中国農業大學
China Agricultural University

National R&D Key Programme of China (No. 2017YFE0110800)
国家重点研发计划政府间国际科技创新合作重点专项

硝基呋喃类药物半抗原设计、合成、单克隆抗体的制备及免疫分析方法的建立

Hapten design and synthesis, monoclonal antibody production and immunoassays development for nitrofurans

Xuezhi Yu 于雪芝

CAU 中国农业大学

2021.10

演讲内容 Contents

研究背景 Research Background

- 本研究来源于“中欧食品安全合作H2020 EU-China-Safe”项目中WP4

  WP4 of the *China EU food safety cooperation H2020 EU China safe* project

- 我方负责四种新型硝基呋喃类药物抗体及快速检测方法的开发

  The development of antibodies and rapid detection methods for four new nitrofuran drugs （CAU）

研究背景 Research Background

- 硝基呋喃类抗生素是一类合成的广谱抗菌药物，广泛应用于家畜、蜜蜂和水产养殖中

  Nitrofurans are a class of synthetic broad spectrum antimicrobial drugs which have been widely used in livestock, bee, and aquaculture

- 自1995年以来，由于其代谢产物对人类健康的致癌和致突变作用，禁止在所有食品动物中使用

  World wide banned, since 1995 in all food producing animals due to carcinogenic and mutagenic effects of their metabolites on human health

- 硝基呋喃类药物由于其低成本和有效性，仍然存在非法使用的情况

  Despite of the prohibition, nitrofurans are still illegally used in veterinary practice due to their low cost, and effectiveness

研究背景 Research Background

- 硝呋索尔、硝呋烯腙、硝呋酚酰肼以及硝呋地腙是目前严重威胁人类健康的四种硝基呋喃类药物

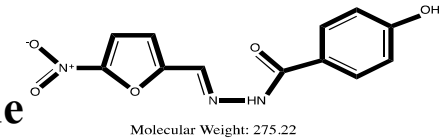
  Nifursol, Nitrovin , Nifuroxazide, and Nifuraldizone are four nitrofuran drugs that seriously threaten human health

- 仅见硝呋索尔代谢物的抗体的研究，但是抗体灵敏度不够理想，其余三种未见报道

  Only antibodies against nifurosol metabolites, but the sensitivity of antibodies is not ideal, and the other three have not been reported

研究内容 Research Contents

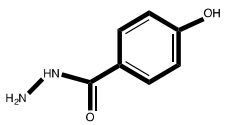
1. **硝呋酚酰肼**
   Nifuroxazide



Molecular Weight: 275.22

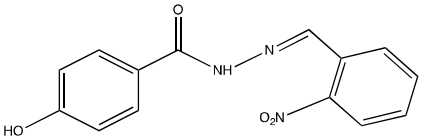
代谢物
Metabolite

HBD



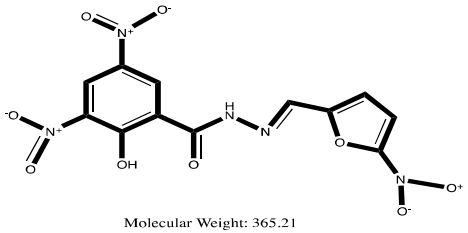
Molecular Weight: 152.15

衍生化
Derivatization



Molecular Weight: 285.26

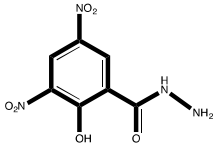
2. **硝呋索尔**
   Nifursol



Molecular Weight: 365.21

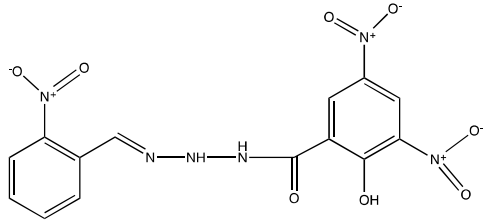
代谢物
Metabolite

DNSH



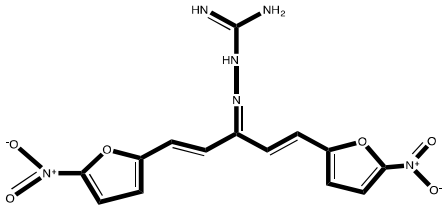
Molecular Weight: 242.15

衍生化
Derivatization



Molecular Weight: 390.27

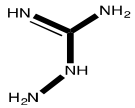
3. **硝呋烯腙**
   Nitrovin



Molecular Weight: 360.29

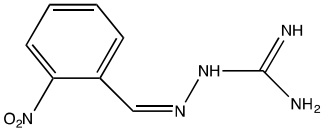
代谢物
Metabolite

AGD



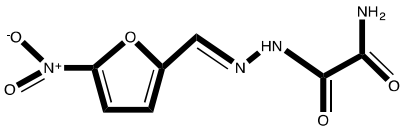
Molecular Weight: 74.09

衍生化
Derivatization



Molecular Weight: 207.19

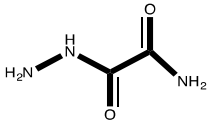
4. **硝呋地腙**
   Nifuraldizone



Molecular Weight: 226.15

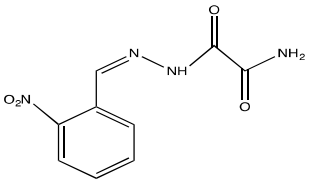
代谢物
Metabolite

OXZ



Molecular Weight: 103.08

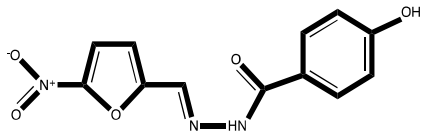
衍生化
Derivatization



Molecular Weight: 236.19

半抗原设计 Hapten Design

1. **硝呋酚酰肼**
   Nifuroxazide

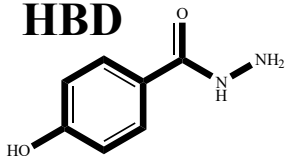


Molecular Weight: 275.22

代谢物

Metabolite

HBD

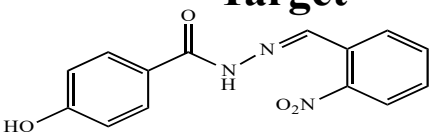


Molecular Weight: 152.15

衍生化

Derivatization

Target



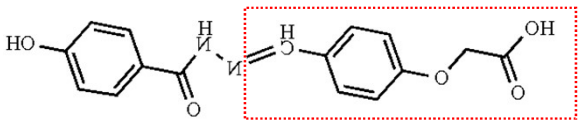
省去衍生化步骤？直接识别代谢物？

Eliminate derivatization steps? Direct identification of metabolites?

?

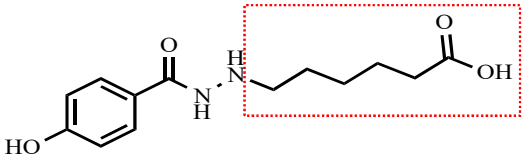
半抗原设计 Hapten Design

A



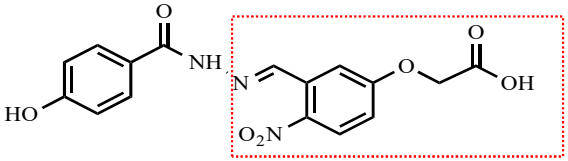
Molecular Weight: 314.30

B



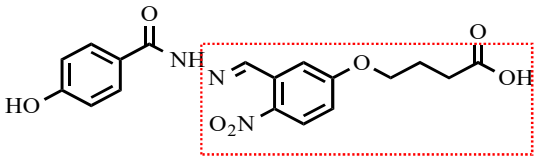
Molecular Weight: 266.30

C



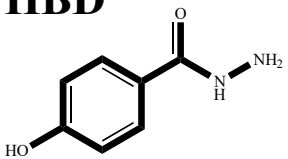
Molecular Weight: 359.29

D



Molecular Weight: 387.35

HBD



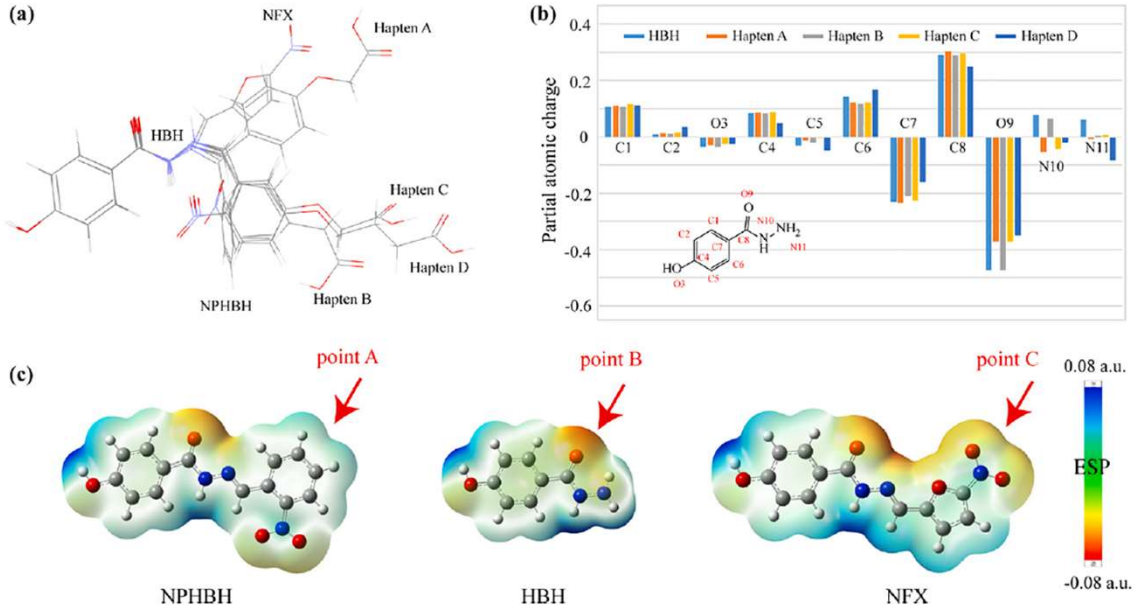
Molecular Weight: 152.15

- 结构、大小、空间构像、电子构型、疏水性等尽量与靶标分子相同
  The structure, size, spatial configuration, electronic configuration and hydrophobicity shall be as much as possible the same as the HBD
- 引入间隔臂，使其远离载体蛋白，最大限度暴露于免疫系统
  The spacer arm is introduced away from the carrier protein to maximize exposure to the immune system
- 由于HBD结构非常小且简单，线性脂肪族间隔臂可能不足以引发显著的抗体反应，需要引入适量且体积大的间隔臂
  For the HBH hapten design, it is envisaged that a linear aliphatic spacer arm may not be enough to elicit a significant antibody response due to the infinitesimal and simple structure. The introduction of appropriately complicated and bulked structure is expected such as phenyl group

半抗原设计 Hapten Design

构象和原子电荷研究半抗原的合理性

- 根据最低构象排列
- 如图2a所示，半抗原和目标化合物HBD的完全重叠，说明在HBD的N11位置引入间隔臂几乎不会影响HBD的构象特征
- 不同间隔臂的引入并未引起HBD原子电荷的显著变化（图2b）
- 四种半抗原可用于制备HBH抗体
- 半抗原C与HBD的原子电荷分布最为相似，推断Hapten C为最佳半抗原



The rationality of the designed haptens was studied by conformational studies and electronic analysis

- Arranged according to the lowest conformation
- As shown in Fig. 2a, the complete overlap of haptens and HBD indicates that the introduction of spacer arm at N11 position of HBD will hardly affect the conformational characteristics of HBD
- The introduction of different spacer arms did not cause significant changes in the charge of HBD atoms (Fig. 2b)
- Four haptens can be used to prepare HBH antibody.
- Hapten C and HBD have the most similar atomic charge distribution, so hapten C may be the best hapten

半抗原设计 Hapten Design

- 质谱结果表明半抗原合成成功
- **Results of the MS shows that haptens were synthesized successfully**

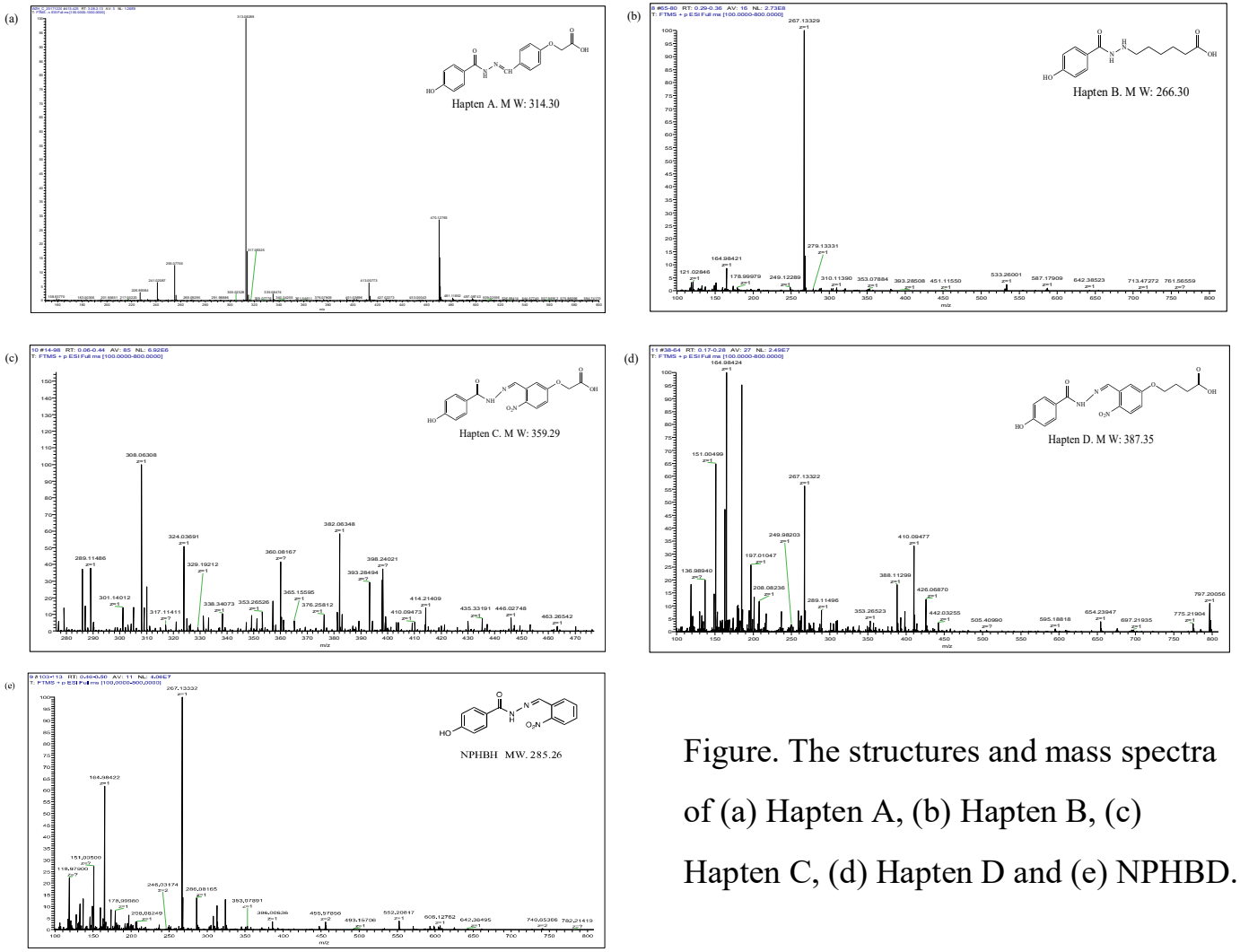


Figure. The structures and mass spectra of (a) Hapten A, (b) Hapten B, (c) Hapten C, (d) Hapten D and (e) NPHBD.

半抗原设计 Hapten Design

- **核磁结果表明半抗原合成成功**
- **Results of the NMR shows that haptens were synthesized successfully**

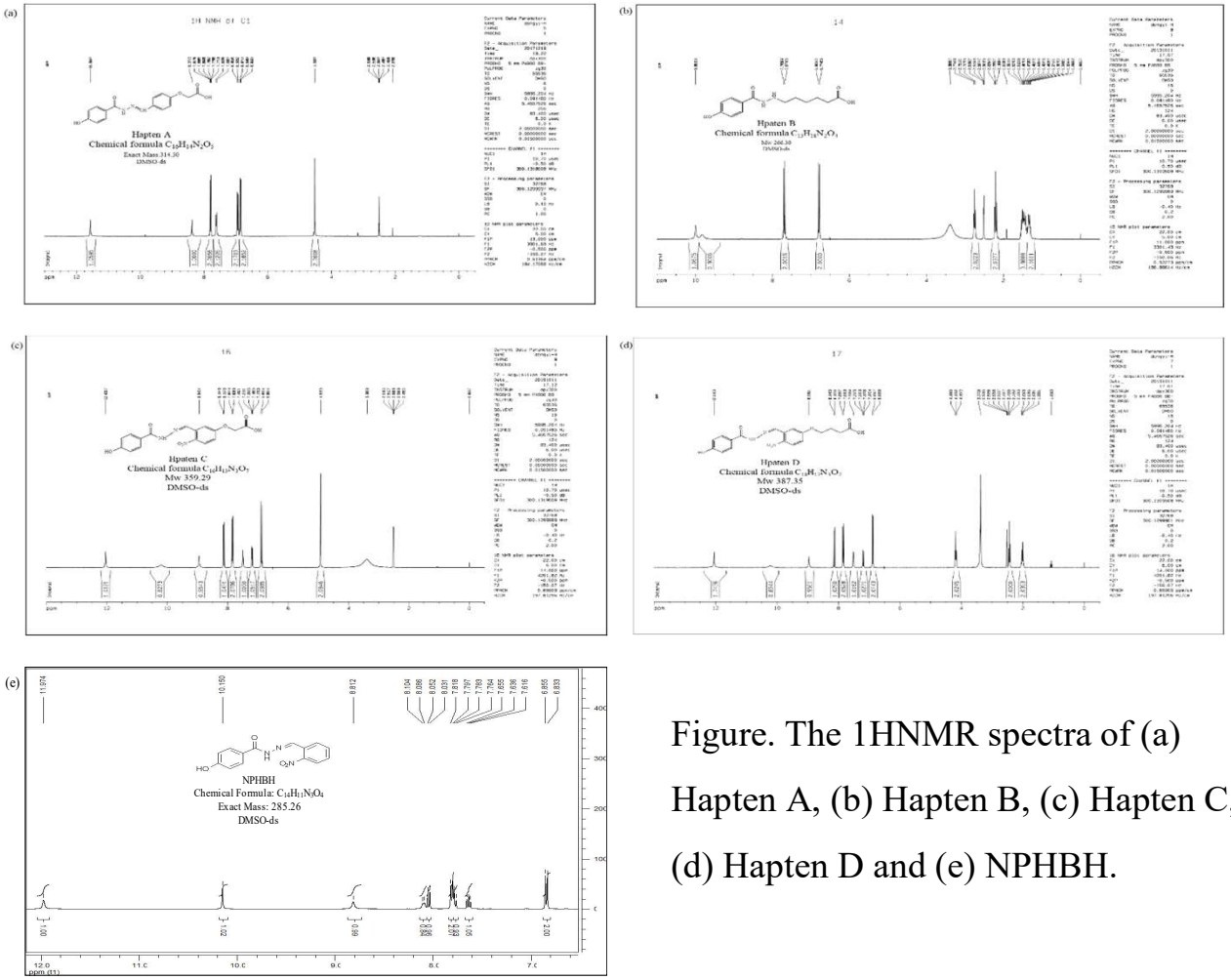


Figure. The 1HNMR spectra of (a) Hapten A, (b) Hapten B, (c) Hapten C, (d) Hapten D and (e) NPHBH.

抗原合成 Antigen Preparation

- **MALDI-TOF-MS结果表明抗原合成成功，偶联比分别为11.4:1 (Hapten A-BSA), 14.4:1 (Hapten B-BSA), 8.2:1 (Hapten C- BSA) 以及 10.4:1**
- **Results of the MALDI-TOF-MS shows that antigens were synthesized successfully. The calculated molar ratios of the hapten-BSA were 11.4:1 (Hapten A-BSA), 14.4:1 (Hapten B-BSA), 8.2:1 (Hapten C- BSA) and 10.4:1 (Hapten D-BSA), respectively.**

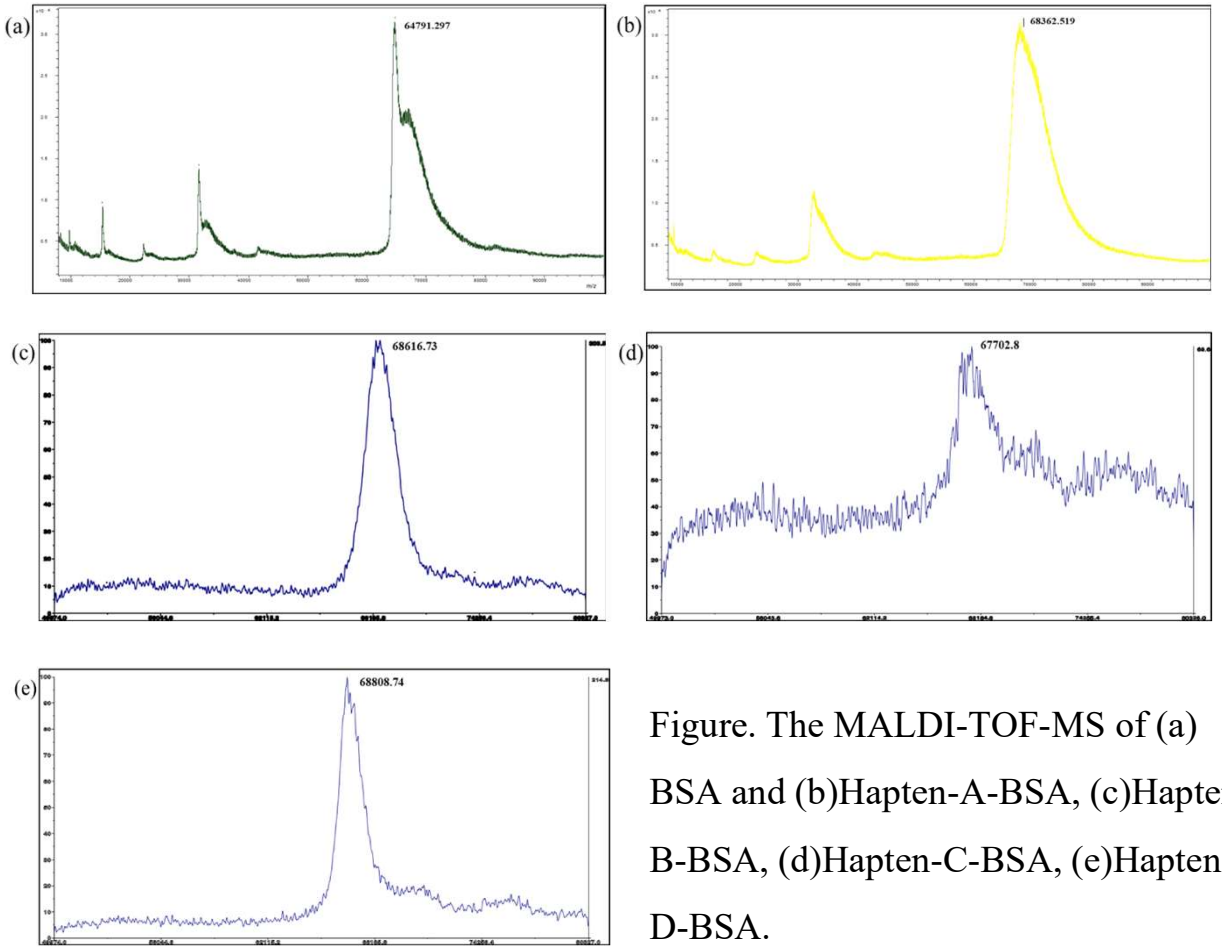


Figure. The MALDI-TOF-MS of (a) BSA and (b)Hapten-A-BSA, (c)Hapten-B-BSA, (d)Hapten-C-BSA, (e)Hapten-D-BSA.

抗体制备 Antibody Preparation

	Coating Antigen A-BSA			Coating Antigen B-BSA			Coating Antigen C-BSA			Coating Antigen D-BSA		
	Titter	IC_{50} for HBD (ng mL^{-1})	IC_{50} for NPHBD) (ng mL^{-1})	Titter	IC_{50} for HBD (ng mL^{-1})	IC_{50} for NPHBD(ng mL^{-1})	Titter	IC_{50} for HBD (ng mL^{-1})	IC_{50} for NPHBD(ng mL^{-1})	Titter	IC_{50} for HBD (ng mL^{-1})	IC_{50} for NPHBD(ng mL^{-1})
Hapten-A-1#	1/1000	758.5	433.2	1/1000	450.4	359.3	1/1000	698.2	472.8	1/1000	689.6	445.4
Hapten-A-2#	1/1000	744.3	443.5	1/1000	562.6	396.6	1/1000	795.3	456.9	1/1000	951.4	529.8
Hapten-A-3#	1/1100	861.5	522.7	1/1000	673.5	441.2	1/1000	994.2	511.5	1/1500	857.7	498.4
Hapten-A-4#	1/1000	753.9	426.1	1/1000	543.3	478.5	1/1000	931.3	519.0	1/1100	858.1	434.2
Hapten-A-5#	1/1200	949.1	589.3	1/1000	869.1	447.6	1/1500	892.5	654.3	1/1000	940.3	541.1
Hapten-B-1#	1/10000	190.5	138.3	1/10000	65.5	48.4	1/8000	80.3	61.4	1/10000	75.8	65.8
Hapten-B-2#	1/10000	187.4	140.2	1/12000	55.4	52.5	1/8000	73.9	69.6	1/8000	62.1	52.1
Hapten-B-3#	1/10000	177.6	135.4	1/10000	48.3	35.3	1/10000	84.5	56.5	1/10000	63.6	53.6
Hapten-B-4#	1/10000	176.9	141.3	1/13000	71.8	63.7	1/10000	75.6	66.4	1/10000	85.8	55.8
Hapten-B-5#	1/10000	157.1	144.1	1/10000	55.9	46.9	1/12000	69.8	49.7	1/11000	74.5	64.5
Hapten-C-1#	1/10000	146.5	92.3	1/12000	18.2	14.1	1/14000	25.2	12.3	1/12000	33.2	14.6
Hapten-C-2#	1/10000	133.5	94.5	1/15000	25.3	13.2	1/15000	21.8	13.5	1/13000	36.3	21.3
Hapten-C-3#	1/10000	126.3	98.7	1/20000	23.2	11.3	1/14000	24.3	18.6	1/12000	34.2	19.3
Hapten-C-4#	1/10000	118.2	93.4	1/20000	11.5	5.5	1/16000	21.8	14.4	1/11000	32.4	15.5
Hapten-C-5#	**1/10000**	**115.8**	**82.7**	**1/20000**	**3.9**	**2.5**	**1/17000**	**23.4**	**15.3**	**1/10000**	**18.9**	**12.6**
Hapten-D-1#	1/10000	181.3	145.2	1/15000	53.8	46.4	1/11000	86.3	69.5	1/13000	71.8	63.6
Hapten-D-2#	1/10000	199.2	162.7	1/11000	46.3	33.2	1/13000	78.5	62.6	1/10000	78.3	53.9
Hapten-D-3#	1/10000	193.1	159.1	1/10000	58.5	48.1	1/10000	72.9	51.8	1/10000	71.4	51.1
Hapten-D-4#	1/11000	179.9	136.3	1/10000	39.5	33.6	1/10000	85.6	53.5	1/10000	67.5	41.3
Hapten-D-5#	1/12000	188.4	141.9	1/10000	53.2	47.4	1/10000	96.4	64.7	1/10000	87.1	44.5

- **经过免疫Hapten C-5#小鼠免疫效果最好，用于后续细胞融合**
- **Hapten C-#5 Mice was selected for cell fusion due to high sensitivity towards HBD and derivative**

抗体制备 Antibody Preparation

mAb	Hapten-A-BSA		Hapten-B-BSA		Hapten-C-BSA		Hapten-D-BSA	
	IC_{50} for HBD(ng mL^{-1})	IC_{50} for NPHBD(ng mL^{-1})	IC_{50} for HBD(ng mL^{-1})	IC_{50} NPHBD (ng mL^{-1})	IC_{50} for HBD (ng mL^{-1})	IC_{50} for NPHBD(ng mL^{-1})	IC_{50} for HBD (ng mL^{-1})	IC_{50} for NPHBD (ng mL^{-1})
2F12	0.46	0.23	**0.25**	**0.10**	0.27	0.19	0.31	0.15
4D11	0.63	0.26	0.33	0.11	0.28	0.23	0.33	0.13
6H6	0.69	0.31	0.33	0.14	0.32	0.21	0.35	0.14
2B12	0.68	0.28	0.34	0.16	0.35	0.24	0.38	0.19

- 共获得4株单克隆抗体，经优化2F12性能最好，Hapten-B-BSA为最优包被原，HBD及其衍生物的IC_{50}值分别为 0.25 和 0.10 ng mL^{-1} 。
- The four mAbs derived from Hapten-C, 2F12 was selected as high sensitive mAb and the Hapten-B-BSA was the best coating antigen. The IC_{50} values were detected 0.25 for HBD and 0.10 ng mL^{-1} for the derivative

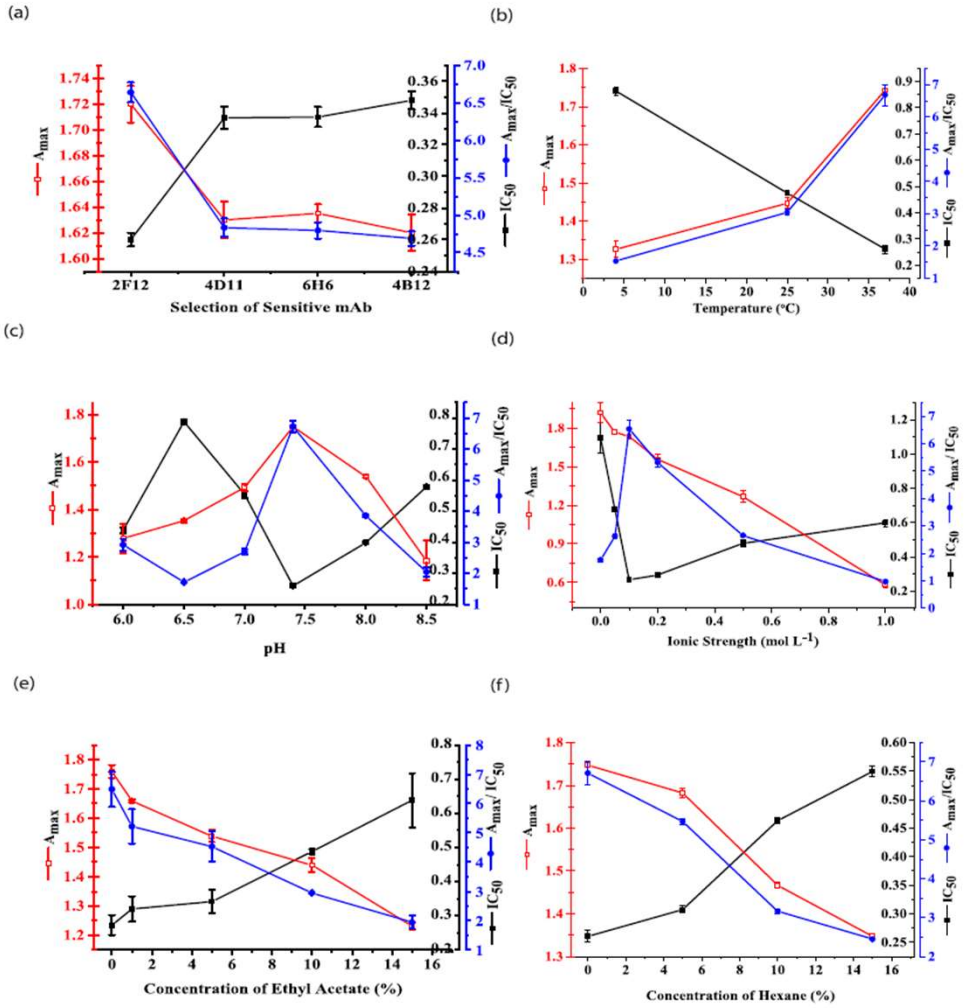
抗体制备 Antibody Preparation

- 单克隆抗体2F12仅与HBD及其衍生物、以及硝呋酚酰肼有交叉，与其他物质无交叉，特异性良好
- 不衍生化，直接检测代谢物
- **The IC_{50} values and cross reactivity of the mAb2F12 evaluated with HBD, its derivative and NFX. The negligible cross reactivity were obtained for other compounds.**
- **Detection of metabolites without derivatization**

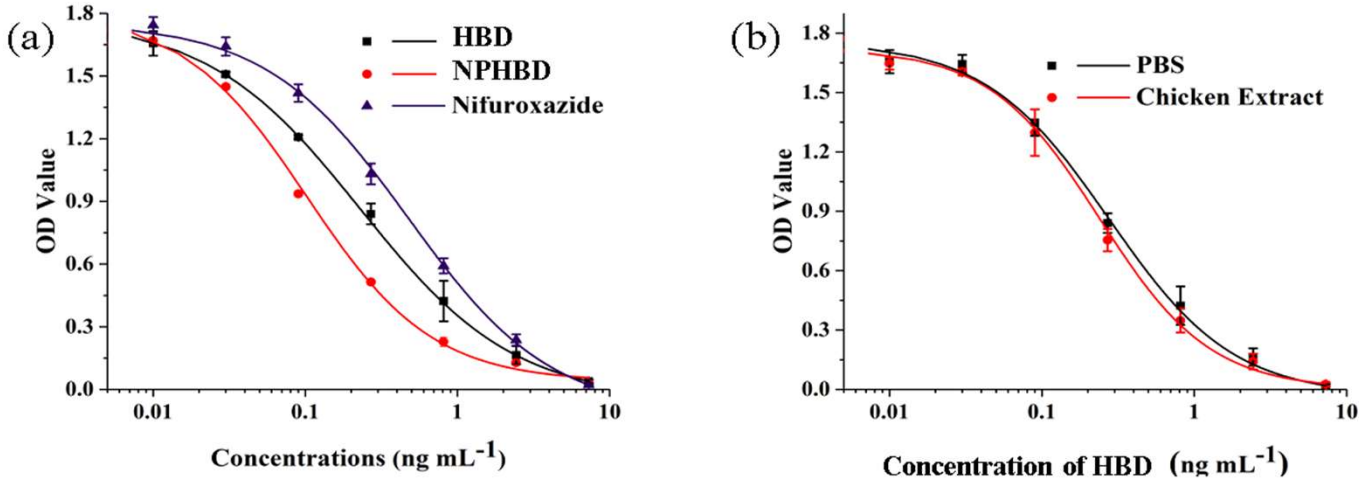
Compounds	IC_{50} (ng mL^{-1})	Cross Reactivity (%)
HBD	0.25	100
NPHBD	0.10	250
Nafuroxazide	0.44	56.81
2-NBA	>1000	<0.02
4-FPA	>1000	<0.02
DNSH	>1000	<0.02
AMOZ	>1000	<0.02
AHD	>1000	<0.02
OXZ	>1000	<0.02
SEM	>1000	<0.02
AGD	>1000	<0.02
AOZ	>1000	<0.02
Nitrovin	>1000	<0.02

试剂盒开发 ELISA Kit Development

- 经过优化，最佳反应条件分别为37℃、pH7.4、离子强度小于0.1 M，不添加有机试剂
- **After optimization, the optimum reaction conditions were 37 ℃, pH 7.4 and ionic strength < 0.1 M, no organic reagent, respectively.**



试剂盒开发 ELISA Kit Development



- 优化后建立标准曲线，HBD 的IC_{50}值为0.25 ng mL^{-1}，可适用于鸡肉中硝呋酚酰肼代谢物的检测
- **The standard curves wre established after optimization. The IC_{50} value of HBD was 0.25 ng ml^{-1}, which was suitable for the detection of nifuraxazide metabolites in chicken**

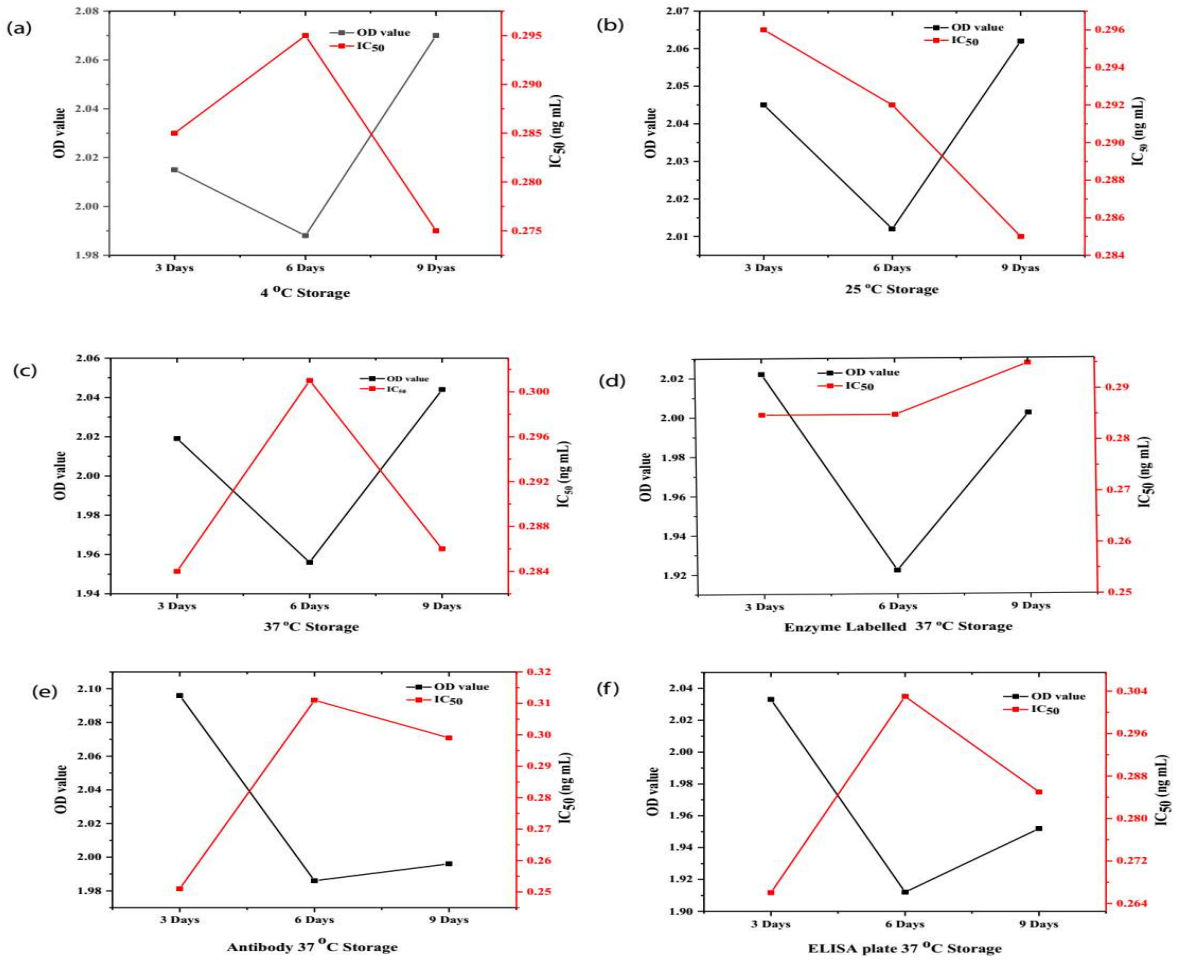
试剂盒开发 ELISA Kit Development

Matrix	Concentrations spiked (μg kg^{-1})	Recoveries (%)	CVs (%)
Chicken	0.5	98.3	10.3
	1.0	99.4	8.5
	2.5	105.6	5.5

- **鸡肉中HBD添加回收率介于98.3-105.6%，CV值小于10.3%**
- **The recovery rate of HBD in chicken was 98.3-105.6%, and the CV value was less than 10.3%**

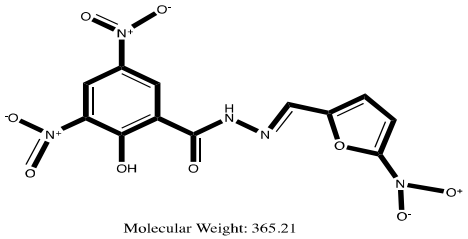
试剂盒开发 ELISA Kit Development

- 试剂盒稳定性试验表明，试剂盒可以在4℃条件下保存一年
- **After assessment it was concluded that the ELISA kit can be store for one year at 4℃.**



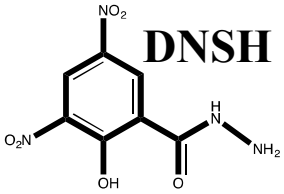
半抗原设计 Hapten Design

2. 硝呋索尔
Nifursol



Molecular Weight: 365.21

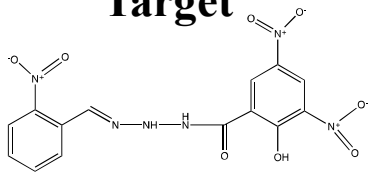
代谢物
Metabolite



Molecular Weight: 242.15

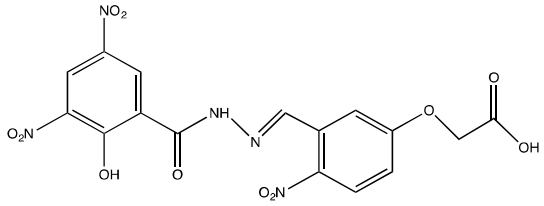
衍生化
Derivatization

Target



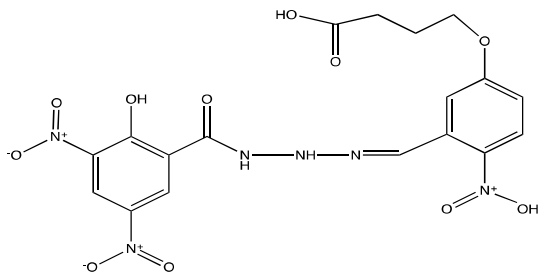
Molecular Weight: 390.27

A



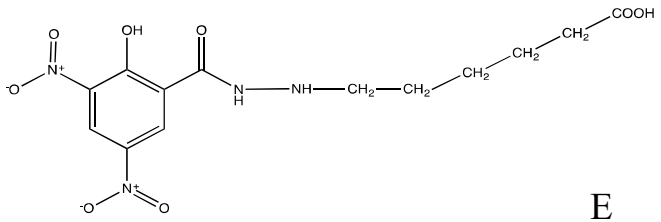
Molecular Weight: 449.29

B



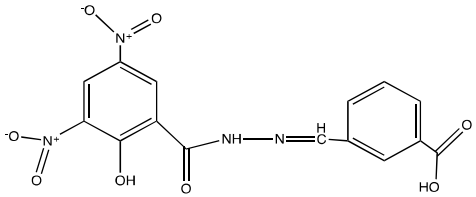
Molecular Weight: 493.36

C



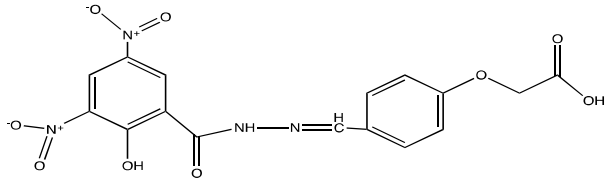
Molecular Weight: 356.29

D



Molecular Weight: 374.27

E



Molecular Weight: 404.29

抗体制备 Antibody Preparation

Haptens/Mice#	Coating Antigen-A-BSA			Coating Antigen-B-BSA			Coating Antigen-C-BSA			Coating Antigen-D-BSA			Coating Antigen-E-BSA		
	Titter	IC_{50} for DNSH $ngml^{-1}$	IC_{50} for NPDNSH $ngml^{-1}$	Titter	IC_{50} for DNSH $ngml^{-1}$	IC_{50} for NPDNSH $ngml^{-1}$	Titter	IC_{50} for DNSH $ngml^{-1}$	IC_{50} for NPDNSH $ngml^{-1}$	Titter	IC_{50} for DNSH $ngml^{-1}$	IC_{50} for NPDNSH $ngml^{-1}$	Titter	IC_{50} for DNSH $ngml^{-1}$	IC_{50} for NPDNSH $ngml^{-1}$
Hapten-A-1#	1/1000	978.4	825.8	1/1000	931.2	747.2	1/500	> 1000	> 1000	1/1000	761.3	853.2	1/1000	943.2	864.3
Hapten-A-2#	1/1000	983.2	826.1	1/1000	875.4	885.5	1/500	> 1000	> 1000	1/1000	866.4	645.3	1/1000	862.3	856.4
Hapten-A-3#	1/1000	956.1	835.6	1/1000	842.3	753.4	1/500	> 1000	> 1000	1/1000	753.1	634.2	1/1000	946.3	843.5
Hapten-A-4#	1/1000	836.2	772.9	1/1000	836.5	657.7	1/500	> 1000	> 1000	1/1000	755.5	645.3	1/1000	948.4	724.5
Hapten-A-5#	1/1000	887.9	877.5	1/1000	778.3	553.2	1/500	> 1000	> 1000	1/1000	845.2	642.1	1/1000	852.1	737.6
Hapten-B-1#	1/1000	883.6	729.1	1/1000	754.5	566.5	1/500	> 1000	> 1000	1/1000	914.5	674.3	1/1000	886.7	898.7
Hapten-B-2#	1/1000	842.1	863.9	1/1000	843.9	643.2	1/500	> 1000	> 1000	1/1000	886.5	643.4	1/1000	846.6	745.6
Hapten-B-3#	1/1000	868.3	757.7	1/1000	757.8	536.4	1/500	> 1000	> 1000	1/1000	943.6	687.7	1/1000	875.7	787.7
Hapten-B-4#	1/1000	843.8	864.8	1/1000	728.4	642.5	1/500	> 1000	> 1000	1/1000	786.5	845.3	1/1000	853.5	863.5
Hapten-B-5#	1/1000	779.4	731.8	1/1000	888.6	535.6	1/500	> 1000	> 1000	1/1000	778.9	756.5	1/1000	766.7	789.8
Hapten-C-1#	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000
Hapten-C-2#	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000
Hapten-C-3#	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000
Hapten-C-4#	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000
Hapten-C-5#	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000	1/500	> 1000	> 1000
Hapten-D-1#	1/1000	764.4	754.2	1/1100	643.2	534.2	1/500	> 1000	> 1000	1/6000	267.8	232.1	1/8000	163.3	114.5
Hapten-D-2#	1/1000	834.8	765.4	1/1200	565.3	555.4	1/500	> 1000	> 1000	1/4000	246.7	255.4	1/4200	389.7	226.4
Hapten-D-3#	1/1000	813.7	742.1	1/2000	464.6	661.2	1/500	> 1000	> 1000	1/3000	376.8	344.5	1/5000	225.4	119.8
Hapten-D-4#	1/1000	766.5	757.3	1/2000	545.7	546.4	1/500	> 1000	> 1000	1/1500	334.7	256.6	1/4000	337.2	231.3
Hapten-D-5#	1/1000	443.5	643.2	1/2000	453.3	426.4	1/500	> 1000	> 1000	1/1200	766.6	542.1	1/4000	336.3	224.7
Hapten-E-1#	1/1500	497.6	355.4	1/1000	566.5	557.5	1/500	> 1000	> 1000	1/5000	544.2	331.9	1/10000	89.2	36.1
Hapten-E-2#	1/1500	343.2	353.7	1/2000	635.2	642.6	1/500	> 1000	> 1000	1/5000	667.4	356.6	1/10000	177.4	52.3
Hapten-E-3#	1/1500	557.8	466.5	1/2000	447.6	587.7	1/500	> 1000	> 1000	1/5000	435.7	238.1	1/12000	76.3	48.3
Hapten-E-4#	1/2000	635.7	655.6	1/2000	532.1	543.5	1/500	> 1000	> 1000	1/5000	236.3	169.5	1/10000	66.4	44.5
Hapten-E-5#	1/2000	357.6	276.5	1/2000	335.4	248.4	1/500	> 1000	> 1000	1/5000	145.4	113.5	**1/15000**	**46.2**	**31.3**

- **经过免疫Hapten E-5#小鼠免疫效果最好，用于后续细胞融合**
- **Hapten E-#5 Mice was selected for cell fusion due to high sensitivity towards DNSH and derivative**

抗体制备 Antibody Preparation

mAb		Coating Antigen-D-BSA				Coating Antigen-E-BSA			
		OD values	mAb dilution	IC_{50} for DNSH ngm^{l-1}	IC_{50} for NPDNSH ngm^{l-1}	OD values	mAb dilution	IC_{50} for DNSH ngm^{l-1}	IC_{50} for NPDNSH ngm^{l-1}
Hapten-D-KLH	2G5	2.66	1/1000	8.39	7.54	2.31	1/1000	6.43	5.78
		2.19	1/3000	6.87	6.86	2.12	1/3000	4.27	4.93
		1.75	1/9000	4.51	3.59	1.73	1/9000	3.35	3.19
		0.78	1/15000	1.01	1.38	1.12	1/15000	1.29	1.02
		0.56	1/20000	0.33	0.27	0.32	1/20000	0.24	0.14
	8D6	5.28	1/1000	7.24	6.17	4.27	1/1000	6.59	5.38
		3.17	1/3000	5.73	5.39	3.58	1/3000	4.23	3.53
		1.93	1/9000	4.29	3.76	2.38	1/9000	2.59	2.17
		0.86	1/15000	1.68	1.02	1.48	1/15000	1.01	1.08
		0.24	1/20000	0.47	0.35	0.22	1/20000	0.57	0.58
Hapten-E-KLH	2B8	4.63	1/3000	10.43	8.35	4.38	1/3000	5.35	4.26
		3.46	1/9000	8.46	7.43	3.78	1/9000	4.54	3.56
		2.24	1/18000	6.59	5.14	2.34	1/18000	3.36	2.47
		1.67	1/27000	4.47	2.06	1.74	1/27000	1.61	0.88
		0.63	1/36000	0.57	0.38	0.46	1/36000	0.70	0.36
	3H10	4.57	1/3000	9.49	8.18	4.37	1/3000	8.27	7.39
		3.46	1/9000	6.58	5.39	3.56	1/9000	6.55	6.57
		2.53	1/18000	3.53	3.12	2.35	1/18000	4.34	4.16
		1.75	1/27000	1.36	1.13	**1.71**	**1/27000**	**0.91**	**0.60**
		0.89	1/36000	0.78	0.29	0.76	1/36000	0.76	0.75

- **共获得4株单克隆抗体，经优化3H10性能最好，Hapten-E-BSA为最优包被原，DNSH及其衍生物的IC_{50}值分别为 0.91 和 0.60 ng mL^{-1} 。**
- **The four mAbs derived from Hapten-E, 3H10 was selected as high sensitive mAb and the Hapten-E-BSA was the best coating antigen. The IC_{50} values were detected 0.91 for DNSH and 0.60 ng mL^{-1} for the derivative**

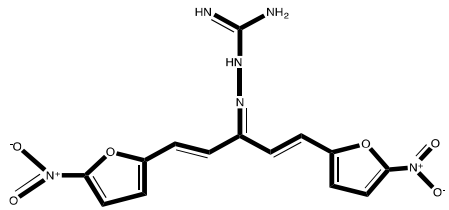
抗体制备 Antibody Preparation

- 单克隆抗体3H10仅与DNSH及其衍生物以及硝呋索尔有交叉，与其他物质无交叉，特异性良好
- 不衍生化，直接检测代谢物
- **The IC_{50} values and cross reactivity of the mAb3H10 evaluated with DNSH, its derivative and nifursol. The negligible cross reactivity were obtained for other compounds.**
- **Detection of metabolites without derivatization**

检测物质	IC_{50} (ng mL^{-1})	CR (%)
DNSH	**0.91**	**100**
NPDNSH	**0.72**	**126.38**
Nifursol	**0.85**	**107.05**
OXZ	>1000	<0.09
NPOXZ	>1000	<0.09
AMOZ	>1000	<0.09
HBH	>1000	<0.09
NPHBH	>1000	<0.09
AOZ	>1000	<0.09
2-NBA	>1000	<0.09
3-CBA	>1000	<0.09
4-FPA	>1000	<0.09
AHD	>1000	<0.09
SEM	>1000	<0.09
AGD	>1000	<0.09
Nifuraldizone	>1000	<0.09
Nitrovin	>1000	<0.09
Nifuroxazide	>1000	<0.09

半抗原设计 Hapten Design

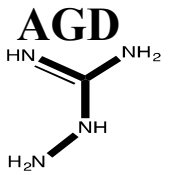
3. 硝呋烯腙
Nitrovin



Molecular Weight: 360.29

代谢物
Metabolite

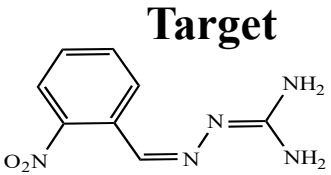
AGD



Molecular Weight: 74.09

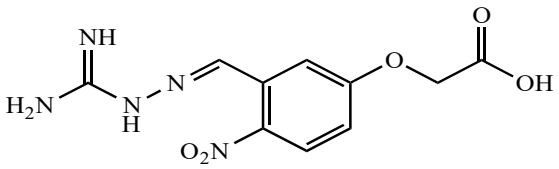
衍生化
Derivatization

Target



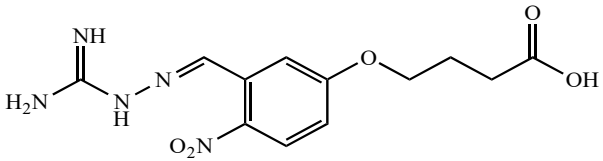
Molecular Weight: 207.19

A



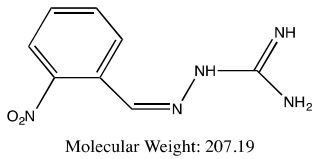
Molecular Weight: 281.23

B



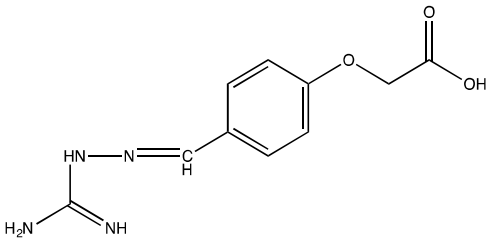
Molecular Weight: 309.28

C



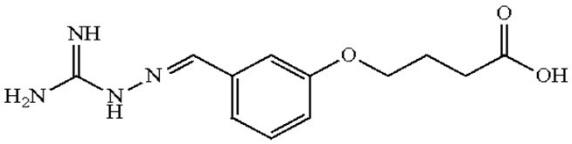
Molecular Weight: 207.19

D



Molecular Weight: 236.23

E



Molecular Weight: 263.29

抗体制备 Antibody Preparation

Haptens/Mice#	Coating Antigen AGD-A-BSA			Coating Antigen AGD-B-BSA		
	Titter	IC_{50} for AGD(ng mL^{-1})	IC_{50} for NPAGD) (ng mL^{-1})	Titter	IC_{50} for AGD (ng mL^{-1})	IC_{50} for NPAGD(ng mL^{-1})
AGD-A-1#	1/1000	865.4	989.3	1/3000	> 1000	> 1000
AGD-A-2#	1/1000	764.5	976.4	1/3000	> 1000	> 1000
AGD-A-3#	1/1000	897.3	895.5	1/3000	> 1000	> 1000
AGD-A-4#	1/1000	888.1	989.8	1/3000	> 1000	> 1000
AGD-A-5#	1/1000	789.2	999.4	1/3000	> 1000	> 1000
AGD-B-1#	1/8000	146.5	178.4	1/5000	564.3	764.4
AGD-B-2#	1/8000	158.3	187.8	1/5000	459.5	832.1
AGD-B-3#	1/8000	49.2	58.5	1/5000	343.2	569.9
AGD-B-4#	1/8000	157.7	179.1	1/5000	431.1	765.3
AGD-B-5#	1/8000	191.9	224.6	1/5000	478.9	345.5

- **AGD-B-3#小鼠免疫效果最好，用于后续细胞融合**

  AGD-B-3# mice was selected for cell fusion due to high sensitivity towards AGD and derivative

抗体制备 Antibody Preparation

mAb	Coating Antigen –AGD-A-BSA				Coating Antigen –AGD-B-BSA			
	OD	Titter	IC_{50} for AGD (ng mL^{-1})	IC_{50} for NPAGD (ng mL^{-1})	OD	Titter	IC_{50} for AGD (ng mL^{-1})	IC_{50} for NPAGD (ng mL^{-1})
2F9	4.32	1/1000	8.44	9.23	5.37	1/1000	4.55	6.28
	3.46	1/3000	6.23	7.66	4.69	1/3000	3.67	5.25
	2.39	1/9000	2.59	3.59	3.48	1/9000	2.36	3.86
	1.23	1/27000	1.13	2.88	1.43	1/27000	1.11	2.12
	0.44	1/81000	0.70	1.49	1.34	1/81000	1.04	1.83
4G8	4.54	1/1000	6.37	7.26	5.38	1/1000	6.18	7.23
	3.36	1/3000	4.55	5.47	4.62	1/3000	4.37	5.39
	2.79	1/9000	3.22	3.65	3.33	1/9000	3.37	4.53
	1.55	1/27000	0.82	0.93	2.12	1/27000	2.42	2.84
	0.43	1/81000	0.34	0.89	0.83	1/81000	0.67	0.83

- 共获得两株单克隆抗体，2F9和4G8，其中4G8性能更优，经优化后，对AGD和NPAGD的IC_{50}分别为0.82，0.93 ng mL^{-1}

  Two mAbs were obtained from the two mice. The 4G8 has the better sensitivity, IC_{50} for AGD and NPAGD were 0.82，0.93 ng mL^{-1}

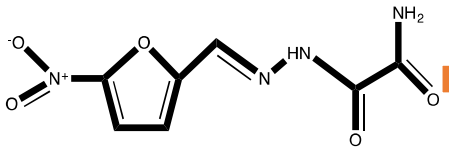
抗体制备 Antibody Preparation

- 单克隆抗体4G8仅与AGD、其衍生物以及母体药物硝呋烯腙有交叉，与其他物质无交叉，特异性良好
- 不衍生化，直接检测代谢物
- **The IC_{50} values and cross reactivity of the mAb 4G8 evaluated with AGD, its derivative and nitrovin. The negligible cross reactivity were obtained for other compounds**
- **Detection of metabolites without derivatization**

Compounds	IC_{50} (ng mL^{-1})	Cross Reactivity (%)
AGD	**0.80**	**100**
NPAGD	**0.87**	**91.95**
Nitrovin	**0.89**	**89.88**
DNSH	>1000	<0.08
NPOXZ	>1000	<0.08
AMOZ	>1000	<0.08
HBD	>1000	<0.08
NPHBD	>1000	<0.08
NPDNSH	>1000	<0.08
2-NBA	>1000	<0.08
AOZ	>1000	<0.08
4-FPA	>1000	<0.08
AHD	>1000	<0.08
SEM	>1000	<0.08
OXZ	>1000	<0.08
Nifuroxazide	>1000	<0.08
Nifuraldizone	>1000	<0.08

半抗原设计 Hapten Design

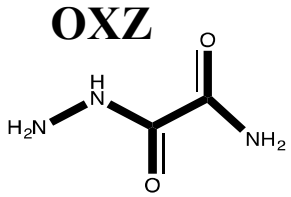
4. 硝呋地腙
Nifuraldizone



Molecular Weight: 226.15

代谢物
Metabolite

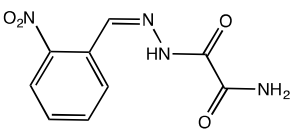
OXZ



Molecular Weight: 103.08

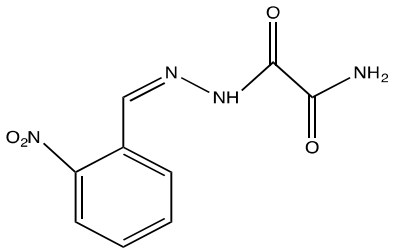
衍生化
Derivatization

Target



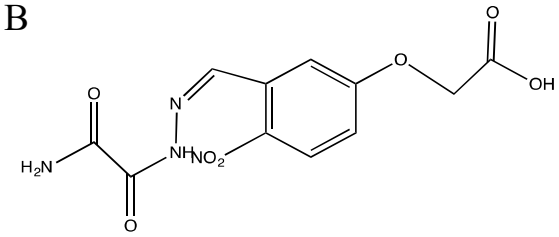
Molecular Weight: 236.19

A



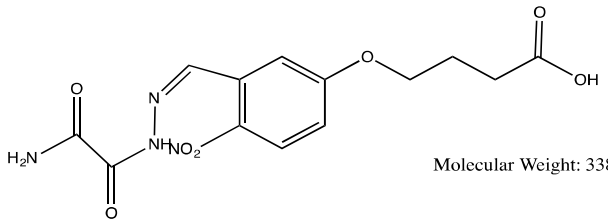
Molecular Weight: 236.19

B



Molecular Weight: 310.22

C



Molecular Weight: 338.28

抗体制备 Antibody Preparation

Haptens/Mice#	Coating Antigen 1-BSA			Coating Antigen 2-BSA		
	Titter	IC_{50} for OXZ (ng mL^{-1})	IC_{50} for NPOXZ) (ng mL^{-1})	Titter	IC_{50} for OXZ (ng mL^{-1})	IC_{50} for NPOXZ(ng mL^{-1})
Hapten-1-1#	1/15000	375.5	133.2	1/10000	121.2	89.3
Hapten-1-2#	1/15000	256.3	143.5	1/12000	189.7	96.6
Hapten-1-3#	1/14000	461.5	122.7	1/14000	211.4	71.2
Hapten-1-4#	1/13000	353.9	126.1	1/10000	135.2	68.5
Hapten-1-5#	1/18000	519.1	189.3	**1/16000**	**88.1**	**47.6**
Hapten-2-1#	1/20000	50.5	31.3	1/11000	278.3	148.9
Hapten-2-2#	**1/27000**	**28.1**	**12.4**	1/20000	158.3	112.6
Hapten-2-3#	1/20000	98.2	68.3	1/19000	198.6	125.8
Hapten-2-4#	1/20000	156.2	65.6	1/15000	176.5	123.2
Hapten-2-5#	1/20000	133.1	46.3	1/15000	146.4	126.1

- **Hapten-1-5#小鼠以及Hapten-2-2#小鼠免疫效果最好，用于后续细胞融合**

  Hapten-1-5# mice and hapten-2-2# mice were selected for cell fusion due to high sensitivity towards OXZ and derivative

抗体制备 Antibody Preparation

	Coating Antigen Hapten-1-BSA				Coating Antigen Hapten-2-BSA			
mAb	OD values	mAb dilutions	IC_{50} for OXZ (ng mL^{-1})	IC_{50} for NPOXZ (ng mL^{-1})	OD value	mAb dilutions	IC_{50} for OXZ (ng mL^{-1})	IC_{50} for NPOXZ (ng mL^{-1})
Hapten-1- 2B6	5.24	1/1000	7.39	7.12	4.29	1/1000	6.22	5.37
	3.23	1/3000	6.51	6.22	3.44	1/3000	4.76	4.89
	2.18	1/9000	3.44	2.35	2.11	1/9000	3.04	2.76
	1.79	1/27000	2.13	1.87	1.90	1/27000	1.78	1.34
	1.45	1/81000	1.89	1.46	**1.53**	**1/81000**	**1.42**	**0.95**
	0.56	1/243000	0.77	0.54	0.49	1/243000	0.76	0.23
Hapten-2-2D9	5.45	1/1000	6.33	6.03	5.44	1/1000	7.36	6.44
	3.33	1/3000	4.62	4.28	4.32	1/3000	5.11	4.62
	2.39	1/9000	3.34	2.97	3.77	1/9000	3.78	3.74
	1.87	1/27000	1.25	1.10	2.12	1/27000	2.14	2.23
	1.68	**1/81000**	**0.66**	**0.21**	1.59	1/81000	0.98	0.61
	0.67	1/243000	0.21	0.09	0.74	1/243000	0.36	0.28

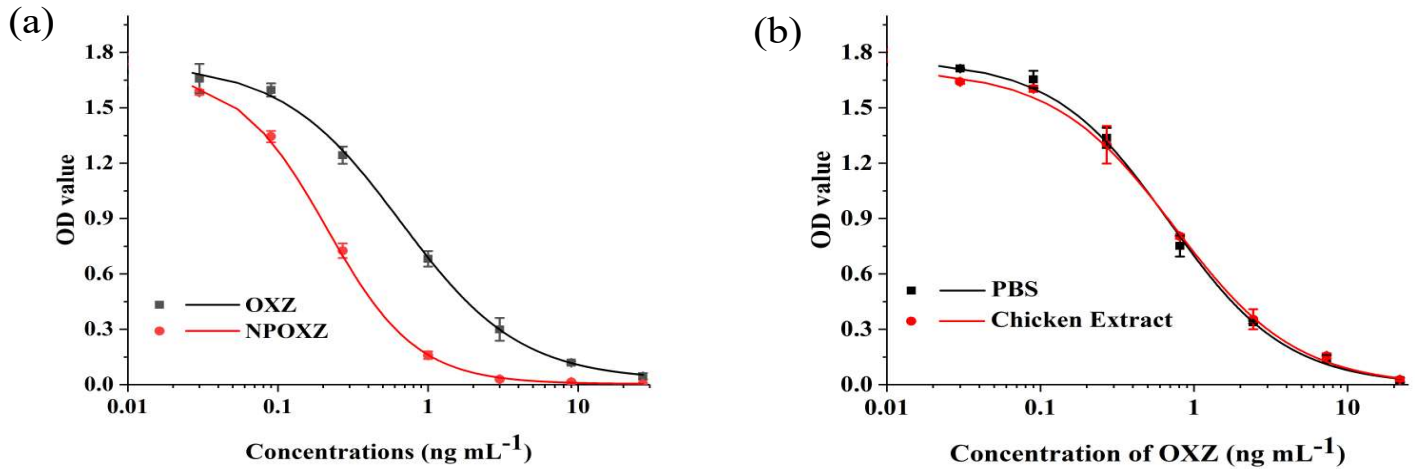
- 从两只小鼠分别获得一株单克隆抗体，2B6和2D9，经优化后，对OXZ和NPOXZ的IC_{50}分别为1.42，0.95ng mL^{-1}及1.42，0.95ng mL^{-1}
  Two mAbs were obtained from the two mice. The IC_{50} for OXZ and NPOXZ were 1.42，0.95ng mL^{-1} and 1.42，0.95ng mL^{-1}

抗体制备 Antibody Preparation

- 单克隆抗体2D9仅与OXZ其代谢物以及母体药物硝呋地腙有交叉，与其他物质无交叉，特异性良好
- **The IC_{50} values and cross reactivity of the mAb 2D9 evaluated with OXZ, its derivative and nifuraldezone. The negligible cross reactivity were obtained for other compounds**

Compounds	IC_{50} (ng mL^{-1})	Cross Reactivity (%)
OXZ	0.66	100
NPOXZ	0.21	314.28
Nifuraldizone	6.91	9.55
AHD	>1000	<0.06
SEM	>1000	<0.06
AOZ	>1000	<0.06
DNSH	>1000	<0.06
AMOZ	>1000	<0.06
AGD	>1000	<0.06
2-NBA	>1000	<0.06
HBD	>1000	<0.06
NPHBD	>1000	<0.06
Nitrovin	>1000	<0.06
Nifuoxazide	>1000	<0.06

试剂盒开发 ELISA Kit Development



- 优化后建立标准曲线，OXZ 的IC_{50}值为0.66 ng mL^{-1}，可适用于鸡肉中硝呋地腙代谢物的检测
- **The standard curves wre established after optimization. The IC_{50} value of OXZ was 0.66 ng ml^{-1}, which was suitable for the detection of nifuraldizone metabolites in chicken**

试剂盒开发 ELISA Kit Development

Matrix	Concentrations spiked (μg kg^{-1})	Recoveries (%)	CV (%)
Chicken	0.5	84.5	8.6
	1.0	91.3	8.4
	2.5	81.7	8.8
	5.5	96.9	8.3

- 鸡肉中OXZ添加回收率介于84.5-96.9%，CV值小于8.3%
- The recovery rate of OXZ in chicken was 84.5-96.9%, and the CV value was less than 8.3%

Compounds	Metabolite	IC_{50} for metabolite (ng/mL)	IC_{50} of derivatives (ng/mL)	IC_{50} for parent drug (ng/mL)
Nifursol	**DNSH**	**0.91**	**0.72**	**0.85**
Nifuroxazide	**HBD**	**0.25**	**0.10**	**0.44**
Nitrovin	**AGD**	**0.80**	**0.87**	**0.89**
Nifuraldizone	**OXZ**	**0.66**	**0.21**	**6.91**

- 针对四种靶标代谢物制备了四株单克隆抗体

  Four monoclonal antibodies were prepared against four target metabolites

- 有望实现不经衍生化，直接检测代谢物

  Detection of metabolites without derivatization

- 对代谢物的 IC_{50} 值分别为0.91、 0.25、 0.80和0.66 ng/mL

  The IC_{50} values of metabolite were 0.91, 0.25, 0.80 and 0.66 ng / ml, respectively

- 对衍生物的 IC_{50} 值分别为0.72 、 0.10、0.87和0.21 ng/mL

  The IC_{50} values of derivatives were 0.72, 0.10, 0.87 and 0.21 ng / ml, respectively

文章和专利 Papers and patents

1. Ghulam Mujtaba Mari, Hongfang Li, Baolei Dong, Huijuan Yang, Aisha Talpur, Jiafei Mi, Liuchuan Guo, Xuezhi Yu, Yuebin Ke, Diangang Han, Zhanhui Wang. Hapten synthesis, monoclonal antibody production and immunoassay development for direct detection of 4-hydroxybenzehydrazide in chicken, the metabolite of nifuroxazide. ***Food Chemistry***. 2021, 355, 129598.
2. Weilin Wu, Shuyu Yang, Jialiang Liu, Jiafei Mi, Leina Dou, Yantong Pan, Ghulam Mujtaba Mari, Zhanhui Wang. Progress in immunoassays for nitrofurans detection. ***Food and Agricultural Immunology***. 2020, 31 (1), 890–909
3. 王战辉，吴伟林，刘佳靓，杨舒宇，朱建宇。硝呋烯腙半抗原和人工抗原及其制备方法与应用。202010158357.8
4. 王战辉，温凯，于雪芝，江海洋，沈建忠，史为民，朱建宇。硝呋酚酰肼半抗原和人工抗原及其制备方法与应用。201910991603.5
5. 王战辉，于雪芝，温凯，沈建忠，江海洋，张素霞，朱建宇。硝呋地腙半抗原和人工抗原及其制备方法与应用。201910991617.7
6. 王战辉，柯跃斌，温凯，于雪芝，余文博，吴伟林，史为民，张素霞.硝呋索尔半抗原和人工抗原的合成及其单克隆抗抗体的制备。202110408006.2

谢谢

敬请专家提出宝贵意见