席 Dr. Joe Boison 即利用簡報內容，針對方法回收率（recovery）及準確度 accuracy (bias)等名詞進行詳細解釋，以確保所有委員在討論過程中採用的定義相同。計算回收率%(絕對)時需考量基質存在對儀器訊號的影響，基質的存在可能顯著增加或抑制訊號，故此時回收率的計算需與基質匹配檢量線（matrix-matched calibration curve）計算，而不可以標準溶液定量，否則結果將高估或低估回收率。而準確度 accuracy (bias)則表示方法與真值接近的程度，可進一步利用添加內部標準品改善之。

孟寧素（Monesin）及那寧素（Narasin）屬離子型抗球蟲劑，此次收集的文獻方法計 14 篇，其中 3 篇爲 LC 方法，其餘則爲 LC/MS(MS)方法。考量此動物用藥的應用範圍，此次選出的方法，需可檢測動物組織（雞、羊、牛）中的 Monesin 及 Narasin A，包括肌肉、肝臟、腎臟、脂肪、牛奶及蛋；檢測範圍 25-400 ppb；回收率視濃度而定，應

落於 60-110%；方法需包括確認部分或另以其他方法確認，此方法將應用於促進國際貿易並需符合法規要求。各委員報告了每篇文獻之回顧結果及評論後，主席即請大家開始選擇最爲可行的方法。由於並未有一篇文獻同時符合所有要求，故針對檢體前處理及檢測兩大部分，進行逐篇討論。前三篇 LC 方法之流程相當類似，皆採用管柱後衍生化（post column derivatization, PCD）以 vanillin 爲衍生化試劑，再檢測 UV 520 nm，淨化時使用二氯甲烷溶劑，基於目前市面上並無現成 PCD 產品，必須自行組裝及最佳化，故決定不採用 PCD 法，而二氯甲烷具致癌性，應以其他溶劑取代。會議中委員投票決定採用 LC/MS/MS 方法，以同時進行定性及定量，主要參考方法 13B 參數。檢體前處理部分則以方法 1, 4, 5, 13 爲主要參考文獻，分組討論出極性溶劑萃取流程及非極性溶劑萃取流程等 3 種草案，詳見總結報告 p.9-12。極性溶劑萃取流程擬應用於豬（牛）肉、肝、腎、蛋等檢體，係使用甲醇/水（87/13）爲萃取溶劑，添加內標(nigericin)，離心後以 C18 SPE 淨化，殘留物以移動相溶解後再以 LC/MS/MS 分析。非極性溶劑萃取流程則使用 isooctane/ethyl acetate 或 ethyl acetae 爲萃取溶劑，擬應用脂肪及牛奶等檢體。

二、泰黴素（Tylosin）（6 月 16 日）

此次選出的方法，需可檢測動物組織（雞、羊、牛）中的 Tylosin A (as marker residue)，包括肌肉、肝臟、腎臟、脂肪、牛奶及蛋；檢測範圍 25-400 ppb；回收率視濃度而定，應落於 60-110%；方法需包括確認部分或另以其他方法確認，此方法將應用於促進國際貿易並需符合法規要求。泰黴素屬 macrolide 類抗生素，此次收集的文獻方法計 15 篇，因爲 macrolide 方法中通常包括泰黴素及 tilmicosin，故委員們決議將 tilmicosin 納入此次方法中。經委員們討論及表決，檢驗方法可採用 LC/UV 定量及 LC/MS/MS 確認或 LC/MS/MS 直接定性及定量；主要採用方法 01, 16 及 17，研擬之檢驗方法草案詳見總結報告 p.7-8，LC/UV 及 LC/MS/MS 方法之萃取流程相同，LC/MS/MS 則需添加內標 (roxithroycin)。

三、培林（Ractopamine）（6 月 17 日）

此次選出的方法，需可檢測動物組織（羊、牛）中的培林，包括肌肉、肝臟、腎臟及脂肪。檢驗方法應包括檢測 free (parent) ractopamine 或經酵素水解流程檢測之總培林量（parent and conjugates）。檢測範圍 1-100 ppb (ng/g)，以符合歐盟 Reference Point for Action (RPA) 1 ppb 及國際間 MRL 最低值 10 ppb；回收率視濃度而定，應落於 60-110%；方法需包括確認部分或另以其他方法確認，此方法將應用於促進國際貿易並需符合法規

要求。培林屬乙型受體素（β-agonists）動物用藥，此次收集的文獻方法計 15 篇，包括 ELISA 方法、Coulometric 方法、LC-FLD 及 LC/MS/MS 方法，而 ELISA 及 Coulometric 方法經委員討論及投票後決定不納入考慮，而 LC-FLD 則因靈敏度足夠被接受作爲定量方法，因此僅有 LC-FLD 及 LC/MS/MS 方法可被選作進一步評估的方法。委員們區分爲兩組，分別進行 LC-FLD 及 LC/MS/MS 方法之討論，最後選定方法 11 作爲 LC-FLD 方法之主要依據，但由於流程中使用了 2 個固相萃取（Solid Phase Extraction, SPE）淨化步驟，耗費時間長，故將流程進行修飾，詳見總結報告 p.16-17。檢體係採用甲醇爲萃取溶劑，並加入適當的內標，LC-FLD 方法添加 IS-ritodine， LC/MS/MS 方法則添加 IS-Ractopamine d5，檢測培林總量時則需以β-glucuronidase 進行水解，主席建議水解條件尚需最佳化（溫度及時間），因爲各文獻採用的條件差距相當大，此部分將由 ELANCO 公司進行測試。

三天會議研擬之檢驗方法流程，將由 ELANCO 公司實驗室進行 single laboratory validation (SLV)，測試結果將再轉知各委員，各委員仍有義務協助後續評估或實驗事宜。

參、心得

本人此次有幸參與國際 AOAC 動物用藥檢驗方法專家評審會議，個人收獲良多，已於 98 年 7 月 29 日進行心得分享，簡報內容見附件三。歷經 3 天辛苦的審查會議，相當佩服參與的各國專家，會議過程中除了展現其專業外，認真的表現亦相當另人激賞。會議進行的步調及方式由主席引導，以便在會議結束前有具體決議。雖然參與會議過程相當辛苦，卻是相當好的學習機會，對於未來有關檢驗方法研擬相關事務可有新的思維，而其運作模式亦可思考改進，以縮減作業時間並增加檢驗方法之公信力。

肆、建議

國際間貿易往來頻繁，各國進行食品衛生安全把關，必須以具有公信力之檢驗方法進行檢驗，始可減少國際貿易糾紛。針對現行食品中殘留藥物檢驗方法公告情形，建議如下：(1)研擬檢驗方法長期計畫，部分工作委外，進行公告方法之更新、研擬、審查及指定等工作。(2)邀集學者、專家及相關實驗室參與檢驗方法研究計畫，整合國內相關領域之研究檢驗量能，定期進行方法回顧、評估、審查及方法確效、實驗室間比對等工作，以增快檢驗方法公告及指定之速度，遇緊急事件時，亦可馬上啓動該系統。(3)持續積極參與國際 AOAC 相關會議，提升檢驗水平及視野，以因應日益複雜之國際食品安全檢驗問題。

ERP Review Sheet

Evaluation of Method Mon-11

Title Determination of amprolium, ethopabate, lasalocid, monensin, narasin, and salinomycin in chicken tissues, plasma, and egg using liquid chromatography-mass spectrometry
Submitted by: Su-Hsiang Tseng (Primary reviewer)
Secondary Review:
Summary of Method: (Figure 2)
Sample (3 g) was extracted with a mixture of methanol (0.5 mL)-water (0.5 mL)-acetone/tetrahydrofuran (6/4) (6 mL) by homogenizer and ultrasonic bath, and then centrifuged for 5 min at 5000 rpm. A 5 mL (corresponding to 1.5 g for egg, liver, muscle) of supernatant or a 2.5 mL of the upper layer for fat (corresponding to 0.75 g for fat), was partitioned with 6 mL of diethyl ether/hexane (6/4), for fat, 0.5 mL of water was also added. After centrifugation (3500 rpm, 3 min), the upper layer (organic phase) was mixed with 5 mL of hexane, and centrifuged for 3 min. The supernatant was evaporated to dryness, and dissolved in 1.5 mL of hexane. One mL of hexane sample solution was apply on a Sep-pac RC Silica column (500 mg), washed with 8 mL of diethyl ether/hexane (6/4), 8 mL of dichloromethane/ethanol (96%) (99.5/0.5), and 8 mL of chloroform, dried for ca. 10 sec, and then eluted with 1 mLX3 of CH_2Cl_2/CH_3OH (90/10). The eluate was evaporated to dryness and reconstituted with 0.4 mL of CH_3OH/H_2O (8/2).
LC/MS conditions: (Perkin-Elmer)
Mobile phase: methanol: 10 mM (0.77 g/L) ammonium acetate mixture (85:15, v/v)
Column: Supelco Discovery C18
Multi-SIM (Positive ion mode):
m/z 693.7 (Monesin, MNa^+); *m/z* 787.5 (Narasin, MNa^+)

Pros/Strengths:

Cons/Weaknesses
- General comments: The analytical procedures were complicated and time-consuming. Some toxic solvents (THF, dichloromethane, chloroform) were used.
- Method Clarity: It's not clear to understand why "a 5 mL volume of the supernatant is corresponding to 1.5 g sample" stated in sample pretreatment paragraph (p.1588).
- Method Scope/Applicability: applicable for egg, fat, meat and liver samples.

Method Validation Report:
- General Comment
  - Method Optimization: The extraction and clean-up procedures could be optimized and some toxic solvents should be replaced.
- Confirmation of identity: the confirmation of identity is week by single MS detector (one ion for one compound).
- Performance Characteristics
  - Analytical Range
    LODs were ranged 1~5 ng/g. LOQs were ranged 2~10 ng/g.
  - Calibration
    The correlation coefficients of calibration curves (5 to 150 ng/g) for monesin and narasin in various tissues were all above 0.998.
    Accuracy/Recovery
    Recoveries of monesin and narasin, at piking levels of 20 and 50 ng/g, were ranged from 85~121% and 86~103%, respectively.
  - Precision
    Repeatability (RSD%) 1.4~6.2%

Recommendation:
This method sounds not proper for further validation study based on the complicated procedures and weak confirmation.

ERP Review Sheet

Evaluation of Method Mon-13A

Title Liquid chromatography-electrospray tandem mass spectrometric method for quantification of monesin in plasma and edible tissues of chicken used in pharmacokinetic studies: Applying a total error approach

Submitted by: Su-Hsiang Tseng (Secondary reviewer)
Secondary Review:
Summary of Method:
Quantification of monesin residues in chicken muscle, liver, and fat was determined by LC/MS/MS (ESI+). Internal standard, narasin, was added at a concentration level of 50 μg/kg. Sample (2 g) was votex-mixed with a mixture of methanol-water (87/13, v/v) (6 mL), placed in a ultrasonic bath for 10 min and centrifuged for 10 min at 4000 xg at 5℃. A 5 mL aliquot was purified on a Varian Bond Elut C18 (200 mg, 3 mL) cartridge (preconditioned with 4 mL of methanol and 2 mL of water), washed with 2 mL of a mixture of methanol-water (80/20, v/v) and eluted with 4 mL of methanol. The eluant was evaporated and redissolved in 300 μL of a mixture of acetonitrile-ammonium acetate 50 mM (80/20, v/v).
LC/MS/MS conditions: (API 2000)
Mobile phase: water, 0.1% formic acid (A); acetonitrile, 0.1% formic acid (B); 80%B to 100% B (6 min), hold 6 min, 100% B to 80% B (2 min), hold 6 min.
Column: Luna C18 with guard column
MRM Transitions: 688.4>635.3 (Monesin, MNH_4^+); 782.6>747.5 (Narasin, MNH_4^+)
Pros/Strengths:
Ammonium precursors were used in this method, compared with sodium precursors, which showed better sensitivity and precision.
Cons/Weaknesses

- General comments: Extraction and clean-up procedures are worth followed, and using ammonium adducts as precursors were recommended to test.
- Method Clarity: R^2 figures of calibration curves for muscle and fat matrices were not shown in Table 2.
- Method Scope/Applicability: applicable for muscle, liver and fat samples. Egg and milk samples were not involved in this method.

Method Validation Report:

- General Comment
  - Method Optimization: Limit of quantification for determining monesin residues in chicken muscle could be discussed further (0.5~2.5 ppb).
- Confirmation of identity: Confirmation was weak. No second mass transitions (qualification ions) for Monesin and Narasin.
- Performance Characteristics
  - Analytical Range
    1~200 μg/kg
  - Calibration
    Matrix matched standards prepared according to the sample preparation protocol used routinely.
  - Accuracy/Recovery
    Muscle (spiking 2.5~100 μg/kg) 88.2~102.2%; Fat (spiking 2.5~200 μg/kg) 93.0~107.0 %; Liver (spiking 1~100 μg/kg) 92.0~105.0%
  - Precision
    Repeatability (RSD%) 1.9~11.8%

Recommendation:
Qualification ions are needed to chosen in this method for confirmation purpose.

ERP Review Sheet

Evaluation of Method Mon-13B

Title Efficient and sensitive screening and confirmation of residues of selected polyether ionophore antibiotics in liver and eggs by liquid chromatography-electrospray tandem mass spectrometry

Submitted by: Su-Hsiang Tseng (Secondary reviewer)

Secondary Review:

Summary of Method:

Sample (5 g) was extracted with a mixture of methanol-water (87/13, v/v) (15 mL) by homogenizer, and centrifuged for 5 min at 3600 xg. A 9 mL of aliquot was purified on an IST Isolute MF C18 (100 mg) cartridge (preconditioned with 4 mL of methanol and 2 mL of water), washed with 800μL of a mixture of methanol-water (80/20, v/v) and eluted with 600 μL of methanol. The eluant was determined by LC/MS/MS.

LC/MS/MS conditions: (Mircromass Quattro)

Mobile phase: acetonitrile: 0.05 M ammonium acetate mixture (8:2, v/v)

Column: Genesis C18 with guard column

MRM Transitions:

Screen: 693>479 (Monesin, MNa^+); 787>431 (Narasin, MNa^+)

Confirmation of Narasin: 787>431, 265, 279, 531

Pros/Strengths:

Optimization of SPE clean-up procedure (Clear information in Table 2). Four daughter ions were detected for narasin with ion ratios meeting EU confirmation criteria, even at 0.2 ng/g concentration. The run time of LC/MS/MS is short (only 4 min).

Cons/Weaknesses

- General comments: Methods for screening 4 polyether ionophore antibiotics (narasin, salinomycin, monensin, and lasalocid) and confirming narasin were introduced.
- Method Clarity: There's no internal standard or any step of making up volume were used in this method. So, the calculation of residue concentration is very important. However, the quantification was not very clear in this study.
- Method Scope/Applicability: applicable for egg and liver.

Method Validation Report: (Only for Narasin)

- General Comment
  - Method Optimization: Limits of quantification for both monesin and narasin were not validated in this study.
- Confirmation of identity: Four daughter ions were used for confirmation of narasin. However, no second mass transitions (qualification ions) for confirmation of monesin.
- Performance Characteristics
  - Analytical Range
    0.026 (LOD)~20 ng/g (narasin in egg)
  - Calibration
    Standard curves (0.2 to 50 ng/mL) typically produced correlation coefficients of 0.99.
    Accuracy/Recovery
    Egg (spiking 0.5~20 ng/g) 94~108%; Chicken liver (spiking 5 and 10 ng/g) 98~105%.
  - Precision
    Repeatability (RSD%) 4~10%

Recommendation:

The fully validation data of this method for determining narasin and monesin residues in various kinds of matrices should be set up. According to the observation of this study (p. 1992), a lager elution volume would be used to improve the stability of clean-up performance when using other types of C18 cartridge.

ERP Review Sheet Example
Evaluation of Method Tylosin-02

Title: Determination of Macrocyclic Lactone Drug Residues in Animal Muscle by Liquid Chromatography/Tandem Mass Spectrometry

Submitted by: Su-Hsiang Tseng (Primary reviewer)

Primary Review:

Summary of Method:

A robust, credible, and practical multi-residue method based on LC/MS/MS (ESI+) was developed for the simultaneous determination of 9 macrocyclic lactone drugs in bovine, porcine, chicken, and sheep muscles. LOD and LOQ of tylosin in this developed method were 0.1 ppb and 0.4 ppb, respectively. Sample (5 g) was extracted with acetonitrile 3 times (15 mL, 10 mL, 10 mL) by homogenizer and ultrasonic bath, and then centrifuged (5000 xg, 5 min) at 4 °C. All supernatants were combined. A 5 mL of the combined extracts was diluted to 15 mL with deionized water, and the solution was defatted by shaking with 5 mL *n*-hexane. The mixture was then centrifuged, and the *n*-hexane layer was discarded. The lower aqueous layer was applied to a Supelco C18 (500 mg/3 mL) cartridge (pre-conditioned with 5 mL acetonitrile and acetonitrile:water (1:3, v/v), washed with 5 mL water and 5 mL acetonitrile:water (1:3, v/v), and eluted with 5 mL 3% ammonium hydroxide in methanol. The eluates were then evaporated to dryness at 50 °C and the residues were redissolved in 1 mL mobile phase A:B (1:1, v/v).
LC/MS/MS: Agilent Model 1100 with API 4000 triple-quadrupole mass spectrometer.
Mobile phase: Acetonitrile+0.15% formic acid (A); Water+0.15% formic acid (B); 10%A hold 1 min, 10%A to 50% A (7 min), 50% A to 95% A (3 min), hold 10 min.
Column: Symmetry C18 (Waters, 3.5 μm, 150 x 2.1 mm)
MRM Transitions: 916>174*; 916>772 * Underlined = quantifier

Pros/Strengths:

A multiresidue LC/MS/MS method capable of simultaneous 9 macrocyclic lactone drugs (belonging to 2 groups) in muscle tissues was developed and suitable for routine determination of residues in livestock products.

Cons/Weaknesses:

- General comments: the extraction procedures are simple and easy to follow.
- Method Clarity: a 5 mL aliquot of the combined extracts was used for clean-up, however, the absolute volume of total extracts was not mentioned (final making up volume).
- Method Scope/Applicability: applicable for muscle tissues; liver, kidney, eggs, milk and fat samples were not involved in this method.

Method Validation Report:

- General Comment:
  - Method Optimization: the cleanup procedure by C18 SPE was optimized fully by discussing the washing and eluting solvents in this paper.
- Confirmation of identity: Two mass transitions were chosen for each compound in this LC/MS/MS method to fulfill the EU criteria for confirmation of target drug residues. The ion ratios of the qualifier ion to the corresponding quantitation ion, at spiking levels of 50-200 μg/kg, were within the acceptable criteria set by the EU guidelines.
- Performance Characteristics:
  - Analytical Range:
    0.4 (LOQ)~200 μg/kg
  - Calibration:
    The matrix matched calibration curve of tylosin was >0.999 (10.0~1000.0 ng/mL).
  - Accuracy/Recovery:
    Muscle (spiked 50~200 μg/kg) 68.4~84.9% (intraday); 69.8~86.6% (interday)
  - Precision:
    Repeatability (RSD %) 3.8~12% (intraday); 4.6~14% (interday)

Recommendation:

The retention time of first peak in figure 2 is about 9 min. That means the LC/MS/MS conditions could be optimized to shorten the run time (<15 min could be possible). Various kinds of matrices, such as fat, liver, milk and egg, should be also validated.

ERP Review Sheet
Evaluation of Method Tylosin-13

Title: Identification and quantification of five macrolide antibiotics in several tissues, eggs and milk by liquid chromatography–electrospray tandem mass spectrometry

Submitted by: Su-Hsiang Tseng (Secondary reviewer)
Secondary Review:

Summary of Method:
A HPLC–MS–MS (ESI+) method capable of determining five macrolides in several tissues (muscle, kidney, liver), eggs and milk from swine, cattle and hens was presented. Roxithromycin was used as an internal standard spiked at a level of 200 μg/kg. Tylosin can be accurately quantified at a level of 50 μg/kg (1/2 MRL) by this multi-residue analysis.
Extraction: a sample (5 g of tissue sample and whole egg) was extracted with Tris buffer (pH 10.5±0.2) (25 mLX2 for muscle; 25 mL and 20 mL for liver and kidneyl; 35 mL for egg) by vigorously mixing for 15 min, centrifuged (3000 xg, 10 min.) at 4 °C. Milk sample (10 mL) was centrifuged for 10 min at 3000 xg and 4 °C to obtain the skim milk (decreamed), and 5 mL of it was diluted with 20 mL Tris buffer, shenken gently and horizontally for 10 min.
Protein precipitation: acetic acid (600 μL for tissue; 1 mL for egg; 15 mL 3 M sodium acetate buffer for milk) and 5 mL sodium tungstate buffer were added to precipitate the proteins (1 h at 4 °C). The samples were centrifuged at 3000 xg for 10 min. The supernatants were further filtered through a plug of glass wool.
SPE clean-up: The sample was applied to an Oasis HLB (200 mg/6 mL) cartridges (conditioned with 10 mL methanol and 10 mL water), washed with 20 mL methanol:water (5:95, v/v), 5 mL hexane, vacuum-dried for 10 min, and eluted with 5 ml methanol:30% ammonia (95:5, v/v). The eluate was then evaporated to dryness. The residue was dissolved in 500 μL NH_4OAC: ACN (80:20, v/v).
LC/MS/MS: Hewlett Packard with Micromass Quattro II mass spectrometer.
Mobile phase: Acetonitrile (A); 0.1 M ammonium acetate (B); 0%A hold 1 min, 0%A to 30% A (3 min), 30% A to 95% A (3 min), 95% A to 0% A (4 min), hold 3 min.
Column: Purospher C18 (5 μm, 125 x 3 mm)
MRM Transitions: 916.3>772.2*; 916.3>318.4, 916.3>407.4, 916.3>598.4
* Underlined = quantifier

Pros/Strengths:
The method required only a simple extraction without organic solvents and with a short run time (<14 min), large sample batches (more than 50 samples) could be processed daily.

Cons/Weaknesses:
- General comments: this method sounds not applicable for fat sample. However, tylosin is a lipophilic compound and residues in fat or whole milk sample should be determined.
- Method Clarity: it not very clear for the role of Roxithromycin, an internal stand. Recoveries of Roxithromycin were also validated in this method, however, the figures ranged from 58 to 76% which are lower than tylosin's. An internal standard should behave the similar distribution as the target compound during the analytical procedures. According to "3.4.1. Quantification step", the macrolides were quantified by means of external standard curves and shown in Table 3 and Table 4.
- Method Scope/Applicability: applicable for muscle, kidney, liver, egg, skimmed milk samples, whole milk and fat sample was not involved in this method.

Method Validation Report:
- General Comment:
  - Method Optimization: the extraction procedures for various matrices are different, and the reasons are not mentioned in the paper.

- Confirmation of identity: Four mass transition ions (qualifier and quantifier ion) were used for confirming tylosin residue in sample. Ion ratios are shown in Table 5 and the RSDs were <12%.
- Performance Characteristics:
  - Analytical Range:
    25~200 μg/kg
  - Calibration:
    Pure standard solutions were used and a correction factor had to be introduced into the chromatogram integration. The correlation coefficients of the calibration curves for were all >0.99.
  - Accuracy/Recovery:
    Matrices (spiking 50~200 μg/kg) 93~115%
  - Precision:
    Repeatability (RSD %) 2.9~7.5%

Recommendation:
The MDL of tylosin was not mentioned in the paper. The LOQ of tylosin could be lower than 50 ppb by further validation.

ERP Review Sheet
Evaluation of Method Tylosin-18

Title: Confirmation of Macrolide/Lincosamide Antibiotics by Ion Trap HPLC/MS/MS
Submitted by: Su-Hsiang Tseng (Primary reviewer)
Primary Review:
Summary of Method:
Sample (5 g) was made basic (by adding 2 M K_2CO_3) and extracted with ethyl acetate (30 mL, 15 mL) by shaking, and centrifuged (2000 rpm, 10 min). Acidify the combined organic phase (ca. 45 mL) with 2 mL of 0.2 M KH_2PO_4, shake and centrifuge (3 times).To the 6 mL of combined aqueous solution, add 5 mL of a ethyl acetate:hexane solution (1:1), invert the tube, and centrifug at 2000 rpm for 4 min. Discard the top organic layer, and adjust the pH of the aqueous solution to 9.8-10.2 by adding 900 to 1200 µL of 2M K_2CO_3. Add 4.0 mL of ethyl acetate to the aqueous solution, shake, centrifuge at 2000 rpm for 4 min, transfer the upper organic layer to another tube, and repeat the step twice. Evaporate the combined organic solution to dryness at 40 °C, and then dissolve the residue in 500 µL of 50:50 (v/v) methanol/water.
LC/MS/MS: Thermo-Finnigan Surveyor HPLC equipped with ion trap mass spectrometer. (APCI+)
Mobile phase: 5/95 Acetonitrile/Water+0.1%Fa (A); 95/5 Acetonitrile/Water+0.1%Fa (B); 0%B to 100% B (15 min), 100% B to 0% B (0.1 min), hold 9.9 min.
Column: Zorbax SB-C18 (5 µm, 150 x 2.1 mm)
Precursor ion: 916.3; spectra range: 240-930; base product ion: 772; qualifying ions: 407, 598,

Pros/Strengths:
The analytical procedures are described fully in this document and easy to follow.

Cons/Weaknesses:
- General comments: The method has been successfully applied for the confirmation of macrolides in tissue samples of liver, kidney and muscle in beef, pork, and poultry. The minimum proficiency level of tylosin is 0.1 ppm. However, LC equipped with ion-trap mass spectrometer is not very suitable for quantification of residues in animal tissues, and give much lower repeatability and sensitivity than LC equipped with a quadrupole tandem mass spectrometer, which is popular nowadays.
- Method Clarity: validation data were not shown in this document.
- Method Scope/Applicability: applicable for muscle, liver and kidney. Egg, milk and fat samples were not involved in this method.

Method Validation Report:
- General Comment:
  - Method Optimization: the complicated extraction procedures could be optimized.
- Confirmation of identity: Two qualifying ions are used for confirming tylosin in this method.
- Performance Characteristics:
  - Analytical Range:
    100~200 µg/kg
  - Calibration:
    No data.
  - Accuracy/Recovery:
    No data.
  - Precision:
    No data.

Recommendation:
This is a confirmatory method, since the recovery, accuracy and precision data are not displayed in this paper, we do not know if this method could be work for quantification. A clean-up procedure by SPE cartridges could replace the complicated muti-liquid/liquid extraction procedures and save time and solvent consumption.

ERP Review Sheet
Evaluation of Method Tylosin-24

Title: Porcine muscle – The confirmatory and quantitative analysis of macrolides – LC/MS/MS
Submitted by: Su-Hsiang Tseng (Secondary reviewer)
Secondary Review:
Summary of Method:
Sample (2 g) was extracted with EDTA-McIlvainbuffer (20 mL) (0.1M, pH=4.0) by shaking, and centrifuged (2700g, 10 min.). Transfer 10 mL from clear upper layer to a tube. Add 275 µL formic acid (100%) and mix thoroughly, check the pH is 2.0±0.5 (if necessary adjust with formic acid). After centrifugation (15000g, 10 min.), the acidified extract (10 mL) was applied to an OASIS® MCX (60 mg/3 mL) cartridge (pre-conditioned with 3 mL MeOH, water and formic acid (0.5%), washed with 3 mL MeOH/ammonia (10/90), and eluted with 3 mL MeOH/ammonia (95/5). The eluate was evaporated to dryness at 40 °C and reconstituted with 700 µL reconstitution solution (acetonitrile/ammonium formate (0.1M, pH=4.0) (2/98, v/v).
LC/MS/MS: Quattro Micromass (ESI+)
Mobile phase: Ammonia formate (0.01M, pH=4.0) (A); ammonia formate (0.01M, pH=4.0)/MeOH (10/90, v/v) (B); 30%B hold 1 min, 30%B to 100% B (16 min), hold 1 min, 100% B to 30% B (1 min), hold 4 min.
Column: Atlantis C18 (Waters, 5 µm, 150 x 3 mm)
MRM Transitions: 946.6>174.2*; 946.6>772.6 * Underlined = quantifier

Pros/Strengths:
Instead of organic solvents, this method used EDTA-McIlvain buffer to extract tylosin. This extraction followed a clean-up on an OASIS MCX cartridge, it requires less time and solvent consumption than traditional liquid/liquid clean-up procedure.

Cons/Weaknesses:
- General comments: The analytical procedures are described fully in this document and easy to follow.
- Method Clarity: "7.4.3 Multi level standard addition (MLSA)" it stated that the detected levels lower than MRL need to be quantified using a MLSA. However, matrix effects should have compensated by both in MMS or MLSA quantification.
- Method Scope/Applicability: applicable for porcine muscle. Liver, kidney, eggs, milk and fat samples were not involved in this method.

Method Validation Report:
- General Comment:
  - Method Optimization: the lowest point for evaluating the detection limit of tylosin was 1/4 MRL (25 ppb), a lower point should be further validated to obtain the real MDL.

Confirmation of identity: this method (SOP) meets the requirement of EU regulation.
- Performance Characteristics:
  - Analytical Range:
    25~500 µg/kg
  - Calibration:
    Matrix matched standards were used in this method. The correlation coefficient of the calibration line should be at least 0.990.
  - Accuracy/Recovery:
    Muscle (spiked 50~150 µg/kg) 86~95%
  - Precision:
    Repeatability (RSD %) 5.7~13.4%

Recommendation:
This method could be modified and applicable to other kind of matrices, such as milk, liver or fat samples. The LC/MS/MS conditions could be optimized to shorten the run time (<15 min could be possible).

ERP Review Sheet

Evaluation of Method RAC-03

Title Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography tandem mass spectrometry
Submitted by: Su-Hsiang Tseng (Primary reviewer)
Secondary Review:
Summary of Method:
Quantification of ractopamine residues in liver, and urine was determined by LC/MS/MS (ESI+). Internal standard, ractopamine-d_5, was added at a concentration level of 2 ppb. Acetate buffer and β-glucoronidase/arylsulphatase were added to 2.5 g liver sample. Enzymatic hydrolysis was carried out overnight at 40℃. Subsequently, sample was centrifuged (3000 rpm, 10 min). Supernatant was adjusted to pH 8.5-9.0, and applied to the Bond-Elut Certify (6 mL, 300 mg) mixed-mode SPE cartridge (preconditioned with 2 mL of methanol and 2 mL of water). The cartridge was washed and eluted with 4 mL dichloromethane/propanol-2 (80:20) with 3% of ammonia 30%. The eluant was evaporated and dissolved in 200 μL of mobile phase. Detection limit (CCα) ranged from 0.08 to 0.15 ppb, and detection capability (CCβ) ranged from 0.27 to 0.52 ppb in liver.
LC/MS/MS conditions: (Quattro micro triple quadruple analyzer, Micromass)
Mobile phase: water, 0.1% acetic acid (A); acetonitrile/water (90:10), 0.1% acetic acid (B). A:B was 99:1 at 0 min, 55:45 at 12 min, 10:90 at 20 min and 99:1 from 20 to 35 min.
Column: Synergi MAX-RP 80 A
MRM Transitions: 302>284; 302>164 (which one is quantifier?)
Pros/Strengths:
This method is sufficient to identify ractopamine by LC/MS/MS.
Cons/Weaknesses
- General comments: Enzymatic hydrolysis was carried out overnight. However, the hydrolysis time was about 1-2 hr based on other papers.
- Method Clarity: The elution solvent of SPE clean-up was dichloromethane/ propanol-2 (80:20) with 3% of ammonia 30%, in which dichloromethane is a carcinogenic solvent.
- Method Scope/Applicability: applicable to liver. Muscle and fat samples were not involved in this method.

Method Validation Report:
- General Comment
  - Method Optimization: the method was developed and validated, including decision limit (CCα) and detection capability (CCβ), according to the Commission Decision 2002/657/EC.
- Confirmation of identity: Two transitions (one parent ion and two different product ions) were used in this method. It meets the confirmation requirement of EU regulation (2002/657/EC).
- Performance Characteristics
  - Analytical Range
    0.5~5.0 ppb
  - Calibration
    Correlation coefficient r in liver (spiked 0.5~5.0 ppb) were >0.99.
  - Accuracy/Recovery
    Liver (spiked 0.5 ppb) 82~126%
  - Precision
    Repeatability (CV%) in liver 10.4%

Recommendation:
Enzymatic hydrolysis was carried out overnight. The hydrolysis time could be shorten to 1-2 hr. Replace toxic solvents used in this method.

ERP Review Sheet Example

Evaluation of Method RAC-09

Title Residue Depletion of Ractopamine and its metabolites in swine tissue, urine, and serum

Submitted by: Su-Hsiang Tseng (Secondary reviewer)
Secondary Review:

Summary of Method:
Quantification of ractopamine residues in swine tissue, urine, and serum was determined by HPLC-FLD. The Limit of detection for tissues was 1 ng/g. Swine tissue samples (5 g) were extracted with acetone (10 mLx2) and centrifuged for 10 min at 2000 xg. After evaporation, the dry residue was dissolved in 1 mL of ammonium acetate buffer (25 mM, pH5.0), and 20 μL of β-glucuronidase was added. Then incubated at 65℃ for 2 h. Sample solutions were mixed with 2 mL of sodium borate buffer (25 mM, pH 10.3±0.1) and extracted twice with 7 mL of ethyl acetate. After centrifugation (2000 xg, 5 min), the combined supernatants were applied to the alumina A cartridge, which previously activated with 5 mL of ethyl acetate. Wash the cartridge with ethyl acetate, dry under vacuum for 2-3 min, elute ractopamine with 10 mL of methanol. The eluate was evaporated to dryness and the residue was reconstituted in 1 mL of 0.2% acetic acid for HPLC anlaysis.
HPLC conditions: (Waters LC system equipped with fluorescence detector)
Fluorescence detector: Ex 226 nm, Em 305 nm
Column: Supelcosil LC-18-DB
Mobile phase: 80% water with 20% acetonitrile with the addition of 2 mL of glacial acetic acid and 0.7 g of 1-pentanesulfonic acid per liter.
Pros/Strengths:
A HPLC-FLD method used in this study is both practical and sensitive.
Cons/Weaknesses
- General comments:
- Method Clarity: Enzymatic hydrolysis was carried out after acetone extraction. However, samples were commonly hydrolyzed prior to extraction in other published methods.
- Method Scope/Applicability: applicable for liver, kidney, muscle and fat samples.

Method Validation Report:
- General Comment
  - Method Optimization: 67℃ sounds not the optimum reaction temperature for β-glucuronidase.
- Confirmation of identity: The confirmation by HPLC-FLD alone is not sufficient.
- Performance Characteristics
  - Analytical Range
    2~50 ng/g
  - Calibration
    Standard curve was built in the concentration range of 5~1000 ng/mL, and the regression coefficient was 0.999.
  - Accuracy/Recovery
    Recoveries for liver, kidney, muscle, and fat were 78.4~85.8%, 78.3~92.2%, 79.1~89.3%, 70.5~74.7%, respectively, at spiking levels of 2~50 ng/g.
  - Precision
    Repeatability (between run precision) (CV%) 5.4~13.0%

Recommendation:
The confirmation by HPLC-FLD alone is not sufficient for routine analysis.

ERP Review Sheet
Evaluation of Method RAC-15

Title Determination of Ractopamine Hydrochloride in Swine and Turkey Tissues by Liquid Chromatography with Coulometric Detection

Submitted by: Su-Hsiang Tseng (Primary reviewer)

Summary of Method:

Ground tissue samples (10 g) were twice extracted with methanol (20 mLX2) and centrifuged for 10 min at 1500 xg. Combine the supernatant, and dilute to 50 mL with distilled water. Transfer 4 mL (samples expected to contain≧25 ppb) or 10 mL (samples expected to contain<25 ppb) aliquot to a screw-cap test tube, add 2 mL water to the test tube, and evaporate the solution, leaving ca 1.5 mL aqueous extract. Add 6 mL water to the tube and adjust pH to 10.5±0.5 by adding 250 μL 2M sodium carbonate. Add ethyl acetate (14 mL, 10 mL) to the buffered extract, hand-shake vigorously for ca 30 s. Evaporate the combined ethyl acetate layer (10 mL+10 mL) to dryness, reconstitute the reside with 5 mL acetonitrile/methanol (95:5, v/v) and clean-up with a active Bond Elut acid-washed cartridge (500 mg). Wash the cartridge with solvents as follows: acetonitrile/methanol (95:5, v/v) 6 mL, dry, acetonitrile/methanol (95:5, v/v) 5 mLX2, dichloromethane 5 mL. Eluate the ractopamine from the cartridge by 8 mL dichloromethane/ methanol/TEA (84/15/1, v/v/v). Evaporate the eluate to dryness and reconstitute the residue in 2 mL LC mobile phase.

HPLC conditions: (Varian Vista 5500 system)

Coulometric detector (electrochemical detector): +600 mv

Column: IBM C_{18}, SB-CN or IBM C_{18}

Mobile phase: 0.05 M ammonium phosphate buffer-acetonitrile (80:20, v/v).

Pros/Strengths:

Various matrices were tested in this study and a sensitive method was developed. The MDL was approximately 0.5 ppb as determined in swine liver.

Cons/Weaknesses

- General comments: the clean-up procedures were complicated, and some toxic and carcinogenic solvents (eg. dichloromethane) were used.
- Method Clarity: Should it quantify of ractopamine HCl, not drug base (ractopame)? HCl mass should be reduced while calculating standard concentration.
- Method Scope/Applicability: applicable for liver, kidney, muscle and fat samples.

Method Validation Report:

- General Comment
  - Method Optimization: the LC conditions were optimized, including columns, mobile phases and electrode potential of detector.
- Confirmation of identity: The confirmation of identity is not sufficient only by HPLC-ECD. LC/MS/MS sounds the best equipment for simultaneous quantification and confirmation of residues in samples.
- Performance Characteristics
  - Analytical Range
    0.5~50 ng/g
  - Calibration
    Standard curve was built in the concentration range of 2~300 ng/mL, and the regression coefficient was > 0.999.
  - Accuracy/Recovery
    Spike 5 ppb in swine liver, recoveries range from 75 to 100%.
  - Precision
    Repeatability (CV%) 2~18%

Recommendation:

HPLC-ECD sounds not a very popular equipment, and hardly to maintain it. According to p. 1401, sample throughput was typically 10-12 samples/day/analyst. We need a high sample throughput equipment to undertake routine analysis.

AOAC INTERNATIONAL Dietary Supplements Task Group
Report of the Expert Review Panel – Monensin/Narasin

Date: June 15, 2009

Location: Gaithersburg, MD

Attendees:

Joe Boison (Chair)	CFIA, Canada
Lindell Ward	University of Indianapolis, US
Leedert van Ginkel	RIVM-CRL, The Netherlands
Matt Rodewald	Covance, US
Valerie Reeves	USFDA, CVM, US
In Suk Kim	USDA, FSIS, US
Katerina Mastovska	USDA, ARS, US
Su-Hsiang Tseng (Emily)	BFDA, Taiwan
Erik Verdon	AFSSA, France
*Guo-Fang Pang	Qinhuangdao Entry-Exit Inspection and Quarantine Bureau, People's Republic of China
*Dr. Pang, was unable to attend, he sent his regrets and his reviews of the methods	

Observers:

Mark Coleman	ELANCO, US
Tom Burnett	ELANCO, US
Kim Lombardi	ELANCO, US

AOAC Staff:

Al Pohland	AOAC INTERNATIONAL, US
Robert Rathbone	AOAC INTERNATIONAL, US

Draft Fitness for Purpose: A fitness of purpose statement has been developed for the Determination and Confirmation of Monensin/Narasin in Animal Tissues:

An AOAC Official Method is needed for the analysis of Monensin and Narasin A as marker residues in animal tissues (major species chickens, swine and cattle), applicable to muscle, liver, kidney, fat, milk (bovine) and eggs. The analytical range should cover 25-400 ppb (ng/g); recoveries, depending on concentration, should lie in the range of 60-110%. Confirmation of analyte identity is required, either as part of the method or as a separate confirmatory method. The method will be used to facilitate international trade and satisfy regulatory requirements.

Methods Reviewed: Listed below are the methods collected by AOAC for consideration by this ERP.

File Name & No.	Author	Manuscript Title
Mon-01A	Elanco B09297-001	Determination of Monensin in edible tissues of turkeys by HPLC
Mon-01B	Ward et al.	Validation of a Method for the Determination of Narasin in the Edible Tissues of Chickens by LC
Mon-01C	Gerhardt et al.	Determination of ionophores in the tissues of food animals by LC
Mon-04 Part1 and Part 2	Elanco A061485	Non-clinical laboratory study (GLP): Validation of an HPLC/MSMS method for the assay of Monensin A in bovine muscle, liver, kidney, fat and milk
Mon-05	Blanchflower & Kennedy	Determination of monensin, salinomycin and narasin in muscle, liver and eggs from domestic fowl using LC-electrospray MS
Mon-06A	Rokka & Peltonen	Simultaneous determination of 4 coccidiostats in eggs and broiler meat: validation of an LC/MSMS Method
Mon-06B	Matabudul et al.	The simultaneous determination of ionophore antibiotics in animal tissues and eggs by tandem electrospray LC/MS/MS
Mon-06C	Matabudul et al.	The determination of 5 anticoccidial drugs (nicarbazin, lasalocid, monensin, salinomycin and narasin) in animal livers and eggs by LC linked with tandem MS (LC/MS/MS)
Mon-06D	Dubois et al.	Efficient and sensitive detection of residues of nine coccidiostats in egg and muscle by LC-electrospray tandem MS
Mon-06E	Jestoi et al	An integrated sample preparation to determine coccidiostats and emerging Fusarium-mycotoxins in various poultry tissues with LC/MS/MS
Mon-11	Hormazabal & Yndestad	Determination of amprolium, ethopabate, lasalocid, monensin, narasin and alinomycin in chicken tissues, plasma, and egg using LC/MS
Mon-12	Heller & Nochetto	Development of Multiclass Methods for Drug Residues in Eggs: Silica SPE Cleanup and LC-MS/MS Analysis of Ionophore and Macrolide Residues
Mon-13A	Cheneau et al	LC-electrospray tandem MS method for quantification of monensin in plasma and edible tissues of chicken used in pharmacokinetic studies: applying a total error approach
Mon-13B	Rosen	Efficient and sensitive screening and confirmation of residues of selected polyether ionophore antibiotics in liver and eggs by LC-electrospray tandem MS

Meeting Minutes:

The Chief Scientific Officer in collaboration with the Subject Matter Expert had chosen 14 Monensin/Narasin methods for evaluation. He opened the meeting by welcoming all the expert review panel members and expressed regrets that Dr. Guo-Fang Pang couldn't join us. The Chairman initiated the meeting with introductions and a brief review of the purpose and expected outcome of the ERP.
To ensure that we were all using the same language in our discussions, the Chair presented a short power point presentation (attached) to clarify the use/misuse of a few term including recovery, accuracy (bias), etc.,

% Recovery (Absolute) is the percentage of analyte experimentally determined to have been recovered from a matrix after due consideration has been given to the effect the presence of matrix may have on the response of the analyte.

(a) MATRIX INDUCED ESI SIGNAL SUPPRESSION EFFECT: In example 1 for Ractopamine, because the responses due to the chemical standard at different concentrations (chemical standard curve) are significantly lowered in the presence of the matrix (matrix matched curve), a clear case of MATRIX INDUCED SIGNAL SUPPRESSION EFFECT, % Recovery must be calculated by comparison of the responses from the matrix fortified sample (Tissue standard curve) to those of the matrix matched samples.

(b) MATRIX INDUCED ESI SIGNAL ENHANCEMENT EFFECT: In example 2 for Clenbuterol, because the responses due to the chemical standard are actually lower than those of the matrix matched samples, a CLEAR CASE OF MATRIX INDUCED SIGNAL ENHANCEMENT EFFECT, % Recovery must be calculated based on comparison of the responses from the matrix fortified samples to those of the matrix matched samples (NOT from the chemical standard)

(c) MATRIX INDUCED ESI SIGNAL ENHANCEMENT EFFECT: In example 3 for zilpaterol, both the responses from the matrix matched and matrix fortified samples demonstrate a SIGNIFICANTLY HIGH SIGNAL ENHANCEMENT compared to those of the chemical standards alone. Once again, % Absolute recovery must be calculated based on the comparison of the responses from the matrix fortified standards to those of the matrix matched samples.

(d) MATRIX INDUCED UV SIGNAL SUPPRESSION EFFECT: In example 4 for tilmicosin UV response in lung tissue, it is clear that the presence of lung tissue results in a significant reduction in the signal of the chemical standard and as such % Absolute recovery must, in this case, also be calculated based on comparison of the responses from the matrix fortified samples with those of the matrix matched samples (NOT CHEMICAL STANDARDS)

Obviously, of the 4 examples given 3 from ESI detector response and 1 from a UV detector, it is VERY IMPORTANT that % Recovery be properly calculated and not be based merely on a comparison of the response to the chemical standard.

As a result of its definition, the calculation of % Recovery is unaffected by the presence or otherwise of internal standards.

Once the concept of recovery was adequately defined, it was decided to distinguish recovery from "accuracy" or "bias" which is a measure of how close or otherwise the method is able to experimentally determine the amount of sample that was theoretically believed to have been added to the matrix of interest.

(Unlike % Absolute recovery, results obtained for "accuracy/bias" can be greatly improved upon by the presence or absence of suitably selected internal standards)

It was also discussed that because GC and LC are traditionally considered to be hyphenated techniques when coupled directly to mass spectrometric detectors that in cases where there is just a single stage mass spectrometer attached to the inlet system the representation will be:

GC-MS, or LC-MS.

Where there is multiple stage MS detector systems usually considered to be in tandem with each other, the designation when coupled directly to a GC or LC inlet system will be:

GC-MS/MS or LC-MS/MS.

As such all our documentation throughout this ERP will reflect these basic concepts/definitions.

Criteria for Vetting Methods to be Considered

Interest in monitoring antibiotic in animal tissues has resulted in a very large number of publications during the past two decades. Therefore, methods were included for review by the AOAC ERP on Monensin Residues based on the following criteria:

Likelihood to meet the fitness of purpose.

- Only methods for animal tissues of interest were included. Preference was given to methods for multiple tissue types.
- Methods that did not include monensin or narasin were excluded.

Literature Review of Monensin/Narasin Tissue Residue Methodology.
v 3.5 AOAC Expert Review Panel on Monensin/Narasin Tissue Methods:
14 Monensin/narasin methods had been selected by the Chief Scientific Officer in collaboration with the Subject Matter Expert for evaluation.

- Only HPLC methodology (with UV or MS detection) was considered to be sufficiently quantitative.

Other considerations

- Only broadly available technology was considered. While LC-MS/MS instrumentation does not necessarily fit this criterion, it is expected to continue to become available.
- Hazardous reagents were avoided. Carbon tetrachloride, in particular, and chlorinated solvents in general. (note that the Elanco method is included for review although it uses methylene chloride)
- Preference has generally been given to methods that use an extraction procedure that is similar to the Elanco method since the MRLs have been set with residue data generated using this method. (However, there are a number of alternatives that are presented for consideration).

Each method under consideration had been assigned to primary and secondary reviewers as follows:

Method	Primary	Secondary
Mon-01A	Leendert van Ginkle	Joe Boison
Mon-01B	Leendert van Ginkle	Joe Boison
Mon-01C	Leendert van Ginkle	Joe Boison
Mon-04 Part1 and Part 2	Lindell Ward	Valerie Reeves
Mon-05	In Suk Kim, Katerina Mastovska	Lindell Ward
Mon-06A	Matt Rodewald	Erik Verdon
Mon-06B	Matt Rodewald	Erik Verdon
Mon-06C	Matt Rodewald	Erik Verdon
Mon-06D	Matt Rodewald	Erik Verdon
Mon-06E	Matt Rodewald	Erik Verdon
Mon-11	Su-Hsiang Tseng	Review not submitted
Mon-12	Joe Boison	In Suk Kim, Katerina Mastovska
Mon-13A	Guo-Fang Pang	Su-Hsiang Tseng
Mon-13B	Guo-Fang Pang	Su-Hsiang Tseng

Lindell Ward was appointed Rapporteur to record the minutes of the meeting. After each reviewer had summarized their review of a method for Monensin/Narasin analysis, the advantages and disadvantages of each method were discussed by the entire panel. Each of the methods was evaluated for completeness (extraction, cleanup, and detection) of validation using the AOAC Acceptance Criteria for Single Laboratory Validation. Additional criteria for evaluation included appropriateness for the intended use, clarity of the method description, ruggedness, reproducibility, recovery, analytical range, and limit of quantification. The written reports of the methods by the reviewers are attached.

Comments on the Strengths and Weaknesses of Each Method – Extraction Procedure

Mon-01A	**LC-Post Column Derivatization; use of CH_2Cl_2; No validation data; 60-110% recovery; 25-100 ppb range; method straightforward; turkey muscle and liver tissues; MON only**
Mon-01B	**LC-Post Column Derivatization; use of CH_2Cl_2; No validation data; 60-110% recovery; 25-100 ppb range; method straightforward; chicken kidney, liver, muscle and skin/fat; NAR only**
Mon-01C	**LC-Post Column Derivatization; use of CH_2Cl_2; No validation data; 60-110% recovery; 25-100 ppb range; method straightforward; turkey/chicken/cattle – edible tissues; LAS, MON, NAR & SAL**
Mon-04 Part1 and Part 2	**Inappropriate use of NAR as internal std; very long, complicated extraction procedure different for each matrix that generated very clean samples; very good recovery, LOQ of 1 ppb for tissue and 0.25 ppb for milk, precision, accuracy and selectivity data.**
Mon-05	**Analytes MON, SAL, NAR from poultry muscle, liver and chicken eggs extracted into hexane/toluene; very complex mobile phase; 2 step extraction, simple sample preparation analyzed on a single quadrupole MS with LOG of 2 ppb;**
Mon-06A	**Small solvent volumes used for extraction of 4 ionophores including MON and NAR in chicken eggs and broiler meat;**
Mon-06B	**Small solvent volumes used for extraction of 4 ionophores including MON and NAR in chicken eggs and broiler chicken, sheep and calf liver;**
Mon-06C	**Same method as above applied to 5 ionophores NIC, LAS, MON, SAL and NAR in eggs, sheep and poultry livers;**
Mon-06D	**Same method as above applied to eggs and poultry muscle tissue**
Mon-06E	**Same method applied to the analysis of Fusarium mycotoxins and coccidiostats including LAS, MON, SAL, NAR, MAD residues in poultry (chicken and turkey) liver and muscle tissue**
Mon-11	**Applicable to amprolium, ethopabate, LAS, MON, NAR, SAL chicken tissues, plasma and eggs by LC-MS; uses multiple extraction solvents and CH_2Cl_2 not environmentally friendly cleanup;**
Mon-12	**Applicable to MON, NAR, LAS, SAL, ERY and TYL residues in eggs at 1 ppb and novobiocin at 3 ppb ; a strategy for method development; a screening method, not determinative; good recoveries** following FDA guidelines at only 1 concentration point; **applied to depletion study**
Mon-13A	**Method uses NAR another ionophore used under similar veterinary practice as internal standard for the analysis of MON (inappropriate); simple, but adequate extraction procedure; applicable for chicken muscle, liver and fat samples; no indication of suitability for eggs and milk.**
Mon-13B	**Applicable for NAR, SAL, MON and LAS in chicken liver and eggs;**

Merits/Demerits of Cleanup procedure

Mon-01A	**Replace methylene chloride; uses perchloric acid; silica-based SPE cleanup**
Mon-01B	
Mon-01C	
Mon-04 Part1 and Part 2	**Complicated extraction procedure changing significantly from matrix to matrix; not recommended**
Mon-05	**Should be considered with possible change of solvent to acetonitrile**
Mon-06A	**No SPE cleanup**
Mon-06B	
Mon-06C	
Mon-06D	
Mon-06E	
Mon-11	**Uses chlorinated solvents**
Mon-12	**Silica-based SPE**
Mon-13A	**C18 SPE cleanup**
Mon-13B	

Detection systems for MON & NAR

Mon-01A	**PCD - avoid** [Panel expressed concern that the technique is too dependent on the pieces of equipment one can put together and there are currently no commercially available reliable instruments that can do the job satisfactorily without significant on-site modifications and optimizations].
Mon-01B	**PCD – avoid [same reason as above]**
Mon-01C	**PCD – avoid[same reason as above]**
Mon-04 Part1 and Part 2	**LC-MS/MS (preferred)** allows for both quantification and confirmation
Mon-05	**LC-MS – could be considered** [allows for quantification and some confirmation]
Mon-06A	**LC-MS/MS – could be considered (preferred)*** allows for both quantification and confirmation
Mon-06B	**LC-MS/MS – could be considered (preferred)** allows for both quantification and confirmation
Mon-06C	**LC-MS/MS - could be considered (preferred)** allows for both quantification and confirmation
Mon-06D	**LC-MS/MS - could be considered (preferred)** allows for both quantification and confirmation
Mon-06E	**LC-MS/MS - could be considered (preferred)** allows for both quantification and confirmation
Mon-11	**LC-MS - could be considered** [allows for quantification and maybe some confirmation]
Mon-12	**LC-MS/MS - could be considered (preferred)** allows for both quantification and confirmation
Mon-13A	**LC-MS/MS - could be considered (preferred)** allows for both quantification and confirmation
Mon-13B	**LC-MS/MS - could be considered (preferred)** allows for both quantification and confirmation

PCD (post column derivatization apparatus)
*LC-MS/MS allows for both quantification and confirmation.

On the basis of the summation in the above 3 Tables the expert panel agreed unanimously, by vote, that:

(a) NO SINGLE method reviewed would meet the requirements for a suitable quantitative and confirmatory method;
(b) Methods 1, (perhaps 4), 5 & 13 would form the basis upon which a method and/or methods for extraction and cleanup would be drafted;

(c) LC-MS/MS (based on Method 13) would be the preferred method of analysis because it can provide both reliable and reproducible quantitative and confirmatory data and with more than the required sensitivity to address the requirements for both drug registration and international trade. It was argued that the PCD, the only other alternate detection system, is too prone to environmental changes and that there are currently no commercially available systems that will address all the needs under consideration including confirmation.

It was agreed to split the expert panel into 2 groups:

(a) Group 1 considered a draft method that considers non-polar extraction approaches (Method 1 and/or 4), cleanup and presenting samples in a form suitable for LC-MS/MS analysis; [In Suk, Katerina, Leen, Valerie]

Observation: No potential problems with LC-MS/MS detection; possible SPE replacement and the need to replace use of methylene chloride. May be suitable for fat and milk samples.

(b) Group 2 considered a draft method that considers polar extraction approaches as detailed in Methods 5 and 13, cleanup and presenting samples in a form suitable for LC-MS/MS. Additionally, Group 2 considered the extraction method used in Method 4 should none of the other extraction procedures considered fail to be suitable for milk extraction [Matt, Joe, Erick, Emily and Lindell].

Observation: Simple MeOH/water extraction; may have to consider CH_3CN /water extraction; C18 SPE cleanup; Can handle all the matrices for cattle and poultry liver, muscle, kidney tissue and eggs but not for fat or milk; no potential problems with LC-MS/MS detection.

Conclusions:

1. Following the reviewer presentations and group discussion, the following methods 6, 11, 12, were not recommended for further consideration based on the unanimous vote of the ERP

2. Monensin and narasin were unanimously selected for simultaneous analysis by LC-MS/MS

3. Methods 1, 4, 5, and 13 were considered for further consideration as follows and Matt Rodewald drafted the initial procedures:

(a) Polar extractions (Method 13) + SPE Cleanup + LC-MS/MS (Method 13B)
(b) Non-polar extractions (Method 1) + SPE Cleanup + LC-MS/MS (Method 13B)
(c) Non-Polar extractions (Method 4) + SPE Cleanup + LC-MS/MS (Method 13B)

Monensin/Narasin ERP-1 (15Jun09)

Draft procedure for the determination of monensin and narasin in poultry and cattle tissues by LC-MS/MS utilizing a polar extraction solvent

Matrices: Poultry liver, kidney, muscle, skin with adhering fat, eggs; cattle liver, kidney and liver

1A. Sample preparation (combination of Method MON-13A and MON-13B)

a) Weigh five grams of homogeneous sample into an appropriate sized centrifuge tube.
b) Add internal standard (ISTD) (nigericin).
c) Add 15 mL of methanol/water (87/13, v/v).
d) Vortex mix in order to homogenize the material and solvent.
e) Place the sample in an ultrasonic bath for 10 min followed by centrifugation for 10 min at 4000 x *g* at 5°C.
f) Pre-condition a Bond Elut C_{18} SPE cartridge (200 mg) with 4 mL of methanol followed by 4 mL of water.
g) Pass 5 mL of extract from step d) through the SPE cartridge.
h) Rinse the cartridge with 2 mL of methanol/water (80/20, v/v).
i) Elute the cartridge with 4 mL of methanol into a 15-mL plastic tube.
j) Evaporate the extract to dryness under N_2 at 45°C.
k) Reconstitute the sample with 300 µL of CH_3CN/50 mM NH_4OAc (80/20, v/v).
l) Transfer the sample into an LC vial for analysis by LC-MS/MS.

1B. LC-MS/MS conditions (Method MON-13B)

m) Mobile phase = CH_3CN/50 mM NH_4OAc (80/20, v/v)
n) HPLC column = Genesis C_{18}, 4 µm, 50 x 2.1 mm
o) Guard column = Genesis C_{18}, 4 µm, 10 x 2.1 mm
p) Flow rate = 0.5 mL/min
q) Injection volume = 10 µL
r) Run time = 4 min
s) LC-MS
   - Positive Electrospray mode
   - Drying gas = N_2, 670 l/h, 500°C
   - Nebulizing gas = N_2, 80 l/h
   - Collision gas = Argon, 2.3 x 10^{-3} mbar
   - Source temperature = 135°C
   - Capillary voltage = 4.0 kV
   - Extractor voltage = 5 V
   - Dwell time 0.2 s
   - SRM transition: 693>479 693>675 (MON)
     787>431, 787>265, 787>279, 787>531 (NAR)
     748>731 (NIG)

Monensin/Narasin ERP-2 (15Jun09)

Draft procedure for the determination of monensin and narasin in cattle fat and milk by LC-MS/MS utilizing a non-polar extraction solvent

2A. Sample preparation for fat tissue

2A-1 Weigh five grams of homogeneous sample into a disposable 50 mL centrifuge tube.
2A-2 Add ISTD (nigericin).
2A-3 Add 15 mL of isooctane/ethyl acetate (90/10, v/v).
2A-4 Homogenize for 15-30 s using a cell disrupter, blender or tissuemizer.
2A-5 Centrifuge for 5-10 min at 2000 - 3000 rpm.
2A-6 Decant the supernatant into a clean 50 mL centrifuge tube containing 1 g of anhydrous sodium sulfate.
2A-7 Repeat steps 2A-3- 2A-5, combining the supernatant with 2A-6.
2A-8 Shake to suspend the sodium sulfate.
2A-9 Centrifuge for 5-10 min at 2000-3000 rpm to pack the sodium sulfate.
2A-10 Place approximately 1 g of anhydrous sodium sulfate onto a silica SPE cartridge.
2A-11 Pre-condition the Waters silica Sep-Pak Plus SPE cartridge with 5 mL of iso-octane.
2A-12 Apply the sample to the cartridge.
2A-13 Rinse the cartridge with 10 mL of CH_2Cl_2. [Note: An attempt will be made to replace CH_2Cl_2 with a less toxic solvent (i.e., EtOAc/hexane, or cyclohexane/EtOAc)].
2A-14 Elute the cartridge with 6 mL of CH_2Cl_2/MeOH (90/10, v/v) [Note: An attempt will be made to replace CH_2Cl_2 with a less toxic solvent (i.e., EtOAc/hexane, or cyclohexane/EtOAc)] into a clean test tube.
2A-15 Evaporate to dryness at 40°C, removing sample from drying apparatus immediately upon reaching dryness.
2A-16 Reconstitute with CH_3CN/50 mM NH_4OAc (80/20, v/v).
2A-17 Transfer the sample into an LC vial for analysis by LC-MS/MS.

i) LC-MS/MS conditions (MON-13B)

a. Mobile phase = CH_3CN/50 mM NH_4OAc (80/20, v/v)
b. HPLC column = Genesis C_{18}, 4 µm, 50 x 2.1 mm
c. Guard column = Genesis C_{18}, 4 µm, 10 x 2.1 mm
d. Flow rate = 0.5 mL/min
e. Injection volume = 10 µL
f. Run time = 4 min
g. LC-MS
   i. Positive Electrospray mode
   ii. Drying gas = N_2, 670 l/h, 500°C
   iii. Nebulizing gas = N_2, 80 l/h
   iv. Collision gas = Argon, 2.3 x 10^{-3} mbar
   v. Source temperature = 135°C
   vi. Capillary voltage = 4.0 kV

vii. Extractor voltage = 5 V
viii. Dwell time 0.2 s
ix. SRM transition: 693>479 693>675 (MON)
787>431, 787>265, 787>279, 787>531 (NAR)
748>731 (NIG)

Monensin/Narasin ERP-3 (15Jun09)

Draft procedure for the determination of monensin and narasin in cattle milk by LC-MS/MS utilizing a non-polar extraction solvent

3A. Sample preparation for milk

3A-1 To 2.0 mL of homogenized milk, add ISTD (nigericin) and then add 2.0 mL of 0.9% NaCl solution and vortex mix for 20 s.
3A-2 Add 6 mL of EtOAc and vortex mix for 20 s.
3A-3 Mix on a reciprocating shaker for 10 min.
3A-4 Centrifuge for 10 min at 4000 x *g* at 5°C.
3A-5 Transfer EtOAc layer into a clean test tube.
3A-6 Repeat steps 3A-2 to 3A-4, combining the EtOAc layers.
3A-7 Evaporate to dryness at 40°C.
3A-8 Add 1 mL of 0.9% NaCl to the tube.
3A-9 Place in an ultrasonic bath for 1 min followed by vortex min for 30 s.
3A-10 Add 3 mL of methyl-t-butyl ether (MTBE) and vortex for 30 s.
3A-11 Mix on a reciprocating shaker for 10 min.
3A-12 Centrifuge for 10 min at 4000 x *g* at 5°C.
3A-13 Transfer the MTBE layer into a clean test tube.
3A-14 Repeat steps 3A-10 to 3A-12, combining the MTBE layers in STEP 3A-13.
3A-14 Evaporate to dryness at 40°C.
3A-15 Add 1.0 mL MeOH/H_2O (80/20, v/v) to the tube.
3A-16 Place in an ultrasonic bath for 1 min followed by vortex min for 30 s.
3A-17 Transfer into a conical ultra-centrifuge tube.
3A-18 Add 100 µL hexane to the tube, vortex 15 sec and vortex 20 s.
3A-19 Centrifuge for 5 min at 13000 x *g* at 5°C.
3A-20 Transfer the lower phase into an LC vial for analysis.

j) LC-MS/MS conditions (MON-13B)
a. Mobile phase = CH_3CN/50 mM NH_4OAc (80/20, v/v)
b. HPLC column = Genesis C_{18}, 4 µm, 50 x 2.1 mm
c. Guard column = Genesis C_{18}, 4 µm, 10 x 2.1 mm
d. Flow rate = 0.5 mL/min
e. Injection volume = 10 µL
f. Run time = 4 min
g. LC-MS
i. Positive Electrospray mode
ii. Drying gas = N_2, 670 l/h, 500°C
iii. Nebulizing gas = N_2, 80 l/h

iv. Collision gas = Argon, 2.3 x 10^{-3} mbar
v. Source temperature = 135°C
vi. Capillary voltage = 4.0 kV
vii. Extractor voltage = 5 V
viii. Dwell time 0.2 s
ix. SRM transition: 693>479 693>675 (MON)
787>431, 787>265, 787>279, 787>531 (NAR)
748>731 (NIG)

Finally, it was unanimously agreed that for the method to satisfy registration requirements and also be suitable to facilitate global trade in foods of animal origin, the low end of the analytical range for the method would need to take into consideration the fact that the lowest MRL defined for monensin is in cattle at 2 ppb by the EU and at 15 ppb for narasin in chicken kidney and liver tissues by JECFA.

Calibration range: 1 – 250 ppb for Monensin;
Calibration range: 10 – 500 ppb for Narasin.

Joe Boison
Chair, MON/NAR ERP
AOAC INTERNATIONAL
Gaithersburg, MD
June 17th, 2009
Revised June 25th, 2009

AOAC INTERNATIONAL Dietary Supplements Task Group
Report of the Expert Review Panel – Tylosin/Tilmicosin

Date: June 16, 2009

Location: Gaithersburg, MD

Attendees:

Lindell Ward (Chair)	University of Indianapolis, US
Leendert van Ginkel	RIVM-CRL, The Netherlands
Matt Rodewald	Covance, US
Valerie Reeves	USFDA, CVM, US
In Suk Kim	USDA, FSIS, US
Katerina Mastovska	USDA, ARS, US
Joe Boison	CFIA, Canada
Su-Hsiang Tseng (Emily)	BFDA, Taiwan
Eric Verdon	AFSSA, France
*Guo-Fang Pang	Qinhuangdao Entry-Exit Inspection and Quarantine Bureau, People's Republic of China

*Dr. Pang, was unable to attend, he sent his regrets and his reviews of the methods

Observers:

Mark Coleman	ELANCO, US
Tom Burnett	ELANCO, US
Kim Lombardi	ELANCO, US

AOAC Staff:

Al Pohland	AOAC INTERNATIONAL, US
Robert Rathbone	AOAC INTERNATIONAL, US

Draft Fitness for Purpose: A fitness of purpose statement has been developed for the Determination and Confirmation of Tylosin A in Animal Tissues:

An AOAC Official Method is needed for the analysis of tylosin A as a marker residue in animal tissues (major species chickens, swine and cattle), applicable to muscle, liver, kidney, fat, milk (bovine) and eggs. The analytical range should cover 25-400 ppb (ng/g); recoveries, depending on concentration, should lie in the range of 60-110%. Confirmation of analyte identity is required, either as part of the method or as a separate confirmatory method. The method will be used to facilitate international trade and satisfy regulatory requirements

Methods Reviewed: Listed below are the methods collected by AOAC for consideration by this ERP.

File Name & No.	Author	Manuscript Title
Tylosin-01	Chan et al	Determination of tylosin and tilmicosin residues in Animal tissues by RPLC
Tylosin-02	He et al	Determination of macrocyclic lactone drug residues in animal muscle by LC/tandem MS
Tylosin-10A	Horie et al.	Simultaneous determination of 5 macrolide antibiotics in meat by HPLC
Tylosin-10B	Leal et al.	Determination of macrolide antibiotics by LC
Tylosin-10C	Codony et al.	Residue analysis of macrolides in poultry muscle by LC-electrospray MS
Tylosin-13	Dubois et al.	Identification and quantification of 5 macrolide antibiotics in several tissues, eggs and milk by LC-electrospray tandem MS
Tylosin-14	Cherlet et al.	Quantitation of tylosin in swine tissues by LC combined with electrospray ionization MS
Tylosin-16	Zhang et al	Residue depletion of tilmicosin in chicken tissue
Tylosin-17	Jiang et al	Residue depletion of tilmicosin in cattle after subcutaneous administration
Tylosin-18	USDA FSIS	Confirmation of macrolide/lincosamide antibiotics by ion trap HPLC/MS/MS
Tylosin-21	De Liguoro et al	Determination of tylosin residues in pig tissues using HPLC
Tylosin-22	Elanco B05086 Rev 4	Determination of tylosin in edible tissues of swine, cattle, chicken and turkey by HPLC
Tylosin-23	Kiehl	Analysis of tilmicosin in swine liver extracts by LC/atmospheric pressure chemical ionization MS
Tylosin-24	RIKILT	Porcine muscle - the confirmatory and quantitative analysis of macrolides LC/MS/MS
Tylosin-25	Horie et al.	Determination of macrolide antibiotics in meat and fish by liquid chromatography–electrospray mass spectrometry

Meeting Minutes:

The Chairman initiated the meeting with introductions and a brief review of the purpose and expected outcome of the ERP. The Chief Scientific Officer in collaboration with the Subject Matter Expert chose 15 Tylosin methods for evaluation:

Criteria for Vetting Methods to be Considered
Interest in monitoring antibiotic in animal tissues has resulted in a very large number of publications during the past two decades. Therefore, methods were included for review by the AOAC ERP on Tylosin Residues based on the following criteria:

Likelihood to meet the fitness of purpose.

- Only methods for animal tissues of interest (cattle: liver, kidney, muscle, fat, and milk; chicken: liver, kidney, muscle, fat, and eggs; swine: liver, kidney, muscle, and fat) were included. Preference was given to methods for multiple tissue types.

Literature Review of Tylosin Tissue Residue Methodology.
Final AOAC Expert Review Panel on Tylosin Tissue Methods 2 of 14

- Methods that did not include tylosin A (or tilmicosin) were excluded. Since macrolide methods almost always include both tylosin A and tilmicosin, methods for tilmicosin were included.
- Methods specific for honey, serum, urine were not included. In some cases, methodology for milk methods were not included when the method did not include an extraction or cleanup step.

Other considerations

- Only broadly available technology was considered.
- Hazardous reagents were avoided: carbon tetrachloride, in particular, and chlorinated solvents in general.
- Preference has generally been given to methods that use an extraction procedure that is similar to the Elanco method since the MRLs have been set with residue data generated using this method. (however, there are a number of alternatives that are presented for consideration).

Each method under consideration had been assigned to primary and secondary reviewers as follows:

Method	Primary	Secondary
Tylosin-01	In Suk Kim, Katerina Mastovska	Valerie Reeves
Tylosin-02	Su-Hsiang Tseng	Matt Rodewald
Tylosin-10A	Joe Boison	Eric Verdon
Tylosin-10B	Joe Boison	Eric Verdon
Tylosin-10C	Joe Boison	Eric Verdon
Tylosin-13	Review not submitted	Su-Hsiang Tseng
Tylosin-14	Matt Rodewald	Valerie Reeves
Tylosin-16	Valerie Reeves	In Suk Kim, Katerina Mastovska
Tylosin-17	Valerie Reeves	In Suk Kim, Katerina Mastovska
Tylosin-18	Su-Hsiang Tseng	Review not submitted
Tylosin-21	Eric Verdon	Leendert van Ginkel
Tylosin-22	Leendert van Ginkel	Joe Boison
Tylosin-23	Eric Verdon	Leendert van Ginkel
Tylosin-24	Matt Rodewald	Su-Hsiang Tseng
Tylosin-25	Matt Rodewald	None

After each reviewer summarized a method for Tylosin analysis, the advantages and disadvantages of each method were discussed by the entire panel. Each of the methods was evaluated for completeness of validation using the AOAC Acceptance Criteria for Single Laboratory Validation. Additional criteria for evaluation included appropriateness for the intended use, clarity of the method description, ruggedness, reproducibility, recovery, analytical range, and limit of quantitation. The written reports of the methods by the reviewers are attached.

Comments on the Strengths and Weaknesses of Each Method

Tylosin-01	**Bovine, porcine muscle and kidney** **Extracted with acetonitrile** **C18 SPE** **HPLC-UV** **Large load on SPE; large volumes for cleanup** **74-84% recoveries** **Used matrix-matched curve** **Can be considered for MS/MS** **Low recovery in kidney**
Tylosin-02	**Multi-residue** **MS/MS** **Extracted with ACN** **C18 SPE** **Good confirmation** **Matrix-matched curve** **Sup-ppb LOQ/LOD** **70-85% recovery (too low)** **Fairly long runtime** **Fairly simple method**
Tylosin-10A	**5.0 g (10A) 2.5 g (B&C) sample**

Tylosin-10B Tylosin-10C	
Tylosin-13	**Extraction is simple, no organics (TRIS buffer)** **Muscle, egg, milk, liver** **Protein ppt by acetic acid** **HLB SPE** **LC-MS/MS** **Seems that the TRIS becomes a buffer only after tissue is added** **Roxithromycin used as an internal standard** **Source must be cleaned after 50 samples, once a day** **Needs better cleanup**
Tylosin-14	**Methanol extraction** **Needs different internal standard** **SCX SPE** **LC-MS/MS** **Very specific for Tylosin A** **30% drift in sensitivity** **50-200 ppb** **LOQ 5 ppb; LOD 0.2-1 ppb** **Can give false positives for Tylosin B** **High internal standard concentrations** **Contains 0,0 as a point in curves is undesirable**
Tylosin-16 Tylosin-17	**Extension of method 01** **Chicken and cattle** **Acetonitrile /phosphate buffer extraction** **C18 SPE** **HPLC-UV** **Quantitation by sum of cis and trans isomers of Tilmicosin** **Lower recoveries than Method 01** **No data for Tylosin** **Large SPE volumes** **Joe Boison states good recovery for Tylosion with this method.**
Tylosin-18	**Confirmation by ion-trap LC-MS/MS** **Multiple liquid extraction steps** **Complicated extraction procedure** **Multi-residue method** **Very labor intensive**
Tylosin-21	**Tylosin in pig tissue** **Phosphate buffer/methanol extraction** **SCX SPE** **LC-UV detection only** **No halogenated solvents** **Low temperature storage (-80°C)** **Single residue** **Simple method** **Extensive validation** **70-90% recovery**

	sub-MRL LOD **Not validated for skin+fat** **Good recovery and precision** **pH 2.5 to avoid losses due to enzyme activity** **Good stability data**
Tylosin-22	**Very well written method** **Methanol/ACN/ascorbic acid extraction** **C18 SPE** **HPLC-UV** **50-250 ppb** **81-85% recovery** **Good screening technique** **Use of dibutylamine phosphate is not ideal due to toxicity** **Chem standard calibration**
Tylosin-23	**Tilmicosin, not Tylosin** **Extraction by previous method (Readnour)** **Methanol/phosphate buffer extraction** **C18 SPE** **HPLC-UV and LC-apci-MS** **Fragmentation pattern for Tilmicosin** **No information for Tylosin** **50-1000 ppb** **83%-86% recovery at 10 ppm** **LOD 50 ppb** **Validated only in swine liver** **Looks like a proof of principle study**
Tylosin-24	**pH 4.0 buffer EDTA extraction** **Multiresidue method** **MCX SPE** **LC-MS/MS** **Needs matrix matched curve** **Porcine muscle only**
Tylosin-25	**Metaphosphoric acid/methanol extraction** **LC-MS** **Not at ½ MRL level of detection** **73-92% recovery** **LOD 10 ppb** **Diluted standard curve** **Not a complex method**

Conclusions:
Following the reviewer presentations and group discussion, the following methods were not recommended for further consideration based on the unanimous vote of the ERP:

A vote was taken proposing that both UV and MS to be used as determination methods, with UV being used for quantitation, and MS being used for confirmation and quantitation if possible. The vote passed 9-0.

A vote was taken to clarify the use of both UV and MS. UV being used for quantitation, and MS being used for confirmation and quantitation if possible. The vote passed 9-0.

A vote was taken for the addition of Tilmicosin to the method as well as Tylosin. The vote passed 9-0.

A vote was taken to use Methods 01, 16, and 17 as working methods for the extraction, cleanup, and UV detection. The vote passed 9-0.

The proposed method (listed below) was voted on as the final method for the detection and confirmation of Tylosin and Tilmicosin. The vote passed 9-0.

Tylosin ERP (16Jun09)

Draft procedure for the determination and/or confirmation of tylosin and tilmicosin in cattle, chicken and swine tissues by LC/UV and/or LC-MS/MS

Matrices: Cattle liver, muscle, kidney, fat and milk
Chicken liver, muscle, kidney, fat and egg
Swine liver, muscle, kidney and fat

1. Sample preparation

a) Weigh five grams of homogeneous sample into an appropriate sized centrifuge tube.
b) Add internal standard (roxithromycin) if performing LC-MS/MS analysis.
c) For fat and milk samples add 10 mL of hexane. For other matrices, proceed to step 1.g.
d) Add 10 mL of ACN and shake for 1 min.
e) Centrifuge for 10 min at 3500 rpm.
f) Discard hexane layer. Proceed to step 1.i.
g) Add 10 mL of ACN and shake for 20 min.
h) Centrifuge for 10 min at 3500 rpm.
i) Transfer supernatant into a 100-mL polypropylene centrifuge tube.
j) Add 5 mL of pH 2.5 KH_2PO_4 and 8 mL ACN to the tissue pellet.
k) Shake 20 min and centrifuge 10 min at 3500 rpm.
l) Combine supernatants and add 40 mL of water, mixing well.
m) Centrifuge at 3500 rpm for 10 min.
n) Pre-condition a Bond Elut C_{18} SPE cartridge (500 mg, 6 mL) with 5 mL of methanol followed by 5 mL of water.
o) Drain the extract through the cartridge with vacuum.
p) Wash the cartridge with 10 mL of water followed by 10 mL of ACN.

q) Dry the cartridge under vacuum for at least 3 min.
r) Elute the cartridge with 2.5 mL of 0.1 M NH_4OAc in 80:20 ACN:MeOH into a clean test tube.
s) Evaporate the extract to dryness under N_2 at 30°C.
t) Reconstitute the sample with 1 mL of mobile phase for analysis.

2. LC conditions (determinative)

a) Mobile phase = 60:30:10 0.02 M ammonium formate (pH 5.0 w/ formic acid):ACN:MeOH
b) HPLC column = Inertsil C_{18}, 3 µm, 100 x 2.1 mm
c) Column thermostat = 30°C
d) Flow rate = 0.5 mL/min
e) Injection volume = 20 µL
f) UV detection = 287 nm

3. LC-MS/MS conditions (determinative/confirmatory)

a) mobile phase = 60:30:10 0.02 M ammonium formate (pH 5.0 w/ TFA):ACN:MeOH
b) HPLC column = Inertsil C_{18}, 3 µm, 100 x 2.1 mm
c) Column thermostat = 30°C
d) Flow rate = 0.5 mL/min
e) Injection volume = 20 µL
f) LC-MS/MS
- Ionization mode: ESI-MS, positive ion mode $[M+H]^+$
- Source temperature: 150°C
- Spray flux:150 µL/min
- Cone voltage: varied from 30 to 50 V
- Drying gas flow: N_2, flow rate ~ 300 L/h
- Nebulizing gas: N_2, flow rate ~ 15 L/h

Compound	Precursor Ion (Da)	Product ion for detection and quantification	Additional ions for confirmation (da/e)	Cone voltage (V)	Collision energy (eV)
Tylosin	916.3	772.2	318.4, 407.4, 598.4	40	33
Tilmicosin	869.6	696.6	522.2, 678.9, 505.7	50	40
Roxithromycin	837.4	158.1	None	45	45

Note: Mobile phase will be diverted away from the LC-MS/MS source when not within the elution band of the compounds of interest.

AOAC INTERNATIONAL Dietary Supplements Task Group
Report of the Expert Review Panel – Ractopamine

Date: June 17, 2009

Location: Gaithersburg, MD

Attendees:

Leendert van Ginkel (Chair)	RIVM-CRL, The Netherlands
Matt Rodewald	Covance, US
Valerie Reeves	USFDA, CVM, US
In Suk Kim	USDA, FSIS, US
Katerina Mastovska	USDA, ARS, US
Joe Boison	CFIA, Canada
Lindell Ward	University of Indianapolis, US
Su-Hsiang Tseng (Emily)	BFDA, Taiwan
Weilin Shelver	USDA, ARS, US
*Guo-Fang Pang *Dr. Pang, was unable to attend, he sent his regrets and his reviews of the methods	Qinhuangdao Entry-Exit Inspection and Quarantine Bureau, People's Republic of China

Observers:

Marc Coleman	ELANCO, US
Tom Burnett	ELANCO, US
Kim Lombardi	ELANCO, US (Scribe)

AOAC Staff:

Al Pohland	AOAC INTERNATIONAL, US
Robert Rathbone	AOAC INTERNATIONAL, US

Fitness for Purpose: A fitness of purpose statement has been developed for the Determination and Confirmation of Ractopamine in Animal Tissues:

An AOAC Official Method is needed for the analysis of ractopamine in animal tissues (swine and cattle), applicable to muscle, liver, kidney and fat. The method should include an option to determine free (parent) ractopamine or total ractopamine (parent plus conjugates) following an enzymatic hydrolysis. The analytical range should cover 1-100 ppb (ng/g) to comply with the EU Reference Point for Action (RPA) of 1 ppb and the lowest MRL values applicable in those countries in which the use of ractopamine is approved of 10 ppb; recoveries, depending on concentration, should lie in the range of 60-110%. Confirmation of analyte identity is required, either as part of the method or as a separate confirmatory method. The method will be used to facilitate international trade and satisfy regulatory requirements.

The outcome of the meeting is not necessarily a single method, two or, if necessary more, individual methods can be selected.

Methods Reviewed: Listed below are the methods collected by AOAC for consideration by this ERP.

File Name & No.	Author	Manuscript Title
RAC-01	Elliott et al.	Screening and confirmatory determination of ractopamine residues in calves treated with growth promoting doses of the β-agonist
RAC-02	Antignac et al.	Identification of ractopamine residues in tissue and urine samples at ultra-trace level using liquid chromatography–positive electrospray tandem mass spectrometry
RAC-03	Blanca et al.	Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography tandem mass spectrometry
RAC-04A	Churchwell et al.	Liquid chormatography/electrospray tandem mas spectrometric analysis of ractopamine residues in livestock tissues
RAC-04B	Doerge et al.	Detection and Confirmation of β-Agonists in Bovine Retina Using LC/APCI-MS
RAC-06A	ELANCO B03903	Determination of Ractopamine Hydrochloride In Turkey Liver and Muscle Tissue by High Performance Liquid Chromatography
RAC-06B Modified	ELANCO B03903 Modified	Determination of Ractopamine Hydrochloride In Chicken Liver by High Performance Liquid Chromatography
RAC-06C	Elanco B06738	Confirmation of Ractopamine Residues In Turkey Liver and Muscle Tissue by Liquid Chromatography/Electrospray Ionization Triple Tandem Quadrupole Mass Spectrometry (Lc/Esi-Ms-Ms)
RAC-08	Fesser et al.	Determination of β-Agonists in Liver and Retina by LiquidChromatography-TandemMass Spectrometry
RAC-09	Qiang et al.	Residue Depletion of Ractopamine and Its Metabolites in Swine Tissues, Urine, and Serum
RAC-10	Shelver & Smith	Tissue Residues and Urinary Excretion of Zilpaterol in Sheep Treated for 10 Days with Dietary Zilpaterol
RAC-11	Shishani et al.	Determination of ractopamine in animal tissues by liquid chromatography-fluorescence and liquid chromatography/tandem mass spectrometry
RAC-14	Sakai et al.	Determination Method for Ractopamine in Swine and Cattle Tissues Using LC/MS
RAC-15	Turberg et al.	Determination of Ractopamine Hydrochloride in Swine, Cattle, and Turkey Feeds by Liquid Chromatography with Coulometric Detection
RAC-17	Williams et al.	Multiresidue confirmation of β-agonists in bovine retina and liver using LC-ES/MS/MS

Meeting Minutes:

The Chairman initiated the meeting with introductions and a brief review of the purpose and expected outcome of the ERP. The Chief Scientific Officer in collaboration with the Subject Matter Expert chose 15 Ractopamine methods for evaluation.

Criteria for Vetting Methods to be Considered
Interest in monitoring β-agonists in animal tissues has resulted in a multitude of publications over the past two decades. Methods were included for review by the AOAC ERP on Ractopamine Residues base on the following criteria:

Likelihood to meet the fitness of purpose

- Only methods for animal tissues were included, with preference given towards ractopamine methods.

Other considerations

- Only broadly available technology was considered. While LC-MS/MS instrumentation does not necessarily fit this criterion, it is expected to continue to become available.
- Hazardous reagents were avoided.

Each method under consideration had been assigned to primary and secondary reviewers as follows:

Method	Primary	Secondary
RAC-01	Valerie Reeves	In Suk Kim, Katerina Mastovska
RAC-02	Weilin Shelver	Review not Submitted
RAC-03	Su-Hsiang Tseng (Emily)	Review not Submitted
RAC-04A	Matt Rodewald	Joe Boison
RAC-04B	Matt Rodewald	Joe Boison
RAC-06A	Joe Boison	Lindell Ward
RAC-06B Modified	Joe Boison	Lindell Ward
RAC-06C	Joe Boison	Lindell Ward
RAC-08	Weilin Shelver	Valerie Reeves
RAC-09	Review not Submitted	Su-Hsiang Tseng (Emily)
RAC-10	In Suk Kim, Katerina Mastovska	Matt Rodewald
RAC-11	Lindell Ward	Matt Rodewald
RAC-14	Matt Rodewald	Valerie Reeves
RAC-15	Su-Hsiang Tseng (Emily)	Weilin Shelver
RAC-17	Valerie Reeves	In Suk Kim, Katerina Mastovska

Methods:

Rac-01: Screening and confirmatory determination of ractopamine residues in calves treated with growth promoting doses of the β-agonist
Authors: Christopher T. Elliott, Colin S. Thompson, Cor. J. M. Arts, Steven R. H. Crooks, Martin J. van Baak, Elwin R. Verheij, G. Andew Baxter
Review:

Multiple or Single Residue	Single
Use of Internal Standard	Clenbuterol-d6
Hydrolysis	Yes
Species (swine, cattle)	Cattle
Tissue Types	Urine
Sample Preparation	Sample incubated with β-glucuronidase , applied to mixed mode SPE, Eluted with NH_4Cl in EtOAC, evaporated and reconstituted in phosphate buffer, applied to C_{18} SPE, eluted with MeOH, dried, dissolved in mobile phase.
Analytical Range	2-250 ppb
Recovery	Precision 5.6% to 3.7%
Confirmation of Identity	Yes, LC-MS/MS, APCI, ELISA
Additional Comments	Standard curve fortified urine samples. Consistent correlation between ELISA and glucaronidated and free Ractopamine. Complex method that uses two SPEs. (If method for liver, easily adaptable to urine). Would not be suitable matrix for N. America because of live animal control.

Rac-02: Identification of ractopamine residues in tissue and urine samples at ultra-trace level using liquid chromatography-positive electrospray tandem mass spectrometry
Authors: Jean-Philippe Antignac, Philippe Marchand, Bruno Le Bizec, Francois Andre
Review:

Multiple or Single Residue	Single
Use of Internal Standard	Yes-isoxsuprine
Hydrolysis	Yes 15 hours using Helix Pomatia 15 hrs
Species	Swine
Tissue Types	Muscle , liver, kidney. In addition urine.
Sample Preparation	Freeze dried, the extracted with MeOH/acetate buffer. Hydrolysis 15 hour incubation. Applied to ChromP SPE, eluted with diethyl ether, then applied to Screen DAU SPE. Evaporated and reconstituted in 0.5% acetic acid.
Analytical Range	Fortified samples. 0- 500 mg/kg
Recovery	Recovery 18%, no precision and accuracy data.
Confirmation of Identity	LC-MS/MS ESI,
Additional Comments	Cumbersome tissue method prep. Used incurred residues.

Rac-03: Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography tandem mass spectrometry
Authors: José Blanca, Patricia Munoz, Miguel Morgado, Nely Méndex, Angel Aranda, Thea Reuvers, Henny Hooghuis
Review:

Multiple or Single Residue	Multiple-clenbuterol, ractopamine, zilpaterol
Use of Internal Standard	Ractopamine d-5
Hydrolysis	Yes-carried out overnight
Species	Cattle and swine
Tissue Types	Liver and urine
Sample Preparation	Acetate buffer and β-glucuronidase/arylsufatase hydrolysis, ph adjusted to 8.5-9, Bond ElutSingle SPE(mix mode), eluted with 3% NH_3 dichloromethane/propanol, evaporated, reconstituted in mobile phase.
Analytical Range	0.5-5.0 ppb
Recovery	82-126%, CV% 10.4%
Confirmation of Identity	LC-MS/MS-electrospray, yes
Additional Comments	Validated according to EU requirements. Long (overnight) hydrolysis.

Rac-04A: Liquid chromatography/electrospray tandem mass spectrometric analysis of incurred ractopamine residues in livestock tissues
Authors: Mona I. Churchwell, C. Lee Holder, David Little, Steve Preece, David J. Smith, Daniel R. Doerge
Rac-04B: Detection and confirmation of β-agonist in bovine retina using LC/APCI-MS
Authors: Daniel R. Doerge, Mona I. Churchwell, Lee Holder, Loyd Rowe, Steve Bajic
Review:

Multiple or Single Residue	Ractopamine, clenbuterol, salbutamol, terbutaline
Use of Internal Standard	None
Hydrolysis	Yes-helix pomatia
Species	Bovine
Tissue Types	Liver and retina
Sample Preparation	Samples homogenize with HCl (hydrolysis here for liver samples), pH adjusted to 7.0, C18 SPE, eluted with MeOH, evaporated reconstituted in mobile phase.
Analytical Range	None given
Recovery	Liver 55-62%, 28-32% retina
Confirmation of Identity	Confirmatory, but not quantitative LC-MS/MS method A ESI, method B APCI
Additional Comments	Some work done at lower levels, method B does not include Ractopamine.

Rac-06A: Determination of ractopamine hydrochloride in turkey liver and muscle tissue by high performance liquid chromatography (B03903)
Authors: Elanco Animal Health
Rac-06B: Determination of ractopamine hydrochloride in chicken liver by high performance liquid chromatography (B03903)
Authors: Elanco Animal Health
Rac-06C: Confirmation of ractopamine residues in turkey liver and muscle tissue by liquid chromatography/electrospray ionization triple tandem quadruple mass spectrometry (LC/ESI-MS-MS)
Authors: Elanco Animal Health
Review:

Multiple or Single Residue	Single
Use of Internal Standard	None
Hydrolysis	No hydrolysis
Species	Turkey, chicken
Tissue Types	Muscle, liver, kidney fat
Sample Preparation	Extracted with methanol (method A: three times; B, two times; C uses A), adjust volume to 90 mls, remove 3 mls and evaporate, reconstitue NaCO3 buffer, add MTBE, perform liquid-liquid extraction, apply MTBE layer (x2)onto Alumina SPE, elute with TFA in MeOH. For Method B: 90 mL extract, take 2 mL directly onto mixed mode SPE, elute with MeOH.
Analytical Range	20-200 ppb muscle, 225-900 ppb liver No range for method C.
Recovery	None
Confirmation of Identity	Methods A and B: HPLC Fluorescence. No confirmation. Method C-uses LC-MS/MS ESI for confirmation only.
Additional Comments	Only 3 mls of extraction solvent used. Method B uses 5 gram tissue. ADI is set as the salt, measured off Ractopamine HCl. Method C well written, can be reproduced by others. Additional work has been done to flourescence method down to 5 ppb, but still needs to be validated. Sample carry over in method B, no carry over with method A.

Rac-08: Determination of β-agonists in liver and retina by liquid chromatography-tandem mass spectrometry
Authors: Adrian C.E. Fesser, Leslie C. Dickson, James D. MacNeil, John R. Patterson, Stephen Lee, Ronald Gedir
Review:

Multiple or Single Residue	Multiple (12 beta-agonists)
Use of Internal Standard	Surrogate, not internal standard, should not be used for quantitation, not added at the beginning
Hydrolysis	Overnight with XIV protease streptomyces griseus
Species	Cattle
Tissue Types	Liver, retina
Sample Preparation	Homogenized and incubated overnight, acidified and extracted with methylene chloride/hexane, pH adjusted to basic. Extracted with t-butyl ether. Evaporated, dissolved in water and applied to HLB SPE, eluted with acidified MeOH, evaporated and reconstituted in ammonium formate buffer/ACN..
Analytical Range	0.5-2 µg/kg liver, 5-20 µg/kg in retina
Recovery	Liver 92-118%, retina 96-117%, variable for molecules other than Ractopamine.
Confirmation of Identity	LC-MS/MS APCI
Additional Comments	Variable recovery range is weakness. Use 2-3 product ions for confirmation. Ion ratios greater than 30%. Needed 2 injections to accomplish, most likely due to old instrumentation. Different enzyme, will not get protein bound.

Rac-09: Residue depletion of ractopamine and its metabolites in swine tissues, urine, and serum
Authors: Zhiyi Qiang, Fenqin Shentu, Bing Wang, Jiangping Wang
Review:

Multiple or Single Residue	Single
Use of Internal Standard	None
Hydrolysis	Yes, B-glucuronidase 2 hr incubation
Species	Swine
Tissue Types	Urine, serum, kidney, muscle, liver, fat
Sample Preparation	Extracted with acetone, incubated, extracted with ethyl acetate 2 times. Alumina A cartridge, elute with methanol.
Analytical Range	2-50 ng/g
Recovery	70-92.29% for all tissues, CV% 5.4-13.0%
Confirmation of Identity	HPLC-FLD, no confirmatory
Additional Comments	Silylated glassware when using borate buffer may be needed. Similar scheme to method 6A-C.

Rac-10: Tissue residues and urinary excretion of zilpaterol in sheep treated for 10 days with dietary zilpaterol
Authors: Weilin L. Shelver, David J. Smith
Review:

Multiple or Single Residue	Zilpaterol
Use of Internal Standard	Cimeterol d7
Hydrolysis	None
Species	Sheep
Tissue Types	Urine, liver, kidney, muscle
Sample Preparation	FLD: 5 g extract with sodium acetate buffer, applied to C18 SPE, eluted with di-ethylamine, evaporated and reconstituted in HCl. LC-MS/MS: Extracted with borate buffer, applied to mixed mode SPE, eluted with methylene chloride/IPA, NH_4OH,evaporated, reconstituted in 10 mM ammonium acetate.
Analytical Range	0.25 – 200 ppb
Recovery	88% in urine FLD, 60% in urine in LC-MS/MS
Confirmation of Identity	LC-MS/MS confirmatory with several ions, UV-FLD, ELISA
Additional Comments	Mixed mode C-18 cation exchange SPE. Used for checking incurred urine samples. No validation for tissues.

Rac-11: Determination of ractopamine in animal tissues by liquid chromatography-fluorescence and liquid chromatography/tandem mass spectrometry
Authors: Eshaq (Isaac) Shishani, Sin Chii Chai, Sami Jamokha, Gene Aznar, Michael K. Hoffman
Review:

Multiple or Single Residue	Single Ractopamine
Use of Internal Standard	Ritodrine
Hydrolysis	Glucuronidase, 2 hrs
Species (swine, cattle)	Swine, cattle
Tissue Types	Muscle
Sample Preparation	Extracted with methanol, incubated, added borate buffer and extracted with ethyl acetate, applied to Alumina SPE, eluted with MeOH, evaporated, reconstituted in 1 M glacial acetic acid, applied to MCX SPE, eluted with NH_4OH in MeOH, evaporated, reconstituted in MeOH or GAA.
Analytical Range	1-4 ppm
Recovery	80-117% swine, 85-114% cattle, precision
Confirmation of Identity	FLD and LC-MS/MS,, confirmatory
Additional Comments	Internal standard could be improved. Overall positive review.

Rac-14: Determination method for ractopamine in swine and cattle tissue using LC/MS
Authors: Takatoshi Sakai, Tomomi Hitomi, Kyoko Sugaya, Shigemi Kai, Mitsunori Murayama, Tamio Maitani
Review:

Multiple or Single Residue	Single
Use of Internal Standard	None
Hydrolysis	None
Species	Swine, cattle
Tissue Types	Muscle, liver and fat
Sample Preparation	Extract with ethyl acetate in presence of K2CO3, evaporated and reconstituted in ACN, extracted with hexane. ACN layer evaporated and reconstituted in MeOH.
Analytical Range	1 ppb LOQ
Recovery	97-109%, precision 6.6-9.5%
Confirmation of Identity	LC/MS, not confirmatory
Additional Comments	Simple

Rac-15: Determination of ractopamine hydrochloride in swine and turkey tissues by liquid chromatography with coulometric detection
Authors: Michael P. Turberg, Thomas D. Macy, Jerry J. Lewis, Mark R. Coleman
Review:

Multiple or Single Residue	Single
Use of Internal Standard	None
Hydrolysis	None
Species	Swine and turkey
Tissue Types	SPE
Sample Preparation	Extracted with MeOH, diluted with water, evaporate aliquot, add sodium carbonate, and extract with ethyl acetate, evaporate, reconstitute in ACN/MeOH and apply to SPE on Bond Elut, elute with dichloromethane/MeOH/TEA, evaporate, reconstitute in mobile phase.
Analytical Range	0.5-50 ng/g
Recovery	75-100%, CV 2-18%
Confirmation of Identity	None. Coulometric detection
Additional Comments	Complicated clean-up. Detection hard to maintain.

Rac-17: Multi-residue confirmation of β-agonist in bovine retina and liver using LC-ES/MS/MS
Authors: Lee D. Williams, Mona I. Churchwell, Daniel R. Doerge
Review:

Multiple or Single Residue	Salbutamol, zilpaterol, terbutalin, cimaterol, fenoterol, 13C clenbuterol, clenbuterol, Ractopamine, brombuterol, mabuterol
Use of Internal Standard	13C clenbuterol
Hydrolysis	Glucuronidase and sulfatase
Species (swine, cattle)	Cattle
Tissue Types	Retina and liver
Sample Preparation	0.2 mg tissue with sodum citrate buffer Incubated, applied to mixed mode SPE HCX 96 well, eluted with NH_4OH in MeOH, evaporated and reconstituted in Formic Acid in MeOH.
Analytical Range	0.25-0.8 ppb
Recovery	Reported 101%, precision <6.4%
Confirmation of Identity	LC-MS/MS ESI, confirmation 2 or more ions.
Additional Comments	Not enough information to make determination for quantitation, mainly screening method. Only 200 mg sample, very small. IS concentration large compared to internal standard.

Discussion around the room regarding pros and cons of using Elisa method, coulometric and flourescence detection.

Vote: ELISA methods do not comply with the objectives of the ERP because of low selectivity and sensitivity.

Yes: 9 No: 0 Abstain: 0

Vote: Coulometric methods do not comply with the objectives of the ERP because the technology is not rugged and general available.

Yes: 9 No: 0 Abstain: 0

Vote: LC-FLD is a good technique for quantitative analysis because it has sufficient sensitivity and has shown to comply with the objectives of the ERP.

Yes: 9 No: 0 Abstain: 0

Determination of Methods for Consideration for further evaluation:

ERP went around the room and discussed criteria for consideration in keeping a method for further evaluation. These criteria included:

- Ability to add/keep the deconjugation step
- Species and matrix applicability
- Applicability to LC-FLD and LC-MS/MS
- Complexity of Extraction
- LOQ
- Recovery/Accuracy/Precision

Methods to be flagged based on criteria 1 for further evaluation:

Green Flags: Methods 3, 11, 17
Yellow Flags: Methods 2 (interesting sample clean-up), 6A, 6B, 6C, 9
Red Flags: Methods 1, 4A, 4B, 8, 10, 14, 15

Review of Dr. Pang's review.
Uses a single SPE clean-up.

Question: When is the optimum time to do the conjugation? Assumption is made that conjugates extract the same way as the parent. There may be a difference in the solubility if conjugation is done on the homogenate vs some other point in the method. CVM, Canada is not interested in the deconjugation step because only marker residue is wanted (i.e. only in countries where there is an approval. EU and other countries where the molecule is not approved, the deconjugation step is needed to quantify total ractopamine, not only the marker residue.

Determining a final method:

Two groups to further discuss methods: LC-MS/MS and LC-FLD
Groups discussed for 45 minutes, then returned back to the larger group for discussion.

There was gravitation by both groups to method 11 for Fluorescence. Concern is with the length of the procedure and the two SPE clean-up steps. The thought process is to separate method 11 into two different methods, 1 using the Alumina A SPE cartridge for FLD detection, and one using the mixed mode, and skipping the Alumina A SPE cartridge for LC-MS/MS. Also wanting to remove the borate buffer and replace with sodium carbonate buffer. Also change the liquid-liquid extraction before the Alumina A cartridge SPE to MTBE instead of Ethyl Acetate. Below is an outline of described methods to be used/evaluated based on method 11:

Modified FLD method for parent only (modified from method 11):

1) 10 g sample
2) IS-ritrodine
3) 20 mL MeOH (x3)
4) 8 mL aliquot and dry down

5) 2 mL 20 mM Na_2CO_3 (pH 10.5)
6) Liquid-liquid 7 mL (x3) with MTBE
7) Alumina A SPE
8) Elute with 10 mL MeOH
9) Dry
10) 4 mL 1 M HOAc
11) MCX Oasis SPE
12) Elute with 4 mL 2% NH_3 in MeOH
13) Dry
14) Recon in 0.5 mL 2% HOAc

Modified LC-MS/MS method for parent only (modified from method 11):

1) 10 g sample
2) IS-Ractopamine d5
3) 20 mL MeOH (x3)
4) 8 mL aliquot and dry down
5) 25 mM NaOAc buffer (pH 5)
6) MCX Oasis SPE
7) Elute with 4 mL 2% NH_3 in MeOH
8) Dry
9) Recon in 0.5 mL 2% HOAc

Modified LC-MS/MS method for total only (modified from method 11):

1) 10 g sample
2) IS-Ractopamine d5
3) 20 mL MeOH (x3)
4) 8 mL aliquot and dry down
5) 25 mM NaOAc buffer (pH 5)
6) B-Glucuronidase 2 hr @ 65°C
7) 2 mL 20 mM Na_2CO_3 (pH 10.5)
8) MCX Oasis SPE
9) Elute with 4 mL 2% NH_3 in MeOH
10) Dry
11) Reconstitute in 0.5 mL 2% HOAc

Vote: Method 11 forms the basis for a 2x2 method for ractopamine, either parent only or total, using FLD or LC-MS/MS.

Yes: 9 No: 0 Abstain: 0

Notes:

- For deconjugation step, would like two options for incubation, one accelerated at an elevated temperature, and one for an overnight incubation. **Note**: reference Rectopamine-01 for information regarding incubation and deconjugation.
- Want to use both glucuronidase and sulfatase (Helix has both).
- Internal Standard, ritrodine can be used for Fluorescence, but for LC-MS/MS needs to be deuterated ractopamine.

Validation Guidance:

- Limit of quantification for banned substance determined by LOC:
  Limit of confirmation (LOC): LOC, 1.5X LOC, and 2.0 x LOC
- Limit of quantification for approved substances:
  MRL is used: 0.5 x MRL, MRL, 1.5 MRL (everywhere else), 2.0 MRL (US)

After having concluded the discussion on the selected analytical method and verifying neither the members of the ERP nor the observers had any further comments or remarks. The chair thanked all for their contributions to the outcome of this meeting and closed the meeting

行政院衛生署藥物食品檢驗局

出國人員出國經驗分享

參加「國際AOAC動物用藥檢驗方法專家評審會議」心得分享

曾素香 副研究員

第四組

2009/07/29

過程

- 此次國際AOAC舉辦之專家評審會議(Expert Review Panel, ERP)，經費出自美國ELANCO公司。目的為選出合適的動物用藥檢驗方法進行後續確效實驗，以作為AOAC公定方法。
- 會議日期及主題

  6月15日：孟寧素(Monensin)/那寧素(Narasin)

  6月16日：泰黴素(Tylosin)

  6月17日：培林(Ractopamine)

2

April 28, 2009
Su-Hsiang Tseng, Ph.D
Associate Researcher
161-2, Kunyang St.,
Nangang, Taipei, Taiwan 11513, R.O.C

AOAC INTERNATIONAL
The Scientific Association Dedicated to Analytical Excellence®

Dear Dr. Tseng:

On behalf of AOAC INTERNATIONAL, we would like to cordially invite you to attend the AOAC Expert Review Panel Panel on Ractopamine, Tylosin, and Monensin/Narasin meeting.

This meeting will take place June 15-17, 2009 at the AOAC INTERNATIONAL Headquarters in Gaithersburg, Maryland, USA.

As a panel member, you will provide expert scientific and technical information to the group and , help the group reach consensus on a candidate method for further validation.

You will be part of a large contingency of scientific experts from US FDA, USDA, CFIA,k RIVM, and AFSSA.

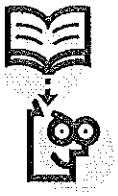
If you need additional information pertaining to this meeting, please do not hesitate to contact me at +1-301-924-7077 extension 123 – apohland@aoac.org or Jennifer Diatz at +1-301-924-7077 extension 107 – jdiatz@aoac.org.

Sincerely,

Dr. Al Pohland
Senior Director,
Chief Scientific Officer
AOAC INTERNATIONAL

May 8, 2009

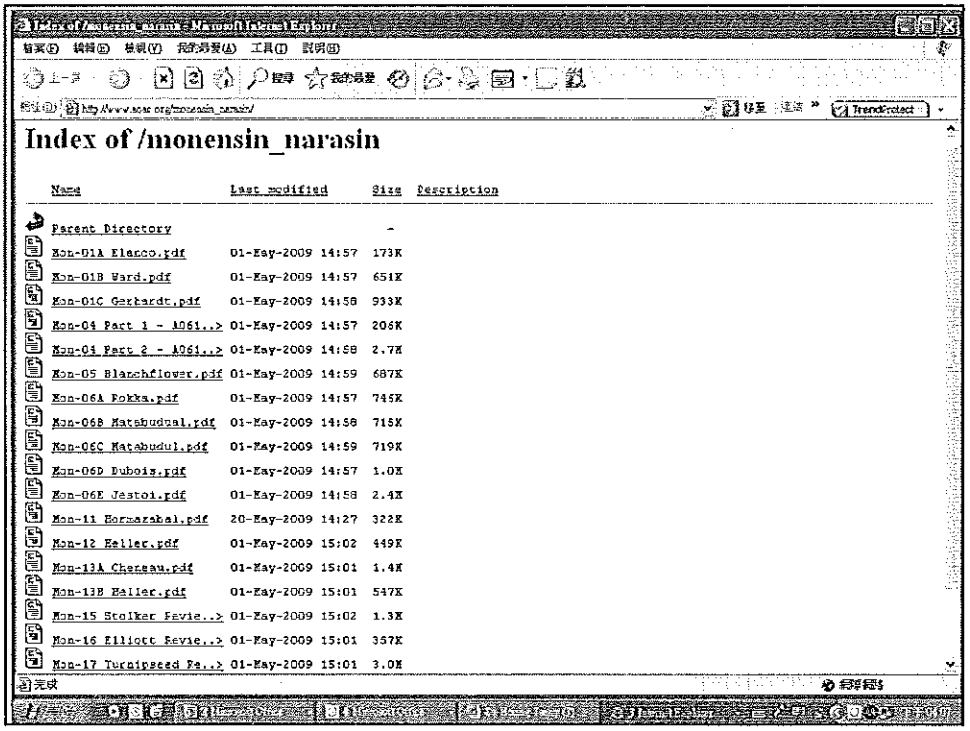
Dear Panel Member,
Below are the method assignments for the Animal Drugs Expert Review Panel, at AOAC headquarters in Gaithersburg, MD. The meeting will begin at 9:00 a.m. I am attaching a review sheet template that you should fill out for each method that you've been assigned. Reviews should be returned no later than June 8, 2009. Reviews will then be compiled, and distributed to the entire panel.



ERP Review Sheet Example
Evaluation of Method X

Title
Submitted by:
Summary of Method:
Pros/Strengths:
Cons/Weaknesses
•General comments
•Method Clarity
•Method Scope/Applicability
Method Validation Report:
•General Comment
--Method Optimization
•Confirmation of identity
•Performance Characteristics
--Analytical Range
--Calibration
--Accuracy/Recovery
--Precision
Recommendation:

Su-Hsiang Tseng (Emily)		
Monensin/Narasin June 15, 2009, 9:00 a.m.	Primary reviewer: Mon-11 Secondary reviewer: Mon-13A&B IMPORTANT NOTE: Mon-13A references the Mon-13B as the source of the method this paper may be reviewed at the reviewer's discretion.	Some additional references (Mon-15, Mon-16 & Mon-17) are provided for general background. The reviews by Elliott and by Stolker provide a general overview of analytical technology. The paper by Turnipseed et al. provides details and insights for ionization and fragmentation of ionophores in mass spectrometry.
Tylosin June 16, 2009, 9:00 a.m.	Primary reviewer: Tylosin-02, Tylosin-18 Secondary reviewer: Tylosin-24, Tylosin-13	A number of reviews (Tylosin-11A, B & C) have been published in the last decade concerning methodology for the analysis of tissues and foods for antibiotics. While there is usually little detail regarding methods, these provide some insight into the higher level needs and uses of available analytical technology.
Ractopamine June 17, 2009, 9:00 a.m.	Primary reviewer: RAC-15, RAC-03 Secondary reviewer: RAC-09	These two reviews Rac-18 & Rac-19 may be useful as a reference to all panel members.



Index of /monensin_narasin

Name	Last modified	Size	Description
Parent Directory		-	
Mon-01A Elanco.pdf	01-May-2009 14:57	173K	
Mon-01B Ward.pdf	01-May-2009 14:57	651K	
Mon-01C Gerhardt.pdf	01-May-2009 14:58	933K	
Mon-04 Part 1 - A061..>	01-May-2009 14:57	206K	
Mon-04 Part 2 - A061..>	01-May-2009 14:58	2.7M	
Mon-05 Blanchflower.pdf	01-May-2009 14:59	687K	
Mon-06A Pokka.pdf	01-May-2009 14:57	745K	
Mon-06B Matsbudpal.pdf	01-May-2009 14:58	715K	
Mon-06C Matsbudul.pdf	01-May-2009 14:59	719K	
Mon-06D Dubois.pdf	01-May-2009 14:57	1.0M	
Mon-06E Jestoi.pdf	01-May-2009 14:58	2.4M	
Mon-11 Bormarabal.pdf	20-May-2009 14:27	322K	
Mon-12 Keller.pdf	01-May-2009 15:02	449K	
Mon-13A Cheneau.pdf	01-May-2009 15:01	1.4M	
Mon-13B Keller.pdf	01-May-2009 15:01	547K	
Mon-15 Stolker Revie..>	01-May-2009 15:02	1.3M	
Mon-16 Elliott Revie..>	01-May-2009 15:01	357K	
Mon-17 Turnipseed Re..>	01-May-2009 15:01	3.0M	

Agenda Expert Review Panel
(Monesin/Narasin, Tylosin and Ractopamine)
June 15-17, 2009
AOAC INTERNATIONAL, Gaithersburg, MD 9:00 a.m.

1. Welcome and Hosekeeping (Pohland)
   a. Policies and Procedures of the Expert Review Panel
   b. Conflict of Interest Policy and Volunteer Acceptance Form
   c. SLV Acceptance Criteria
2. Introductiion of Panelists: (Chair)
3. Discussion of Methods
   a. Receipts of Primary and Secondary Reviewers' Reports
   b. Completion of Method Selection Worksheets
4. Selection of Top Method
5. Discussion of Materials for Use in SLV Study
6. Preparation of Chairman's Summary Report
7. Adjournment

Attendees

- 美國食品藥物管理局(USFDA) 1人：Dr. Valerie Reeves
- 美國農業部(USDA) 3人：Dr. In Suk Kim (FSIS); Dr. Katerina Mastovska (ARS); Dr. weillin Shelver (ARS)
- 美國Covance 公司1人：Dr. Matt Rodewald
- 美國University of Indianalpolis 1人：Dr. Lindell Ward
- 加拿大食品檢驗局(CFIA) 1人：Dr. Joe Boson
- 法國食品安全局(AFSSA) 1人：Dr. Erick Verdon
- 荷蘭國家公共衛生及環境研究所(RIVM) 1人：Dr. Leendert van Ginkel
- 台灣藥物食品檢驗局(BFDA, Taiwan) 1人：Dr. Su-Hsiang Tseng

Observers

- Marc Coleman (ELANCO, US)
- Tom Burnett (ELANCO, US)
- Kim Lombardi (ELANCO, US)

AOAC Staff

- Al Pohland (Senior Director, Chief scientific Officer)
- Robert Rathbone

AOAC INTERNATIONAL Dietary Supplements Task Group Report of the Expert Review Panel – Ractopamine by Leendert van Ginkel (Chair)

Fitness for Purpose: A fitness of purpose statement has been developed for the Determination and Confirmation of Ractopamine in Animal Tissues:

An AOAC Official Method is needed for the analysis of ractopamine in animal tissues (swine and cattle), applicable to muscle, liver, kidney and fat. The method should include an option to determine free (parent) ractopamine or total ractopamine (parent plus conjugates) following an enzymatic hydrolysis. The analytical range should cover 1-100 ppb (ng/g) to comply with the EU Reference Point for Action (RPA) of 1 ppb and the lowest MRL values applicable in those countries in which the use of ractopamine is approved of 10 ppb; recoveries, depending on concentration, should lie in the range of 60-110%. Confirmation of analyte identity is required, either as part of the method or as a separate confirmatory method. The method will be used to facilitate international trade and satisfy regulatory requirements. The outcome of the meeting is not necessarily a single method, two or, if necessary more, individual methods can be selected.

File Name & No.	Author	Manuscript Title
RAC-01	Elliott et al.	Screening and confirmatory determination of ractopamine residues in calves treated with growth promoting doses of the b-agonist
RAC-02	Antignac et al.	Identification of ractopamine residues in tissue and urine samples at ultra-trace level using liquid chromatography–positive electrospray tandem mass spectrometry
RAC-03	Blanca et al.	Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography tandem mass spectrometry
RAC-04A	Churchwell et al.	Liquid chormatography/electrospray tandem mas spectrometric analysis of ractopamine residues in livestock tissues
RAC-04B	Doerge et al.	Detection and Confirmation of b-Agonists in Bovine Retina Using LC/APCI-MS
RAC-06A	ELANCO B03903	Determination of Ractopamine Hydrochloride in Turkey Liver and Muscle Tissue by High Performance Liquid Chromatography
RAC-06B Modified	ELANCO B03903 Modified	Determination of Ractopamine Hydrochloride in Chicken Liver by High Performance Liquid Chromatography
RAC-06C	Elanco B05738	Confirmation of Ractopamine Residues in Turkey Liver and Muscle Tissue by Liquid Chromatography/Electrospray Ionization Triple Tandem Quadrupole Mass Spectrometry (Lc/Esi-Ms-Ms)
RAC-08	Fesser et al.	Determination of b-Agonists in Liver and Retina by LiquidChromatography-TandemMass Spectrometry
RAC-09	Qiang et al.	Residue Depletion of Ractopamine and Its Metabolites in Swine Tissues, Urine, and Serum
RAC-10	Shelver & Smith	Tissue Residues and Urinary Excretion of Zilpaterol in Sheep Treated for 10 Days with Dietary Zilpaterol
RAC-11	Shishani et al.	Determination of ractopamine in animal tissues by liquid chromatography-fluorescence and liquid chromatography/tandem mass spectrometry
RAC-14	Sakai et al.	Determination Method for Ractopamine in Swine and Cattle Tissues Using LC/MS
RAC-15	Turberg et al.	Determination of Ractopamine Hydrochloride in Swine, Cattle, and Turkey Feeds by Liquid Chromatography with Coulometric Detection
RAC-17	Williams et al.	Multiresidue confirmation of b-agonists in bovine retina and liver using LC-ES/MS/MS

Method	Primary	Secondary
RAC-01	Valerie Reeves	In Suk Kim, Katerina Mastovska
RAC-02	Weilin Shelver	Review not Submitted
RAC-03	Su-Hsiang Tseng (Emily)	Review not Submitted
RAC-04A	Matt Rodewald	Joe Boison
RAC-04B	Matt Rodewald	Joe Boison
RAC-06A	Joe Boison	Lindell Ward
RAC-06B Modified	Joe Boison	Lindell Ward
RAC-06C	Joe Boison	Lindell Ward
RAC-08	Weilin Shelver	Valerie Reeves
RAC-09	Review not Submitted	Su-Hsiang Tseng (Emily)
RAC-10	In Suk Kim, Katerina Mastovska	Matt Rodewald
RAC-11	Lindell Ward	Matt Rodewald
RAC-14	Matt Rodewald	Valerie Reeves
RAC-15	Su-Hsiang Tseng (Emily)	Weilin Shelver
RAC-17	Valerie Reeves	In Suk Kim, Katerina Mastovska

Rac-01: Screening and confirmatory determination of ractopamine residues in calves treated with growth promoting doses of the β-agonist

Multiple or Single Residue	Single
Use of Internal Standard	Clenbuterol-d6
Hydrolysis	Yes
Species (swine, cattle)	Cattle
Tissue Types	Urine
Sample Preparation	Sample incubated with β-glucuronidase , applied to mixed mode SPE, Eluted with NH_4Cl in EtOAC, evaporated and reconstituted in phosphate buffer, applied to C_{18} SPE, eluted with MeOH, dried, dissolved in mobile phase.
Analytical Range	2-250 ppb
Recovery	Precision 5.6% to 3.7%
Confirmation of Identity	Yes, LC-MS/MS, APCI, ELISA
Additional Comments	Standard curve fortified urine samples. Consistent correlation between ELISA and glucaronidated and free Ractopamine. Complex method that uses two SPEs. (If method for liver, easily adaptable to urine). Would not be suitable matrix for N. America because of live animal control.

Discussion around the room regarding pros and cons of using Elisa method, coulometric and flourescence detection.

Vote: ELISA methods do not comply with the objectives of the ERP because of low selectivity and sensitivity.
Yes: 9 No: 0 Abstain: 0

Vote: Coulometric methods do not comply with the objectives of the ERP because the technology is not rugged and general available.
Yes: 9 No: 0 Abstain: 0

Vote: LC-FLD is a good technique for quantitative analysis because it has sufficient sensitivity and has shown to comply with the objectives of the ERP.
Yes: 9 No: 0 Abstain: 0

Determination of Methods for Consideration for further evaluation:

ERP went around the room and discussed criteria for consideration in keeping a method for further evaluation. These criteria included:
- Ability to add/keep the deconjugation step
- Species and matrix applicability
- Applicability to LC-FLD and LC-MS/MS
- Complexity of Extraction
- LOQ
- Recovery/Accuracy/Precision

Methods to be flagged based on criteria 1 for further evaluation:
Green Flags: Methods 3, 11, 17
Yellow Flags: Methods 2 (interesting sample clean-up), 6A, 6B, 6C, 9
Red Flags: Methods 1, 4A, 4B, 8, 10, 14, 15

Review of Dr. Pang's review.
Uses a single SPE clean-up.

Question: When is the optimum time to do the conjugation?
Assumption is made that conjugates extract the same way as the parent. There may be a difference in the solubility if conjugation is done on the homogenate vs some other point in the method. CVM, Canada is not interested in the deconjugation step because only marker residue is wanted (i.e. only in countries where there is an approval. EU and other countries where the molecule is not approved, the deconjugation step is needed to quantify total ractopamine, not only the marker residue.

Determining a final method:
Two groups to further discuss methods: LC-MS/MS and LC-FLD Groups discussed for 45 minutes, then returned back to the larger group for discussion.

There was gravitation by both groups to method 11 for Fluorescence. Concern is with the length of the procedure and the two SPE clean-up steps. The thought process is to separate method 11 into two different methods, 1 using the Alumina A SPE cartridge for FLD detection, and one using the mixed mode, and skipping the Alumina A SPE cartridge for LC-MS/MS. Also wanting to remove the borate buffer and replace with sodium carbonate buffer. Also change the liquid-liquid extraction before the Alumina A cartridge SPE to MTBE instead of Ethyl Acetate.

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8. Elute with 10 mL MeOH
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11. MCX Oasis SPE
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13. Dry
14. Recon in 0.5 mL 2% HOAc

Modified LC-MS/MS method for parent only (modified from method 11):

1. 10 g sample
2. IS-Ractopamine d5
3. 20 mL MeOH (x3)
4. 8 mL aliquot and dry down
5. 25 mM NaOAc buffer (pH 5)
6. MCX Oasis SPE
7. Elute with 4 mL 2% NH3 in MeOH
8. Dry
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Modified LC-MS/MS method for total only (modified from method 11):

1. 10 g sample
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3. 20 mL MeOH (x3)
4. 8 mL aliquot and dry down
5. 25 mM NaOAc buffer (pH 5)
6. β-Glucuronidase 2 hr @ 65°C
7. 2 mL 20 mM Na2CO3 (pH 10.5)
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9. Elute with 4 mL 2% NH3 in MeOH
10. Dry
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Vote: Method 11 forms the basis for a 2x2 method for ractopamine, either parent only or total, using FLD or LC-MS/MS.

Yes: 9 No: 0 Abstain: 0

Notes:

•For deconjugation step, would like two options for incubation, one accelerated at an elevated temperature, and one for an overnight incubation. **Note**: reference Rectopamine-01 for information regarding incubation and deconjugation.

•Want to use both glucuronidase and sulfatase (Helix has both).

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After having concluded the discussion on the selected analytical method and verifying neither the members of the ERP nor the observers had any further comments or remarks. The chair thanked all for their contributions to the outcome of this meeting and closed the meeting

心得

- 此次為國際AOAC首次邀請亞洲國家參與，本人有幸代表台灣BFDA實際參與開會，收獲良多。
- 國際AOAC研擬公定方法之過程，值得未來TFDA借鏡。

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建議

- 研擬長期計畫，進行公告方法之研擬、審查及指定等工作。邀集學者、專家、相關實驗室參與，進行方法回顧、評估、審查及方法確效、實驗室間比對等工作。
- 持續積極參與國際AOAC相關會議，提升檢驗水平及視野，以因應日益複雜之國際食品安全檢驗問題。

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