Testing of Stilbenes in Foods of Animal Origin

Government Laboratory

Introduction

- Diethylstilboestrol (DES) and hexoestrol, examples of stilbenes (二苯乙烯) which is a group of synthetic oestrogen hormones.
- DES is considered carcinogenic to humans by the International Agency for Research on Cancer (IARC) in its evaluation in 1987.
- Stilbenes including DES are currently banned to be used in poultry in places such as Hong Kong, the Mainland, Australia, the European Union and the US.

Source : https://www.cfs.gov.hk/english

Introduction (2)

CODEX (食品法典委員會) regulatory level

RISK MANAGEMENT RECOMMENDATIONS (RMRs) FOR RESIDUES OF VETERINARY DRUGS

Stilbenes (growth promotor)

"In view of the available scientific information, there is no safe level of residues of stilbenes or their metabolites in food that represents an acceptable risk to consumers. For this reason, competent authorities should prevent residues of stilbenes in food. This can be accomplished by not using stilbenes in food producing animals."

Source : MRLs AND RMRs FOR RESIDUES OF VETERINARY DRUGS IN FOODS (CX/MRL 2-2021)

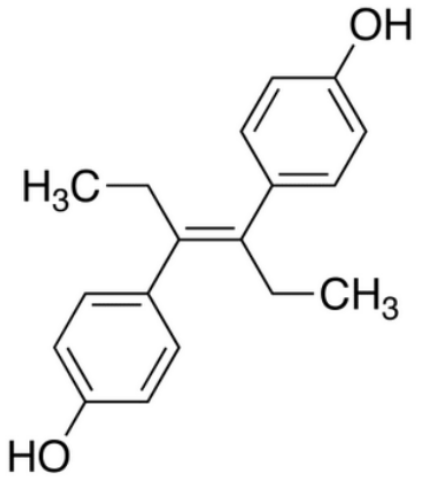
Introduction (3)

Local Regulatory Control

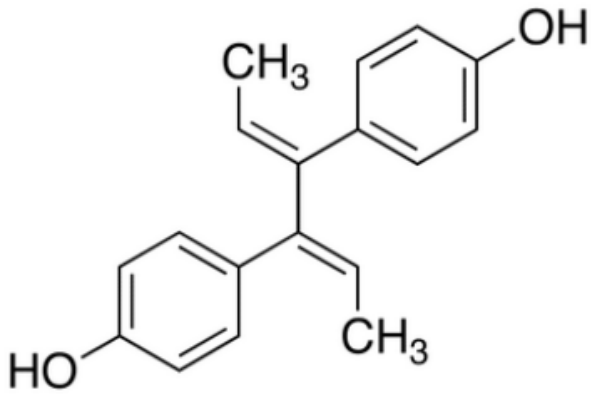
- Harmful Substances in Food Regulations (**Cap 132AF**)
- Public Health (Animals and Birds) (Chemical Residues) Regulation (**Cap 139N**)
- Three stilbenes, namely (1) **diethylstilboestrol** ((E)-αβ-diethylstilbene-4,4'-diol), (2) **hexoestrol** (meso-4,4'-(1,2-diethylethylene) and (3) **dienoestrol** ((E,E)-4,4'-(diethylideneethylene) diphenol), including their salts and esters, are **prohibited chemicals/substances**.

Source : https://www.cfs.gov.hk/english

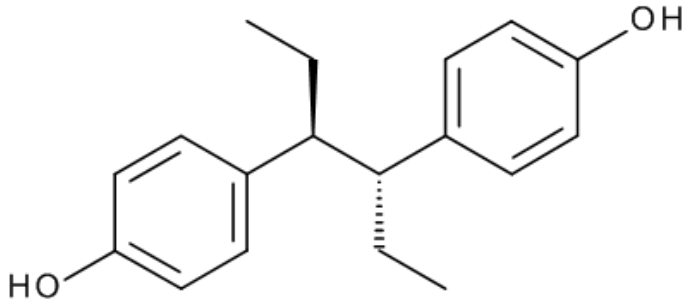
Structures



Diethylstilboestrol
(乙二基已烯雌酚)



Dienoestrol
(乙二烯雌酚)



Hexoestrol
(已烷雌酚)

Methods for Stilbenes

Available analytical methods:

■ GB 31658.9-2021

Determination of estrogen residues in animal derived foods and animal urine by liquid chromatography-tandem mass spectrometry method

■ GB/T 21981-2008

Determination of hormone multiresidues in foodstuffs of animal origin – LC-MS/MS method

■ US Department of Agriculture (CLG-ANA.02)

Determination and Confirmation of Diethylstilbestrol (DES) and Zeranol by GC/MS

EURL Guidance on Minimum Method Performance Requirements (MMPRs)

The purpose of this technical guidance is to improve and harmonise the performance of analytical methods used for the analysis of residues of unauthorised or prohibited pharmacologically active substances and for authorised pharmacologically active substances in matrices for which no use is authorised, taking into account state of the art analytical methods.

Substances	Matrix	MMPR
Diethylstilbestrol (DES)	Liver Meat (including fish)	1ppb (for all substances)
Dienestrol (DE)		
Hexestrol (HEX)		

Source : EURL Guidance on Minimum Method Performance Requirements (MMPRs) for Specific Pharmacologically Active Substances in Specific Animal Matrices (September 2020)

Test method (GB 31658.9-2021)

- **Scope:** Porcine, Bovine, Sheep, Chicken (muscle, liver & kidney), eggs, milk, pig urine, bovine urine
- **Limit of Quantitation:** 1.0 ug/kg
- **Samples were first treated with** β-Glucuronidase Hydrolysis (葡萄醣醛酸酶)
- **Extraction with acetonitrile**
- **Extract cleaned through** solid phase extraction (SPE), **eluate evaporated to dryness**
- **Residue redissolved in methanol/water solution**
- LC-MS/MS **analysis**

β-Glucuronidase Hydrolysis

- **Numerous β-glucuronidase enzymes available (e.g. abalone, bovine liver, E. coli, Helix pomatia)**

Product Information

β-Glucuronidase
Type HP-2
from *Helix pomatia*

Optimal pH: glucuronidase activity	4.5 to 5.0

ProductInformation

This β-Glucuronidase product from *E. coli* is supplied as a powder lyophilized from 10 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid, and 1 mM dithiothreitol. Polyethylene glycol is added as a stabilizer.

Molecular Weight: ~290 kDa (tetramer)[5]
68,259 Da (monomer)[6]

Optimal pH[5]: 6-7

SPE Clean-up

Benefits

- Clean-up, Concentrate, Solvent Switch

Choices of SPE

- HLB SPE & NH2 SPE (GB 31658.9-2021)
- ENVI-Carb & NH2 SPE (GB/T 21981-2008)

Test method (GB 31658.9-2021) (2)

LC condition

Column	C18 column (100 mm x 2.1 mm, 1.7 um) or equivalent
Mobile phase	Mobile phase A: Water, Mobile phase B: Acetonitrile
Flow rate	0.4 mL/min
Injection volume	10 µL

Gradient

Time	Mobile phase A (%)	Mobile phase B (%)
0	80	20
1.0	80	20
7.0	45	55
8.0	45	55
8.1	80	20
10	80	20

Test method (GB 31658.9-2021)

MS/MS condition

Ionization	Electrospray ionization (ESI)
Polarity	Negative

MRM condition

Time	MRM Transition
Diethylstilbestrol (DES)	267.0 > 251.1
	267.0 > 237.1
Dienestrol (DE)	265 > 93.0
	265 >249.1
Hexestrol (HEX)	269.0 > 134.1
	269.0 > 119.1

Internal Standards

Stilbenes	Labelled Internal Standards
Hexoestrol	Hexoestrol –d4
Diethylstilboestrol	Diethylstilboestrol-d8
Dienoestrol	Dienoestrol-d2; Dienoestrol-d6

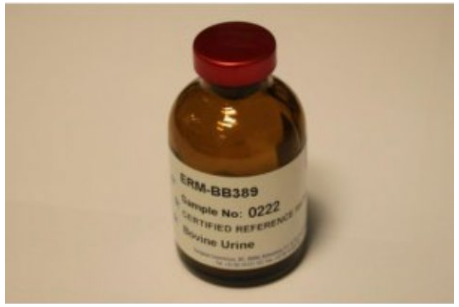
Certified Reference materials

Item	Description
Source	European Reference Materials
CRM code	ERM-BB389
Matrix	Bovine Urine

Certified values

BOVINE URINE		
	Mass Fraction	
	Certified value [µg/kg]	Uncertainty [µg/kg]
Diethylstilbestrol (DES)[1, 2)]	1.1 [3)]	0.5 [5)]
Dienestrol (DE) [1)]	5.5 [4)]	1.4 [6)]
Hexestrol (HEX) [1)]	6.1 [4)]	0.9 [6)]

1) As obtained by enzymatic deconjugation, clean-up and subsequent chromatography in combination with mass spectrometry.



Proficiency Test Program

Available proficiency test program in FAPAS

Item code	Matrix	Analyte(s)	Product Code	Approx. Size	Start Date
02512	Bovine Urine	Synthetic hormones	FCVD21-MRP9	25mL	19/06/2023

Available proficiency test program in Progetto Trieste

Item code	Matrix	Analyte(s)	Approx. Size	Start Date
U3802	Bovine Urine	Stilbenes	18mL x Test Materials	April 2023
M3408	Chicken muscle	Stilbenes	20g x 1 Test Material	November 2023

The End

Thank you for your attention !