Mycotoxin Analysis in Animal Feed by LC/MS/MS

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February 29, 2012 at OTSC

OFFICE OF THE TEXAS STATE CHEMIST

Texas Feed and Fertilizer Control Service • Agriculture Analytical Service

AgriLIFE RESEARCH
Texas A&M System

Mycotoxins

- Mycotoxins
  - Secondary metabolites of certain species of fungi (molds)
  - Approximate 300 listed mycotoxins
  - Genera of toxigenic fungi:
    - *Aspergillus, Penicillium* and *Fusarium*
  - Mycotoxins of greatest importance worldwide:
    - aflatoxins, fumonisins, deoxynivalenol ochratoxin A and zearalenone etc.

Mycotoxins are a Potential Risk to both Humans and Livestock

Guidance Levels for Total Fumonisins in Animal Feeds

Class of Animal	Feed Ingredients & Portion of Diet	Levels in Corn & Corn By-products
Equids and Rabbits	Corn and corn by-products not to exceed 20% of the diet **	5 ppm
Swine and Catfish	Corn and corn by-products not to exceed 50% of the diet**	20 ppm
Breeding Ruminants, Breeding Poultry and Breeding Mink*	Corn and corn by-products not to exceed 50% of the diet**	30 ppm
Ruminants ≥3 Months Old being Raised for Slaughter and Mink being Raised for Pelt Production	Corn and corn by-products not to exceed 50% of the diet**	60 ppm
Poultry being Raised for Slaughter	Corn and corn by-products not to exceed 50% of the diet**	100 ppm
All Other Species or Classes of Livestock and Pet Animals	Corn and corn by-products not to exceed 50% of the diet**	10 ppm

- Includes lactating dairy cattle and hens laying eggs for human consumption. ** Dry weight basis.
- *http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ChemicalContaminantsandPesticides/ucm109231.htm*

Action Levels for Total Aflatoxins in Livestock Feed

Class of Animal	Feed	Aflatoxin Level
Finishing beef cattle	Corn and peanut products	300 ppb
Beef cattle, swine or poultry	Cottonseed meal	300 ppb
Finishing swine over 100 lb.	Corn and peanut products	200 ppb
Breeding cattle, breeding swine and mature poultry	Corn and peanut products	100 ppb
Immature animals	Animal feeds and ingredients, excluding cottonseed meal	20 ppb
Dairy animals, animals not listed above, or unknown use	Animal feeds and ingredients	20 ppb

http://www.fda.gov/AnimalVeterinary/Products/AnimalFoodFeeds/Contaminants/ucm050974.htm

Advisory levels for Deoxynivalenol in livestock feed

Class of Animal	Feed Ingredients & Portion of Diet	DON Levels in Grains & Grain By-products and (Finished Feed)
Ruminating beef and feedlot cattle older than 4 months	Grain and grain by-products not to exceed 50% of the diet	10 ppm (5 ppm)
Chickens	Grain and grain by-products not to exceed 50% of the diet	10 ppm (5 ppm)
Swine	Grain and grain by-products not to exceed 20% of the diet	5 ppm (1 ppm)
All other animals	Grain and grain by-products not to exceed 40% of the diet	5 ppm (2 ppm)

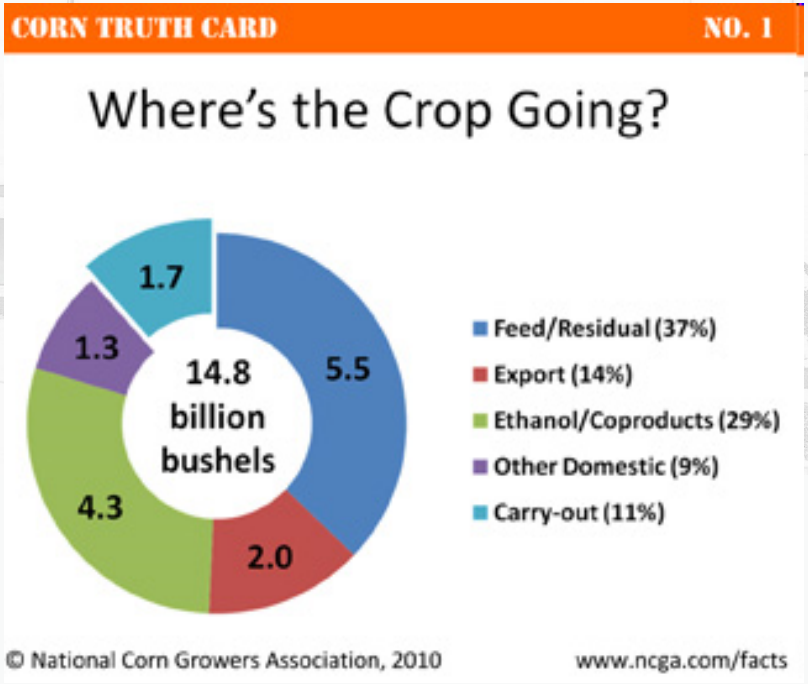
http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/NaturalToxins/ucm120184.htm

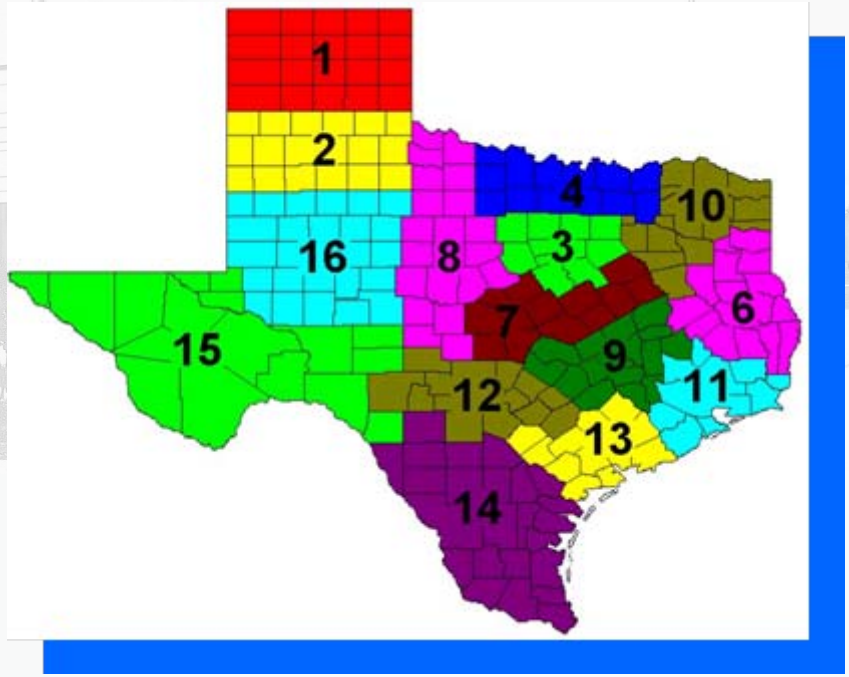
TX Corn Production

- **Year: 2009**
  Harvest: 2.10 million acres
  Yield: 130 bushels/acre

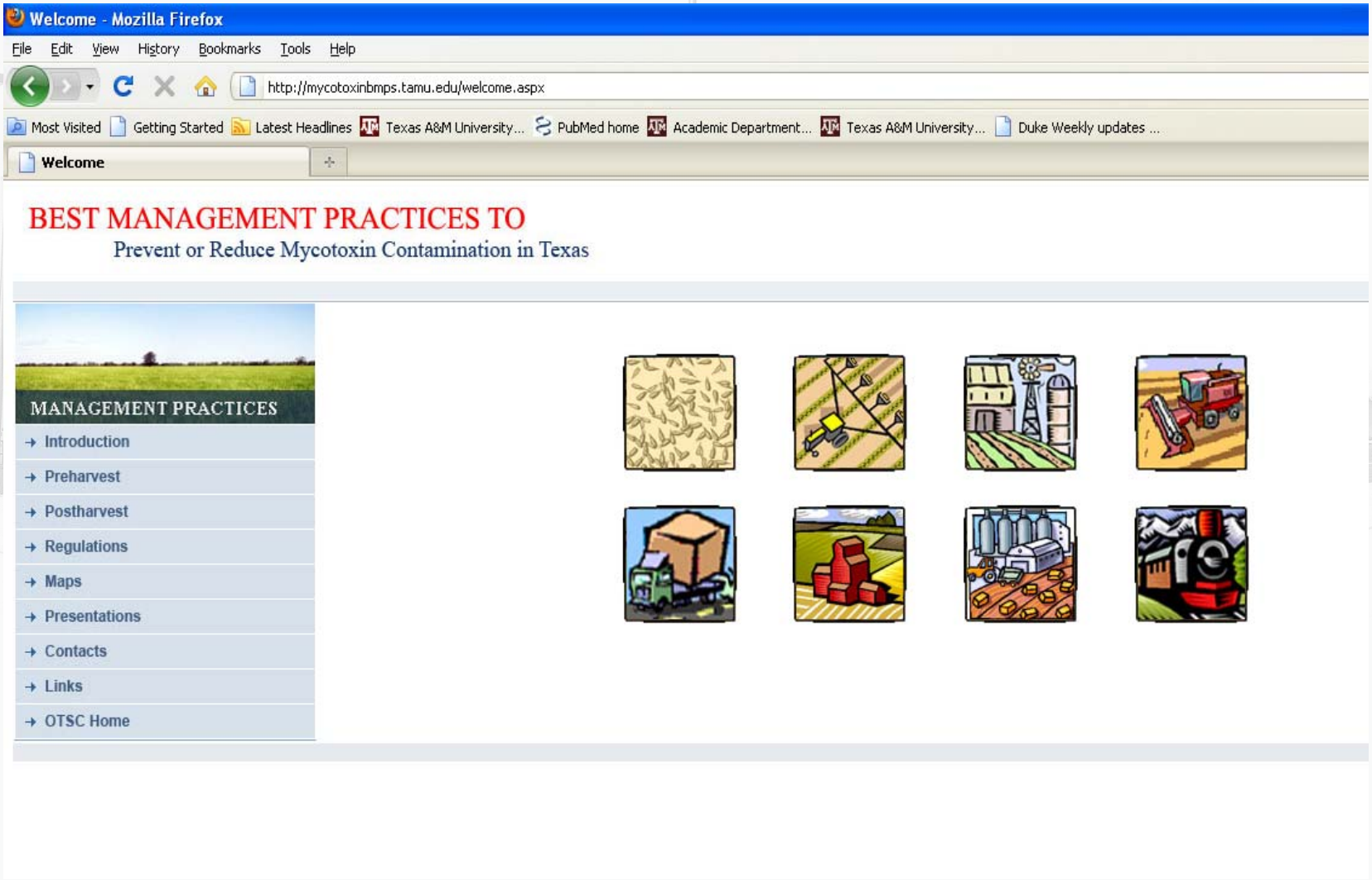
Total Yield:

- **Cash value to farmers:$514 million,$1.7 billion for Texas**





OTSC: Mycotoxin Control



Mycotoxin Action Levels (OTSC Regulations)

- Fumonsins: total Level of Fumonisins ($FB_1+FB_2+FB_3$) are over 5 ppm

  Grain, oilseeds, processed grain, and oilseed meal containing fumonisin above 5 parts per million (ppm) except that with proper labeling as approved by the Office of the Texas State Chemist and targeted for animal species as follows: <20 ppm for swine and catfish not to exceed 50% of diet; <30 ppm for breeding ruminants, breeding poultry and breeding mink not to exceed 50% of diet; <60 ppm for ruminants > 3 months old being raised for slaughter, and mink being raised for pelt production not to exceed 50% of diet; <100 ppm for poultry being raised for slaughter not to exceed 50% of diet; all other species or classes of livestock and pet animals <10 ppm not to exceed 50% of diet except equids and rabbits which should not exceed 5 ppm and 20% of diet; >100 ppm requires a blending permit issued by the Office of the Texas State Chemist. (§61.61 Poisonous or Deleterious Substances)

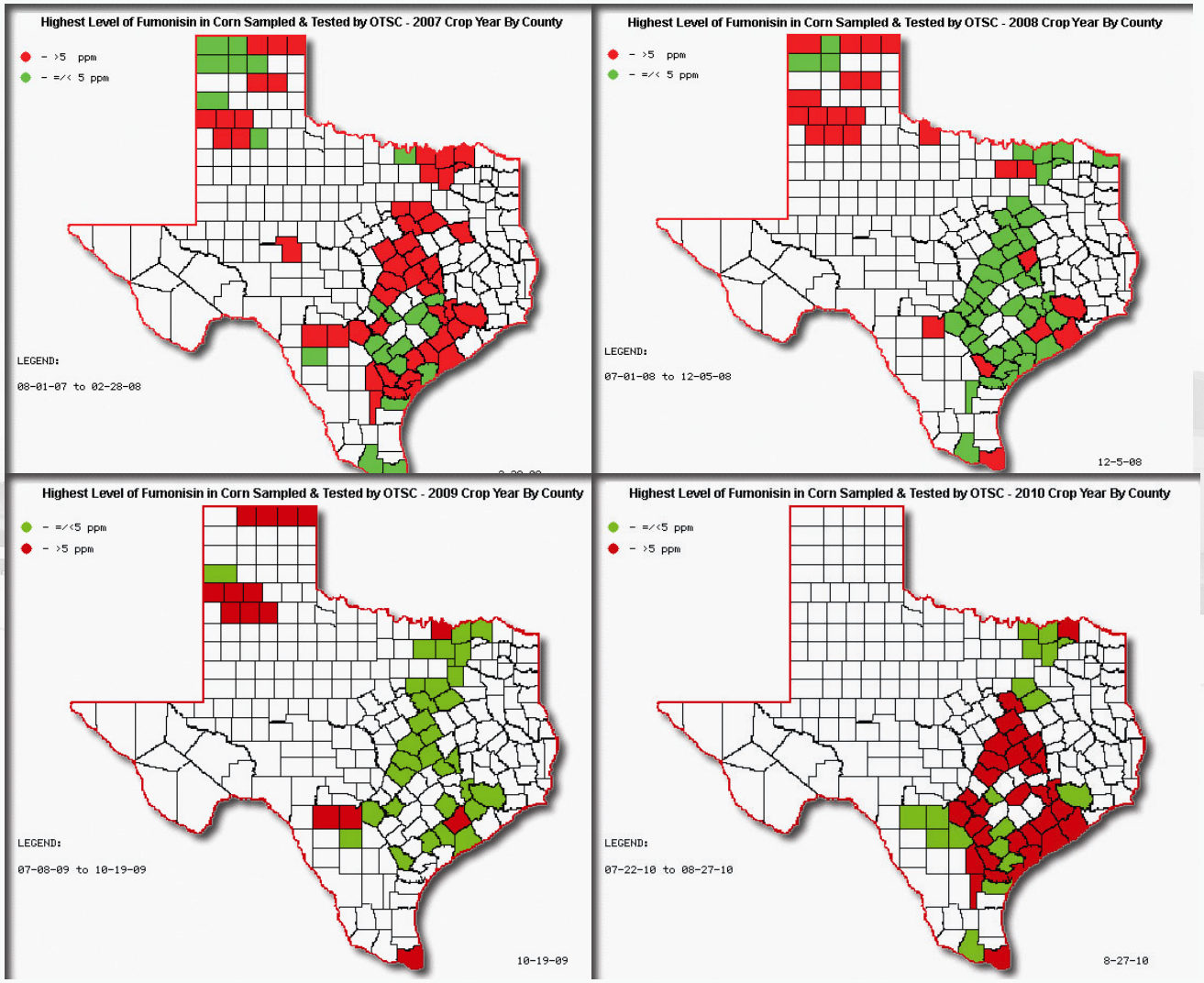
- Aflatoxins: total Aflatoxins ($AFB_1+AFB_2+AFG_1+AFG_2$) are over 20 ppb

  Grain, oilseeds, processed grain and oilseed meals containing aflatoxin B1, B2, G1, G2 above 20 parts per billion (ppb) individually or total except that with proper labeling as approved by the Office of the Texas State Chemist as follows: <50 ppb may be distributed when destined for wildlife; <100 ppb may be distributed when destined for breeding cattle and breeding goats not used in production of milk for human consumption, breeding swine, mature poultry, and sheep; <200 ppb may be distributed when destined for finishing swine (more than 100 lbs. body weight); <300 ppb may be distributed when destined for finishing cattle in confinement; grain containing >300 to <500 ppb requires a blending permit issued by the Office of the Texas State Chemist; aflatoxin >500 ppb in grain and >300 ppb in oilseed, processed grain, and oilseed meal may not enter commerce and a record of disposition shall be submitted to the Office of the TexasState Chemist. (§61.61 Poisonous or Deleterious Substances)

- Other mycotoxins including DON, OTA and ZON

  No action limit has been established yet.

OTSC: Fumonisin Surveillance Program



≤ 5PPM

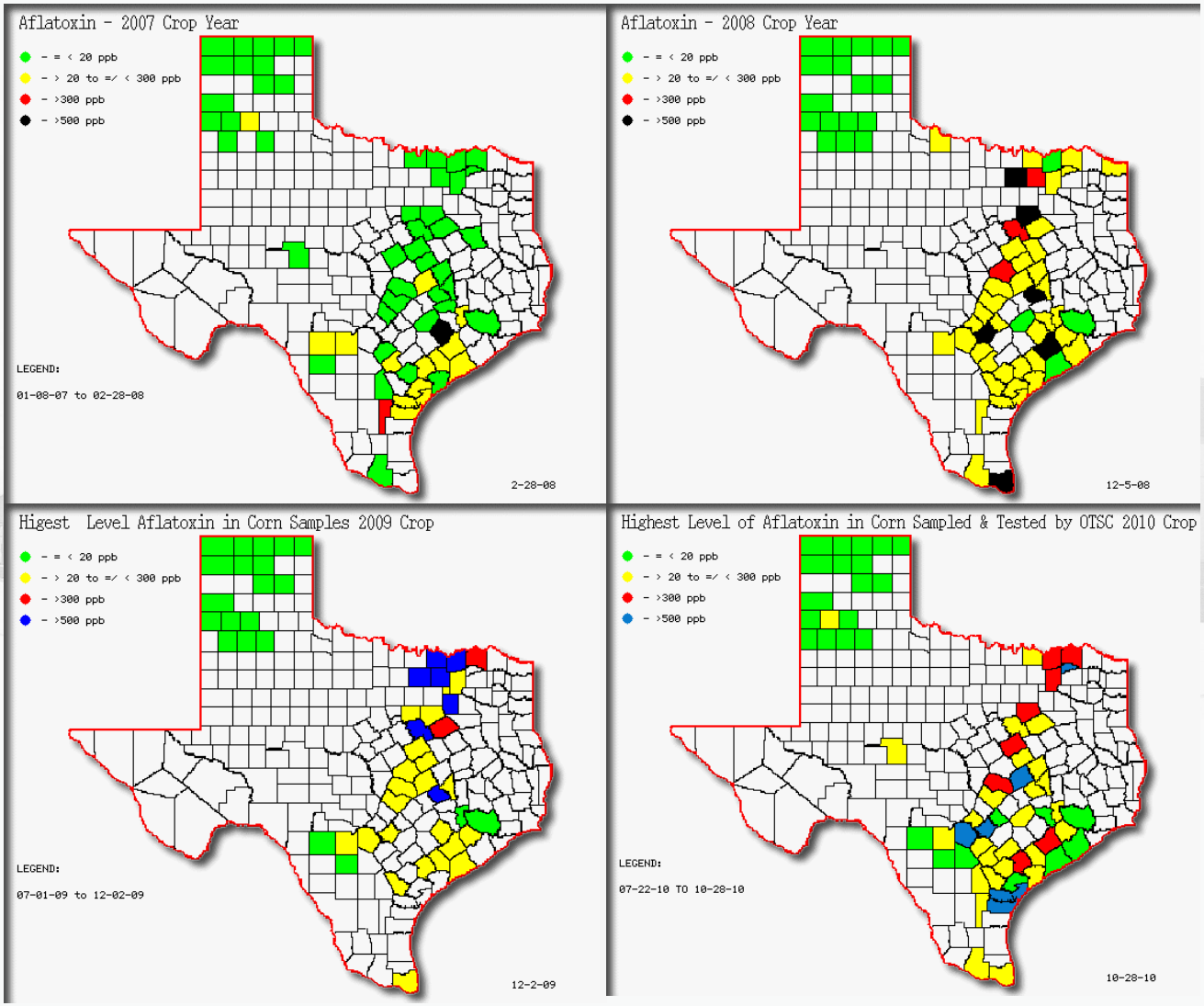
> 5PPM

Hot

2007

2010

OTSC: Aflatoxin Surveillance Program



- ≤ 20 ppb
- >20 ppb to ≤ 300 ppb
- > 300 ppb
- >500 ppb

Analytical Methods

- **Immunoassays**

Pros: High throughput, Semi-quantitative,

Cons: Low sensitivity, Sample purification required, Poor linearity.

(AOAC OMA 2001.06-FB, 990.34-AF, 994.01-ZON, 986.17-DON)

- **LC/Fluorescence Methods**

Pros: Matrix effect independent, robust

Cons: Time consuming, Error-prone cleanup and derivatization steps involved in sample preparation.

(AOAC OMA 995.15-FB, 991.31-AF, 985.18-ZON, 991.44-OTA)

- **LC / MS / MS methods**

Pros: Robust, Sensitive, High throughput, no extra cleanup.

Cons: Matrix effect.

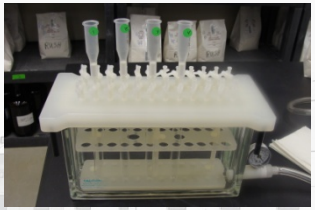
AOAC OMA ?

Sample Preparation and Analysis (Fumonisins)

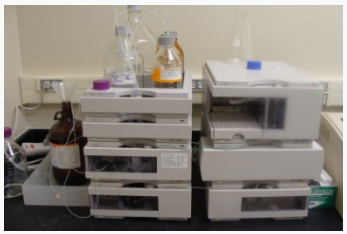
Ground Corn Samples → Extraction → Filtration

Filtration → Adjust pH of the extract

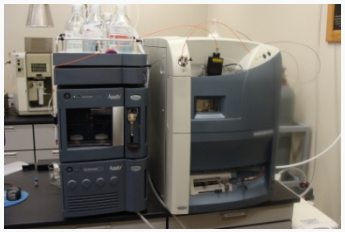
Filtration → Addtion of Internal Standard



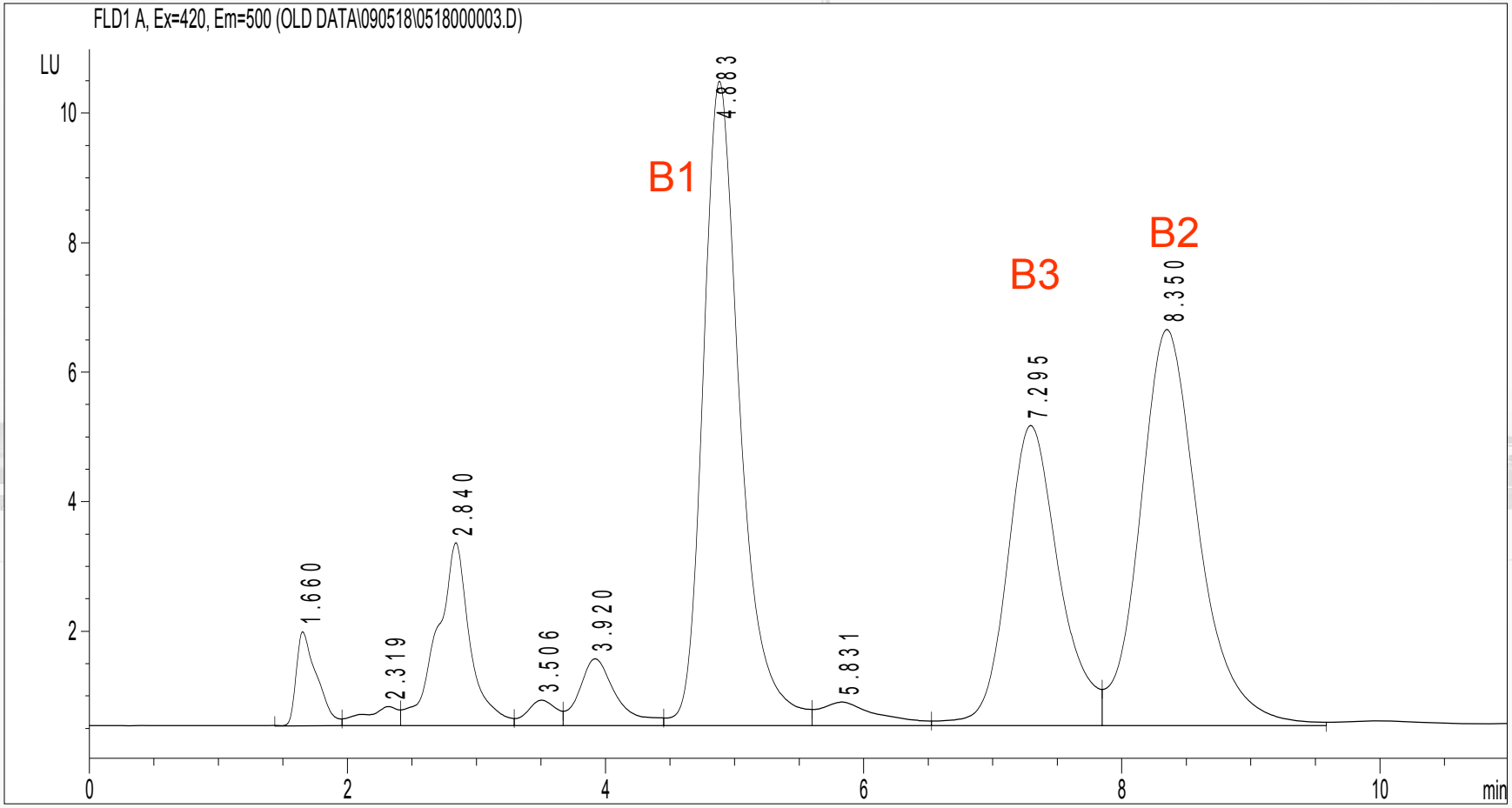
Adjust pH of the extract → Solid Phase Extraction → Derivatization by NDA → LC/Fluorescence



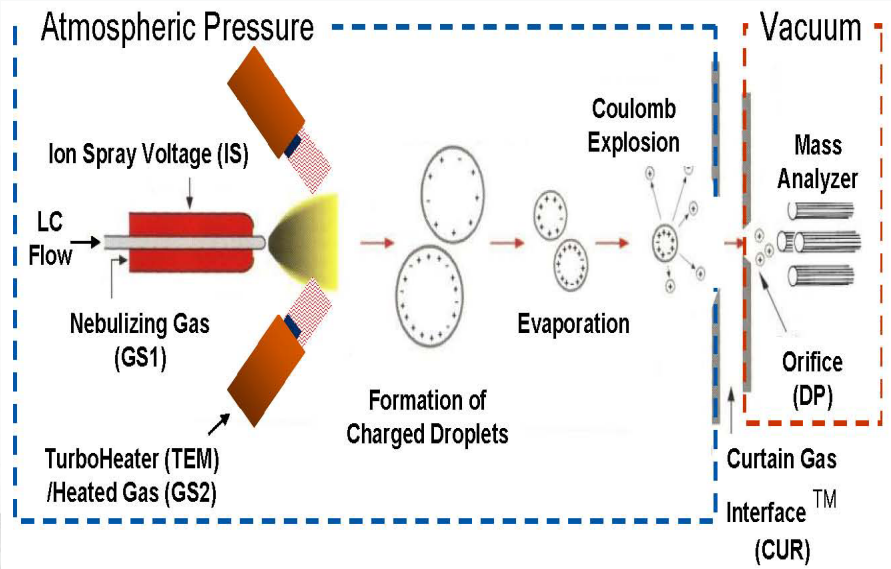
Addtion of Internal Standard → LC/MS/MS



HPLC Chromatogram (Fumonisins)

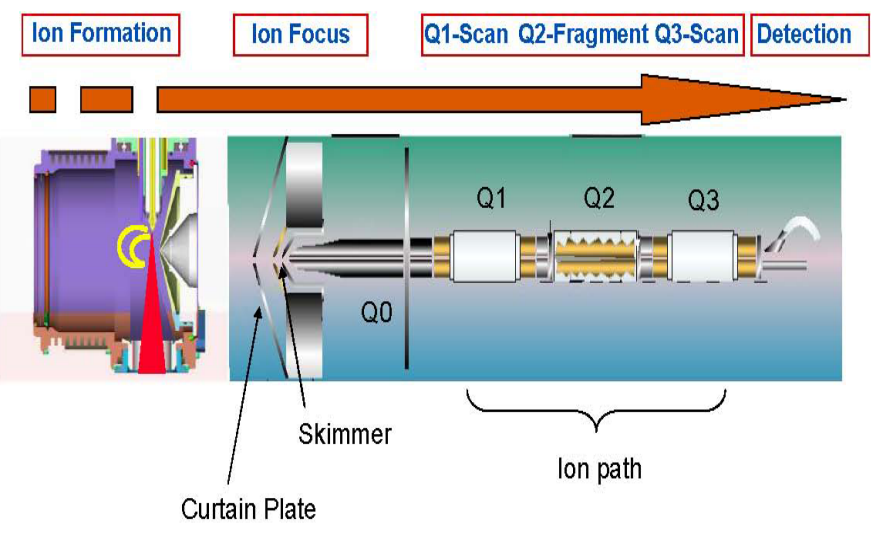


LC/ESI/MS/MS

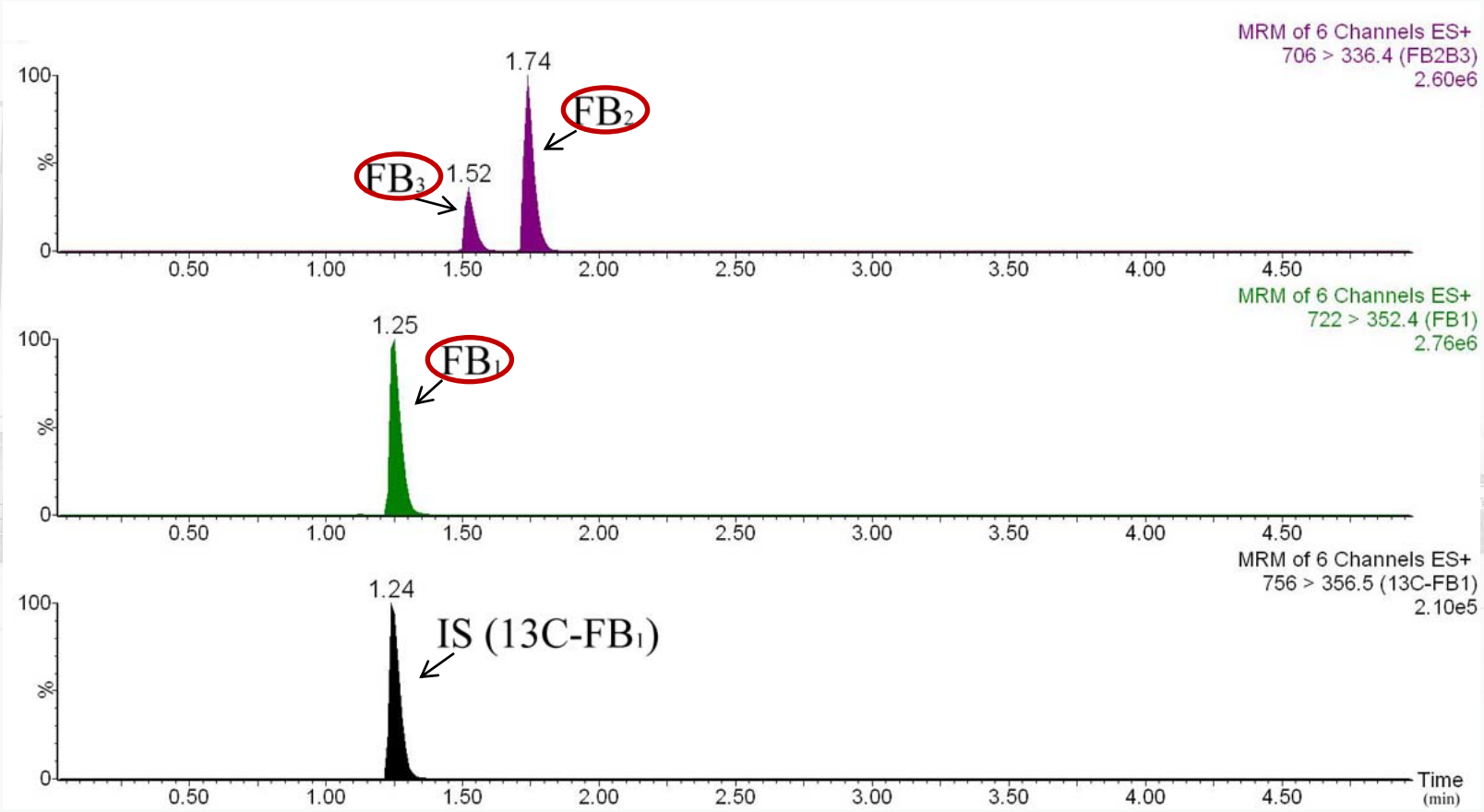


Electrospray Ionization (ESI)

Triple Quadrupole System (Tri-Q)

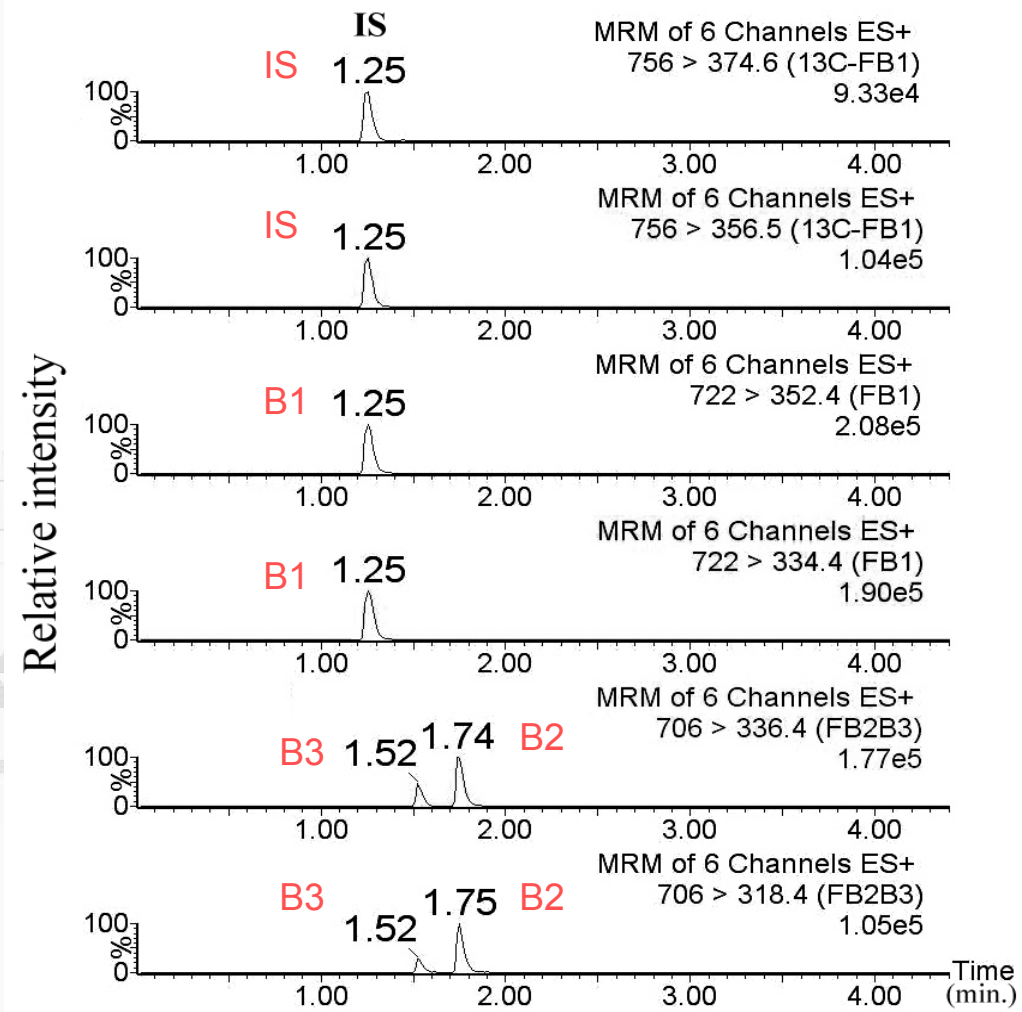


LC/MS/MS Analysis of Fumonisins



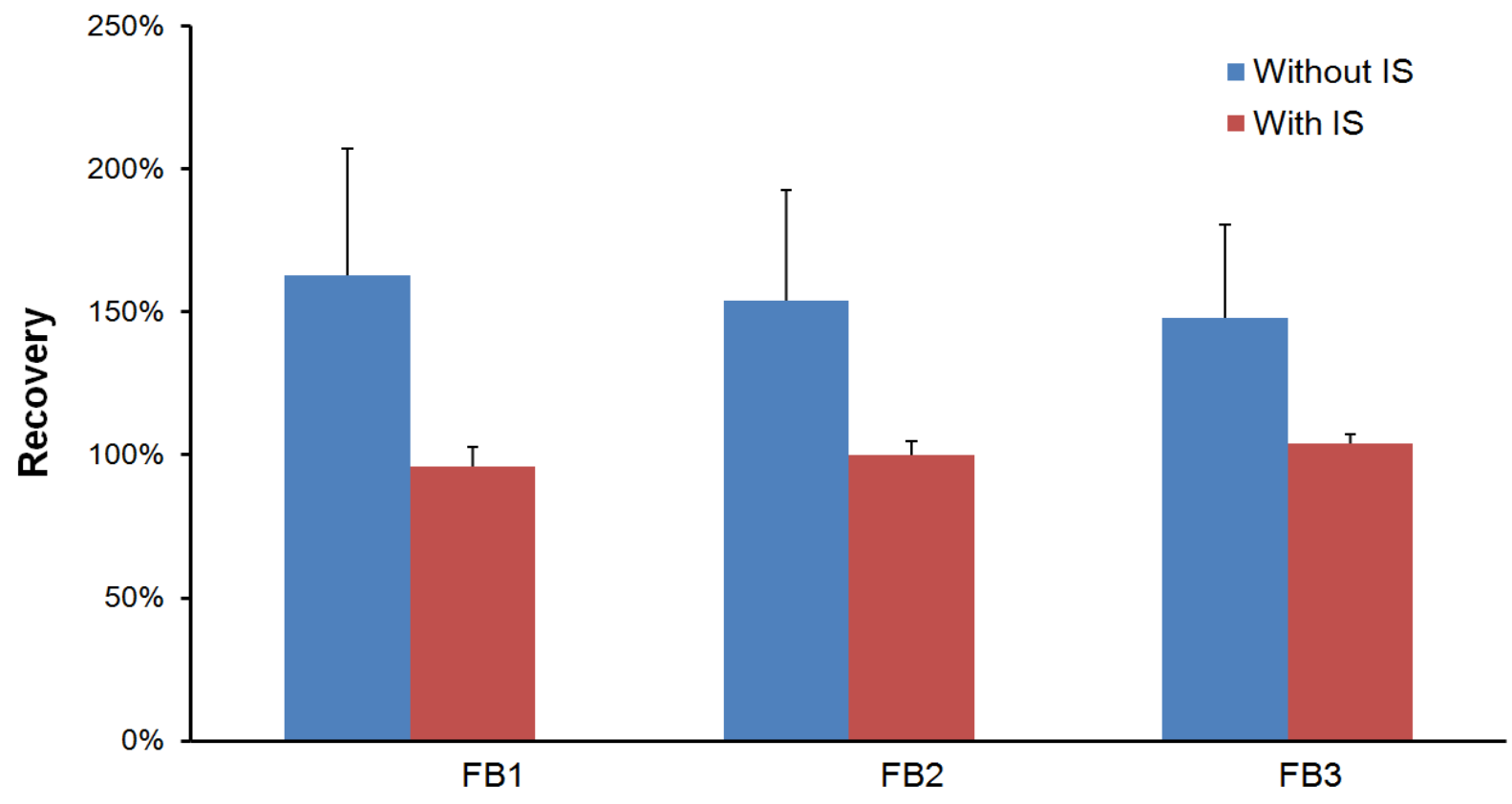
MRM transitions of fumonisins and isotope labeled internal standard. Total analysis time: 5 min

LC/MS/MS Analysis of Fumonisins



MRM transitions of fumonisins and isotope labeled internal standard. Total analysis time: 5 min
(Li, Wei; Herrman, Tim J.; Dai, Susie Y., The Journal of AOAC International, 2010, 93(5): 1472-81)

Matrix Effect Corrected by Internal Standard



Comparison of recovery rates without internal standard (IS) and those with IS when testing blank cornmeal samples fortified with 1 µg g^{-1} for FB_1, 0.5 µg g^{-1} for FB_2 and 0.25 µg g^{-1} for FB_3.
(Li, Wei; Herrman, Tim J.; Dai, Susie Y., The Journal of AOAC International, 2010, 93(5): 1472-81)

Validation of LC/MS/MS Method (Fumonsins)

Analyte	Nominal concentration (µg g^{-1})	Recovery (%)	Accuracy (%)	Intra-day RSD (%) (n=5)	Inter-day RSD (%) (n=3)
Human food					
FB_1	0.2	95	-5	7.2	4.8
	0.5	93	-7	7.9	5.5
	1.0	95	-5	3.5	3.0
FB_2	0.2	107	7	4.3	3.6
	0.5	108	8	3.1	3.1
	1.0	105	5	1.8	2.3
FB_3	0.1	108	8	3.1	4.0
	0.25	103	3	2.4	4.3
	0.50	98	-2	2.7	2.3
Animal feed					
FB_1	0.2	97	-3	7.5	6.2
	0.5	98	-2	6.8	5.8
	1.0	93	-7	7.1	5.5
FB_2	0.2	105	5	5.7	4.8
	0.5	107	7	4.2	3.6
	1.0	104	4	2.8	4.0
FB_3	0.1	94	-6	4.4	5.4
	0.25	96	-4	2.4	2.1
	0.50	96	-4	2.3	3.1

Comparison of the HPLC and LC/MS/MS (Fumonisins)

Sample ID	Analyte	HPLC/Florescence measurement (µg g^{-1})	LC/MS/MS measurement (µg g^{-1})	RSD (%)for LC/MS/MS measurements (N=5)
1[a]	FB_1	**1.40**	**1.49**	**2.0**
	FB_2	**0.35**	**0.37**	**6.6**
	FB_3	**0.27**	**0.26**	**7.7**
2[a]	FB_1	**4.10**	**3.98**	**3.1**
	FB_2	**1.17**	**1.25**	**3.7**
	FB_3	**0.69**	**0.65**	**3.5**
3[a]	FB_1	**9.30**	**8.82**	**3.6**
	FB_2	**2.67**	**2.86**	**3.2**
	FB_3	**1.38**	**1.50**	**5.0**
4[b]	FB_1	**44.60**	**45.59**	**2.4**
	FB_2	**13.07**	**13.36**	**6.6**
	FB_3	**6.43**	**5.95**	**7.4**

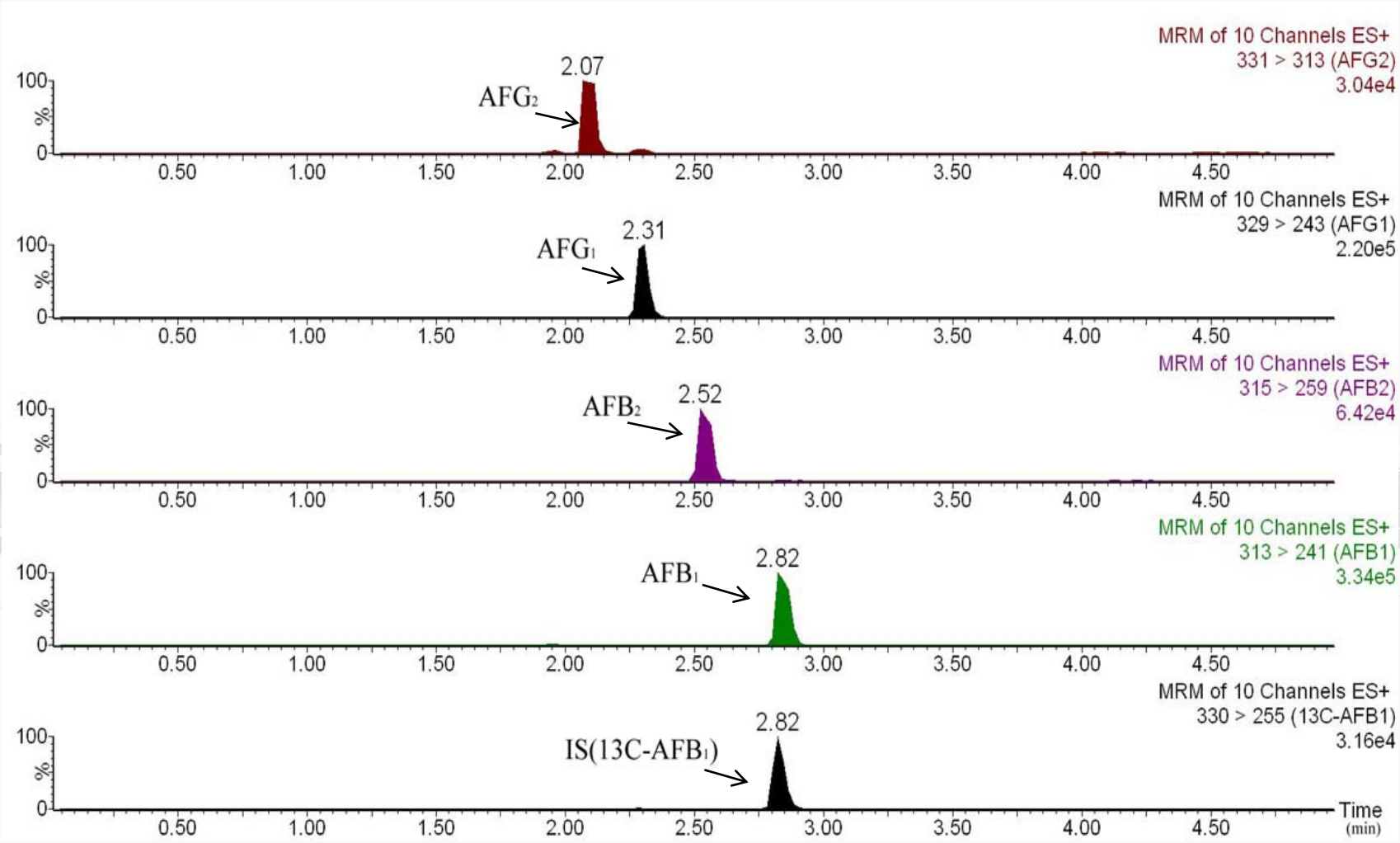
a: dilution factor is 5; b: dilution factor is 50

Which method is better?

Aspects	HPLC/Fluorescence	HPLC/MS/MS
Sample preparation	10 h	2.5 h
Instrumentation	6 h	2 h
Protocol	2 days	Half day

Note: Time was calculated based on fumonsin analysis of a set of **24 regulatory samples**.

LC/MS/MS Method for Aflatoxin Analysis



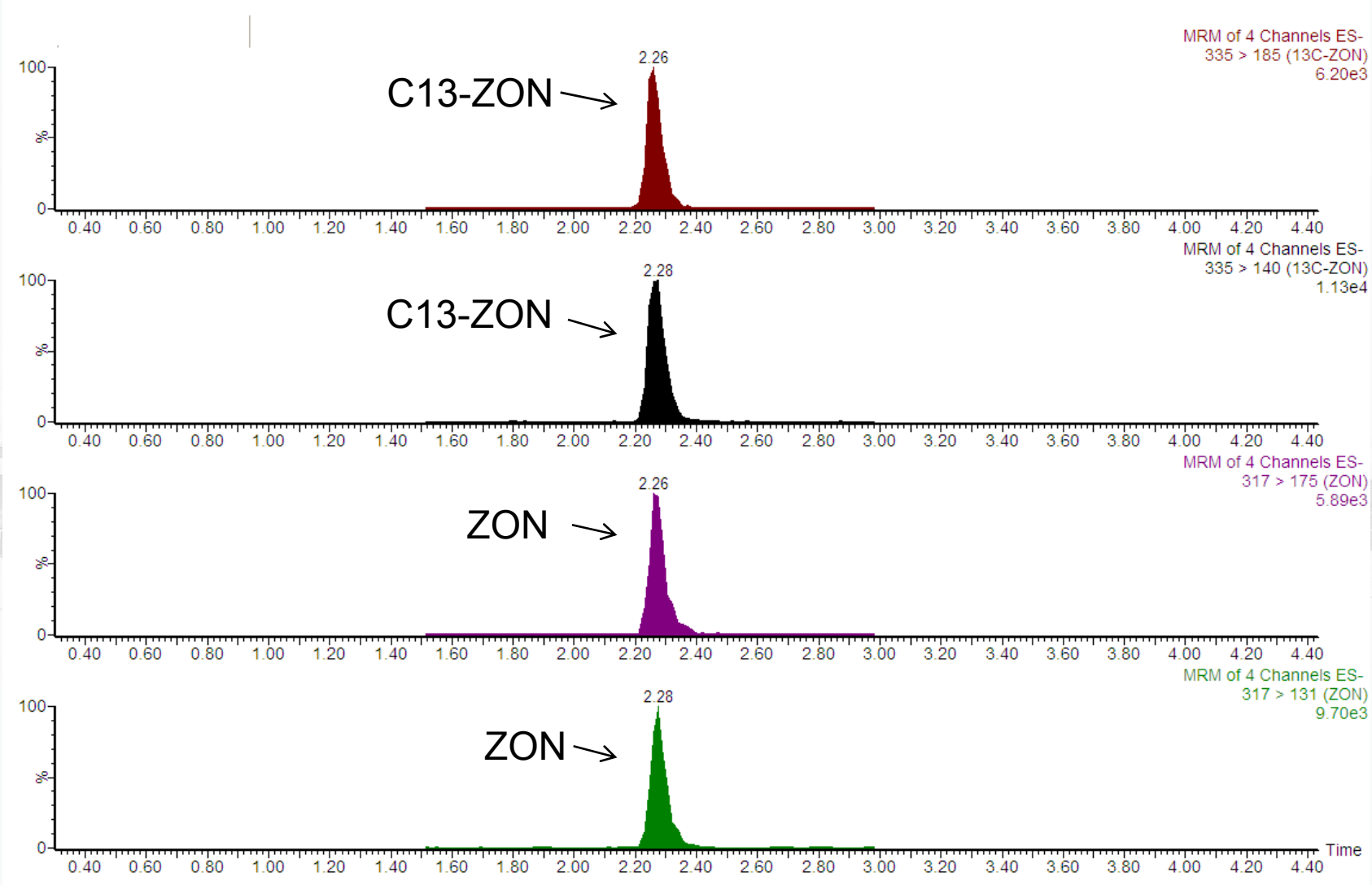
Li, Wei; Herrman, Tim J.; Dai, Susie Y., *Rapid Communications in Mass Spectrometry*, 2011, 25, 1222-30

Validation of LC/MS/MS Method (Aflatoxins)

Analyte	High Level[a] (%)			Medium Level[b] (%)			Low Level[c] (%)		
	Recovery	Intra-day RSD	Inter-day RSD	Recovery	Intra-day RSD	Inter-day RSD	Recovery	Intra-day RSD	Inter-day RSD
Corn Meal									
AFB_1	105	*2*	*3*	102	*3*	*5*	108	*6*	*7*
AFB_2	95	*7*	*9*	99	*7*	*9*	105	*7*	*9*
AFG_1	99	*4*	*5*	103	*4*	*6*	106	*7*	*9*
AFG_2	102	*9*	*9*	95	*8*	*10*	108	*12*	*12*
Milo									
AFB_1	94	*4*	*6*	95	*3*	*5*	93	*6*	*8*
AFB_2	102	*7*	*8*	103	*9*	*10*	82	*10*	*10*
AFG_1	93	*5*	*8*	107	*6*	*9*	108	*7*	*9*
AFG_2	107	*8*	*10*	113	*10*	*13*	122	*13*	*14*
Dog Food									
AFB_1	85	*5*	*8*	89	*5*	*9*	87	*9*	*9*
AFB_2	103	*7*	*12*	91	*8*	*9*	80	*10*	*11*
AFG_1	89	*6*	*10*	91	*5*	*8*	88	*8*	*12*
AFG_2	108	*9*	*13*	121	*10*	*10*	118	*12*	*14*
Peanut Meal									
AFB_1	103	*4*	*5*	80	*7*	*8*	88	*10*	*11*
AFB_2	100	*9*	*14*	82	*9*	*11*	78	*13*	*15*
AFG_1	94	*6*	*7*	96	*9*	*13*	89	*9*	*11*
AFG_2	103	*8*	*15*	114	*11*	*15*	112	*14*	*17*
Dairy Cattle									
AFB_1	97	*7*	*9*	95	*9*	*10*	109	*10*	*11*
AFB_2	104	*9*	*12*	98	*11*	*13*	88	*13*	*14*
AFG_1	101	*7*	*10*	103	*8*	*12*	98	*10*	*14*
AFG_2	87	*10*	*11*	80	*12*	*14*	83	*12*	*16*
Cottonseed									
AFB_1	93	*6*	*7*	89	*7*	*9*	94	*9*	*11*
AFB_2	85	*10*	*12*	87	*11*	*14*	89	*13*	*15*
AFG_1	94	*8*	*8*	95	*10*	*11*	88	*8*	*10*
AFG_2	86	*11*	*14*	92	*10*	*14*	109	*10*	*13*

Li, Wei; Herrman, Tim J.; Dai, Susie Y., *Rapid Communications in Mass Spectrometry*, 2011, 25, 1222-30

LC/MS/MS Method for ZON Analysis



Li, Wei; Herrman, Tim J.; Dai, Susie Y., *Paper In Preparation*

LC/MS/MS Method for ZON Analysis

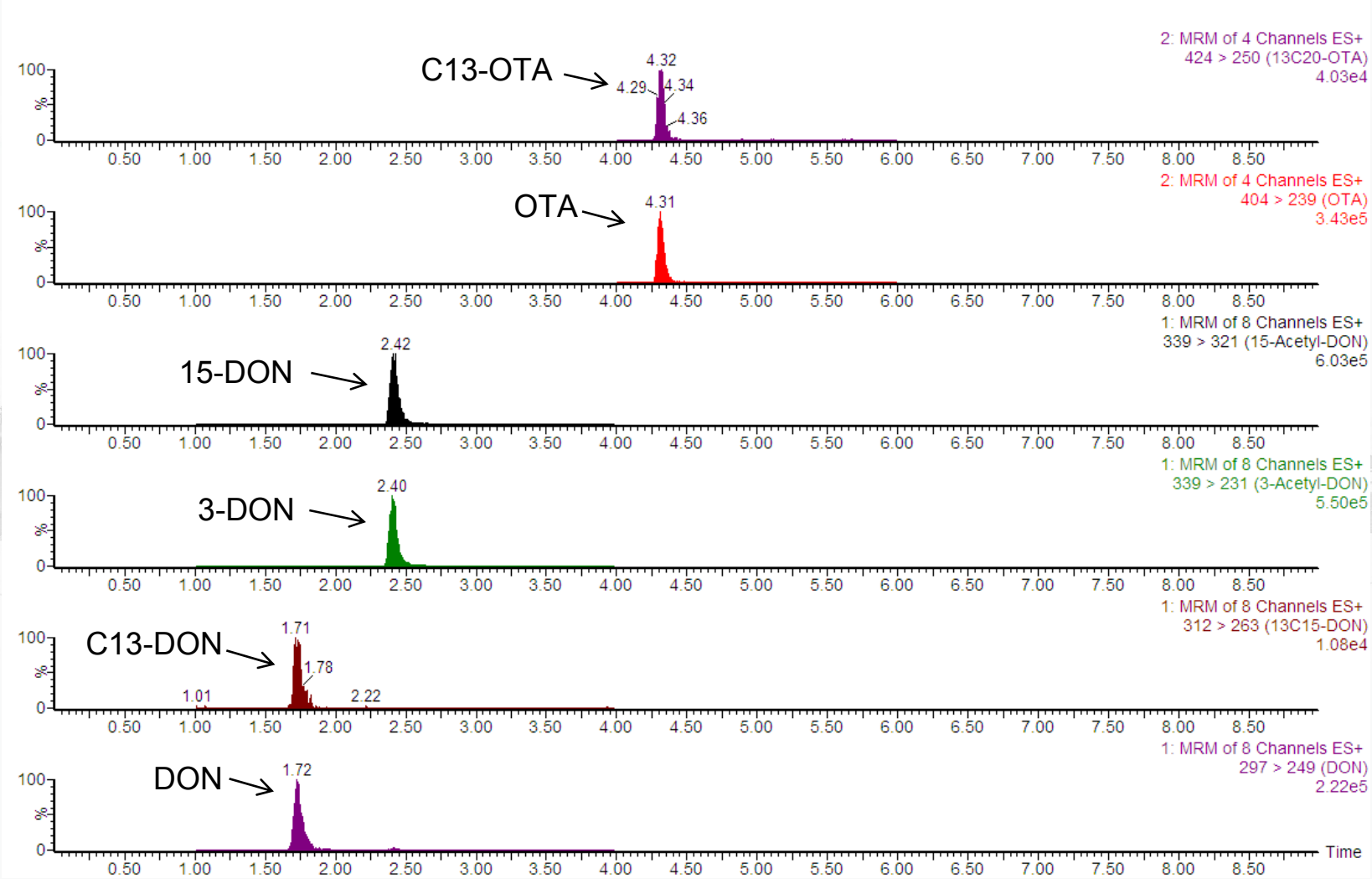
Analyte	Nominal concentration (ng g^{-1})	Recovery (%)	RSD (%) (n=9)
ZON	10	95	11
	20	89	9
	40	86	11

Table 1: Recoveries and precision of spiking cornmeal samples by LC/MS/MS method.

	Sample ID	LC/MS/MS results (ng g^{-1})
Work Control	1	39.0
	2	30.0
	3	25.5
	4	35.5
	5	31.5
	6	35.5
Mean		32.8
SD		4.8
RSD (%)		15

Table 2: Data of Work control samples

LC/MS/MS Method for DON and OTA Analysis



Applications of LC/MS methods

- SOPs

  Fumonisin, Aflatoxin, Zearalenone analysis

- Regulatory sample analysis

  Fumonisin test since 2010

- Collaborative Studies

  2011 International Multiple mycotoxins PT
  2011 FERN Zearalenone PT
  Sample analysis for research communities in TAMU Sys.

Mycotoxin Screening in 2011 (June-October)

	DON (ppb)	OTA (ppb)	ZON (ppb)	AF(ppb)	FB(ppm)
EC Regulation	1250	5	200	20	5
FDA Guidance	2000	-	-	20	5
Number of Violations	0	17	0	82	38
Percentage of Violations in total 266 samples	0	6%	0	31%	14%

266 regulatory samples were analyzed in total.
Note: The results for DON and OTA are preliminary because the methods are not validated.

Summary

- LC/MS/MS methods for quantifying fumonisins, aflatoxins and zearalenone in animal feed were developed and validated.
- With the application of the isotope-labeled internal standard, the matrix effect in LC/MS/MS analysis was effectively eliminated, and the performance of our quantification method met the requirements of FDA regulation criteria.
- The developed method could be satisfactorily applied as a routine procedure to identify and quantify mycotoxins in laboratories performing food/feed quality and safety control when a large sample load is required.

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