



Catching the Cheats

Liquid Chromatography-Mass Spectrometry in Sports Drug Testing



Historical Aspects





Historical Aspects

“Straub tail response”

-15 min from sampling
to analytical result



UCI – list of prohibited substances 1.1.1967

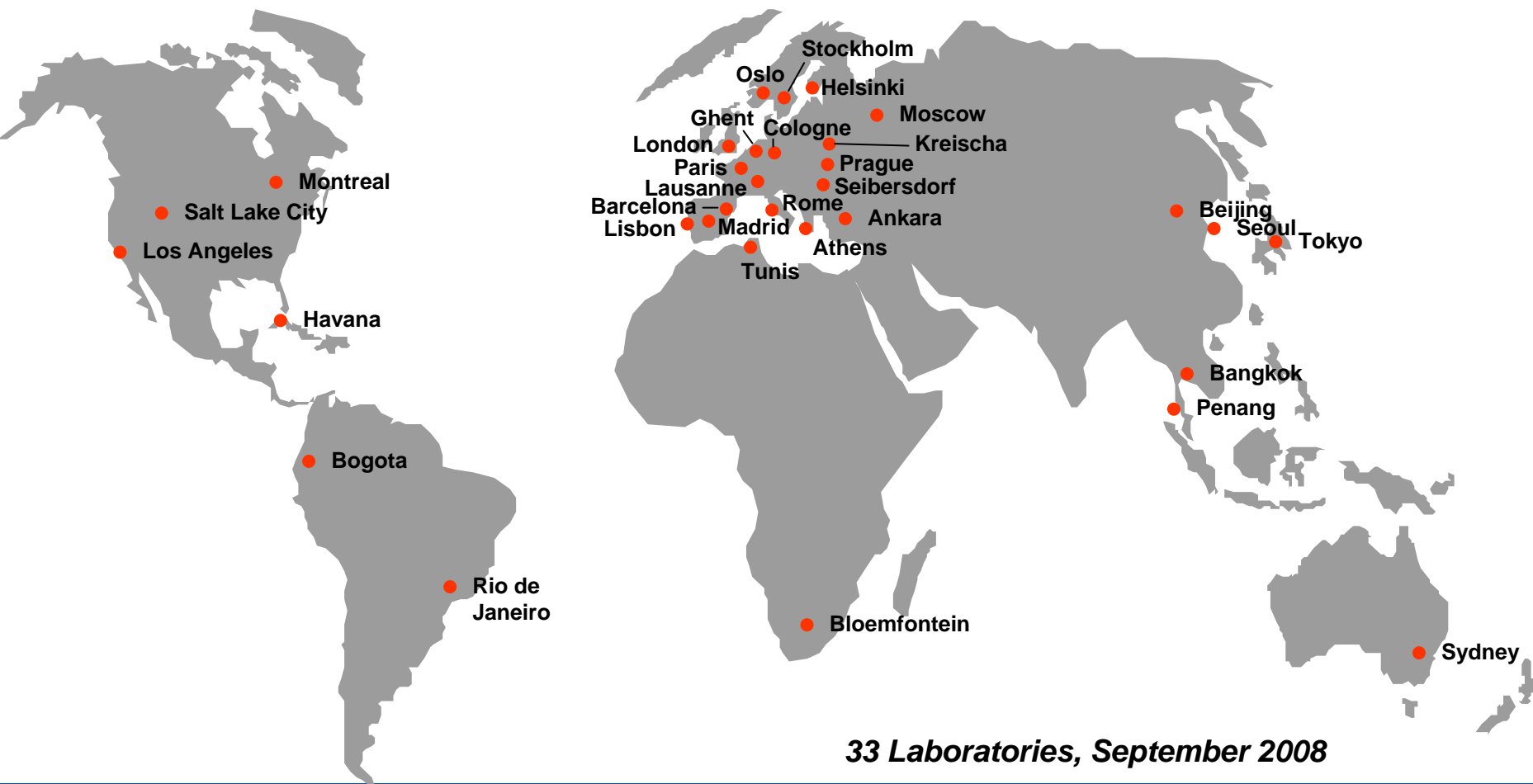
UCI

- Narcotics
(Morphine, Heroine, etc.)

- Substances of the class of
amphetamines



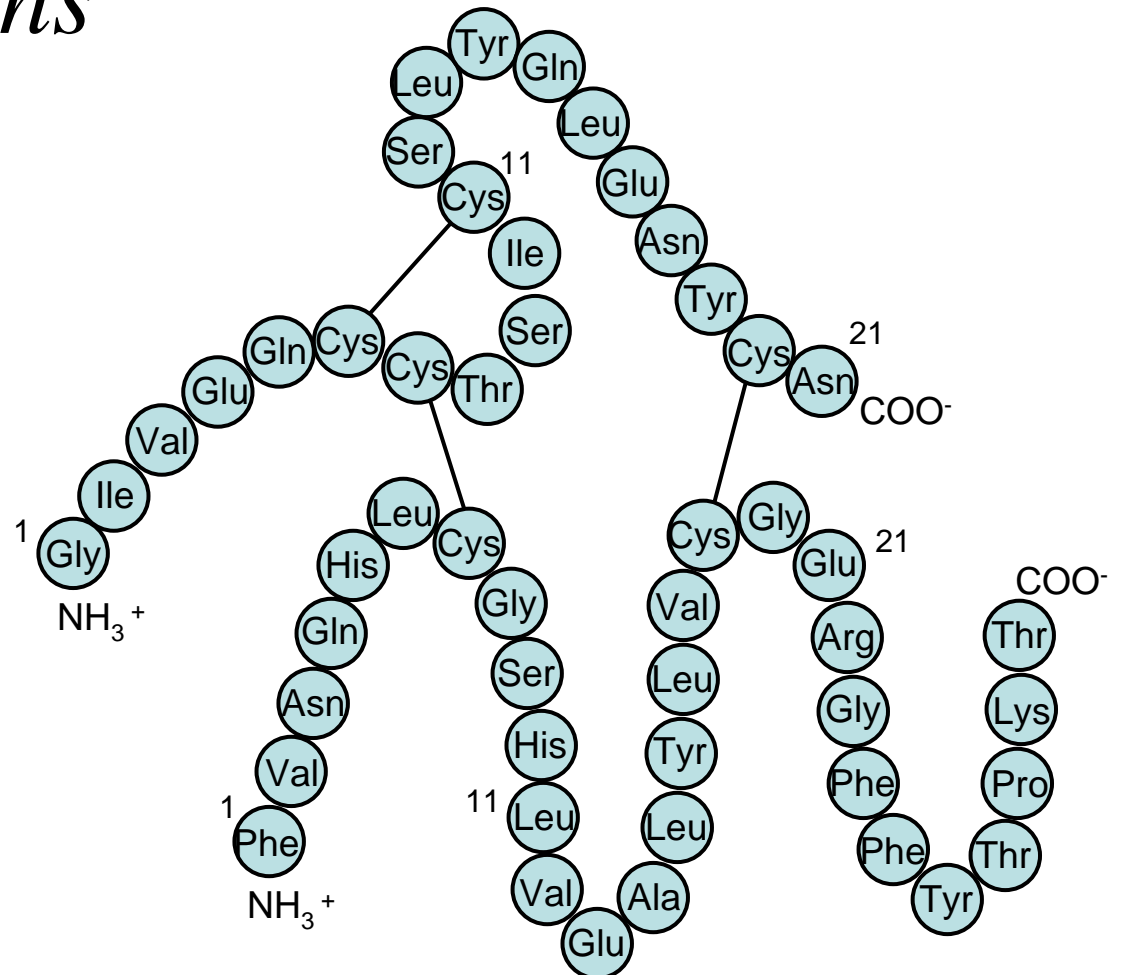
Doping control laboratories accredited by IOC and WADA



33 Laboratories, September 2008



Selected Detection Assays: *Synthetic insulins*





Selected Detection Assays:

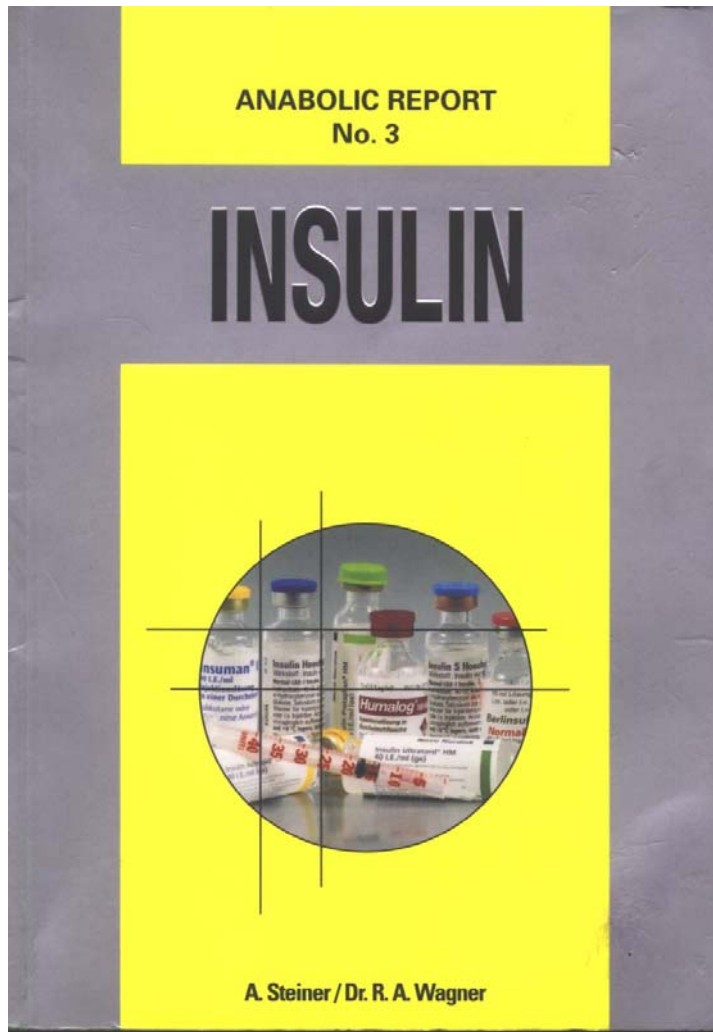
Synthetic insulins

-1998 (Nagano, Olympic Winter Games) request whether the use of insulin is limited to athletes suffering from *diabetes mellitus*

-1999 Insulin was added to the IOC list of prohibited substances



Misuse indicators



Dear Dwain,

...

Your performance enhancing drug program included the following seven prohibited substances:

THG,

testosterone/epitestosterone cream,

EPO (Procrit),

HGH (Serostim),

insulin (Humalog),

modafinil (Provigil)

and liothyronine...

Yours sincerely, Victor Conte



Misuse indicators

Evans, P. J.; Lynch, R. M. **Insulin as a Drug of Abuse in Body Building.** *Br J Sports Med* **2003**, *37*, 356-357.

Rich, J. D.; Dickinson, B. P.; Merriman, N. A.; Thule, P. M. **Insulin Use by Bodybuilders.** *JAMA* **1998**, *279*, 1613.

Dawson, R.T.; Harrison, M. W. **Use of insulin as an anabolic agent** *Br. J. Sports Med.*, **1997**; *31*: 259

Reverter, J. L.; Tural, C.; Rosell, A.; Dominguez, M.; Sanmarti, A. **Self-Induced Insulin Hypoglycemia in a Bodybuilder.** *Arch Intern Med* **1994**, *154*, 225-226



WADA – prohibited list 2008

Substances and Methods Prohibited at All Times (In- and Out-Of-Competition)

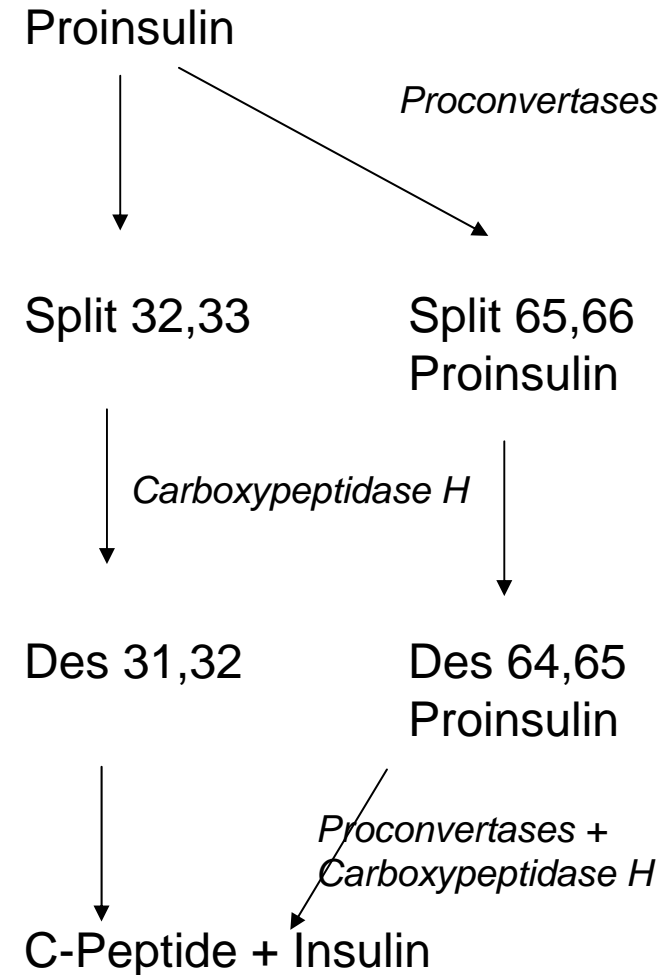
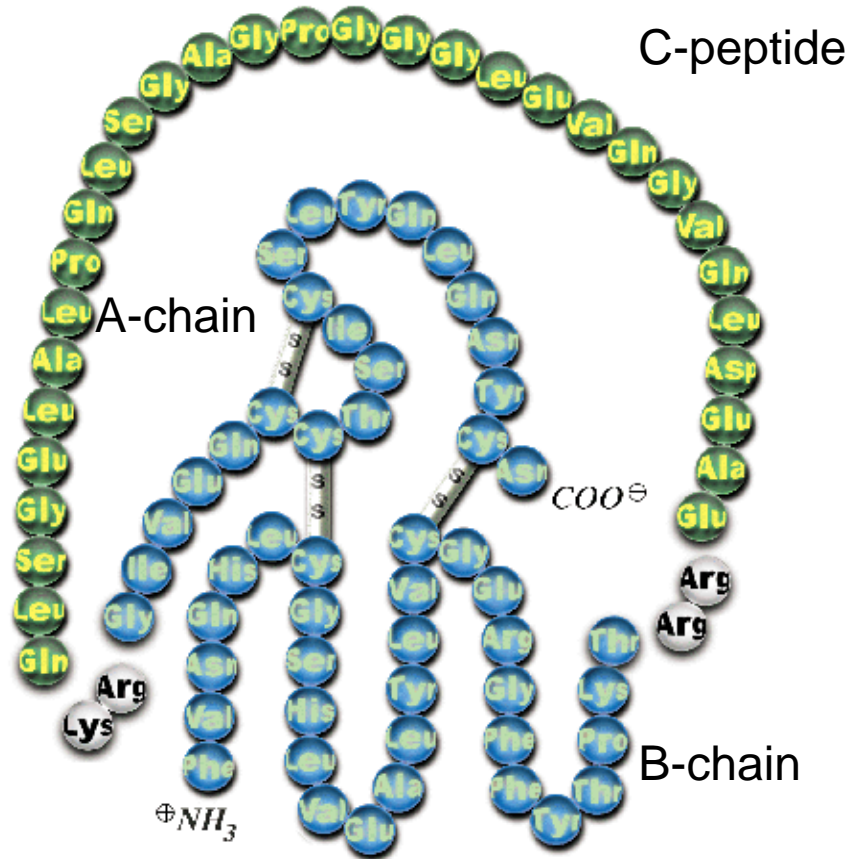
S2. Hormones and related substances

The following substances, including other substances with a similar chemical structure or similar biological effect(s), and their releasing factors, are prohibited:

- 1. Erythropoietin (EPO)**
- 2. Growth Hormone (hGH), Insulin-like Growth Factors (e.g. IGF-1), Mechano Growth Factors (MGFs);**
- 3. Gonadotrophins (LH, hCG), prohibited in males only;**
- 4. Insulin;**
- 5. Corticotrophins.**



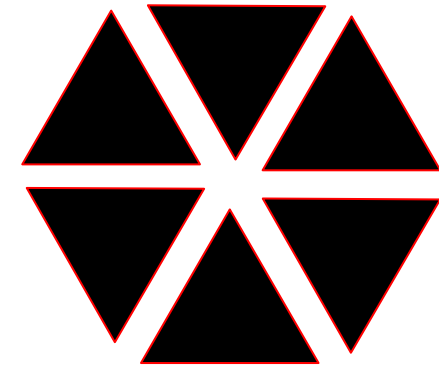
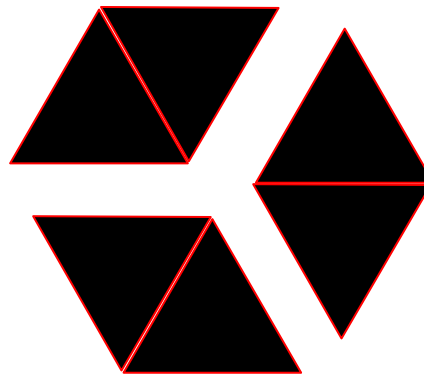
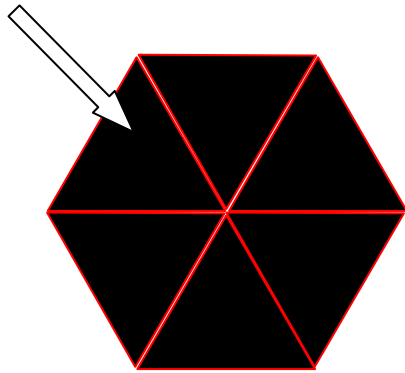
Generation of Human Insulin from Proinsulin ($M_w = 9388$)





Self-Association of Human Insulin to Non-Covalent Hexamer

Insulin monomer



Subcutaneous
injection

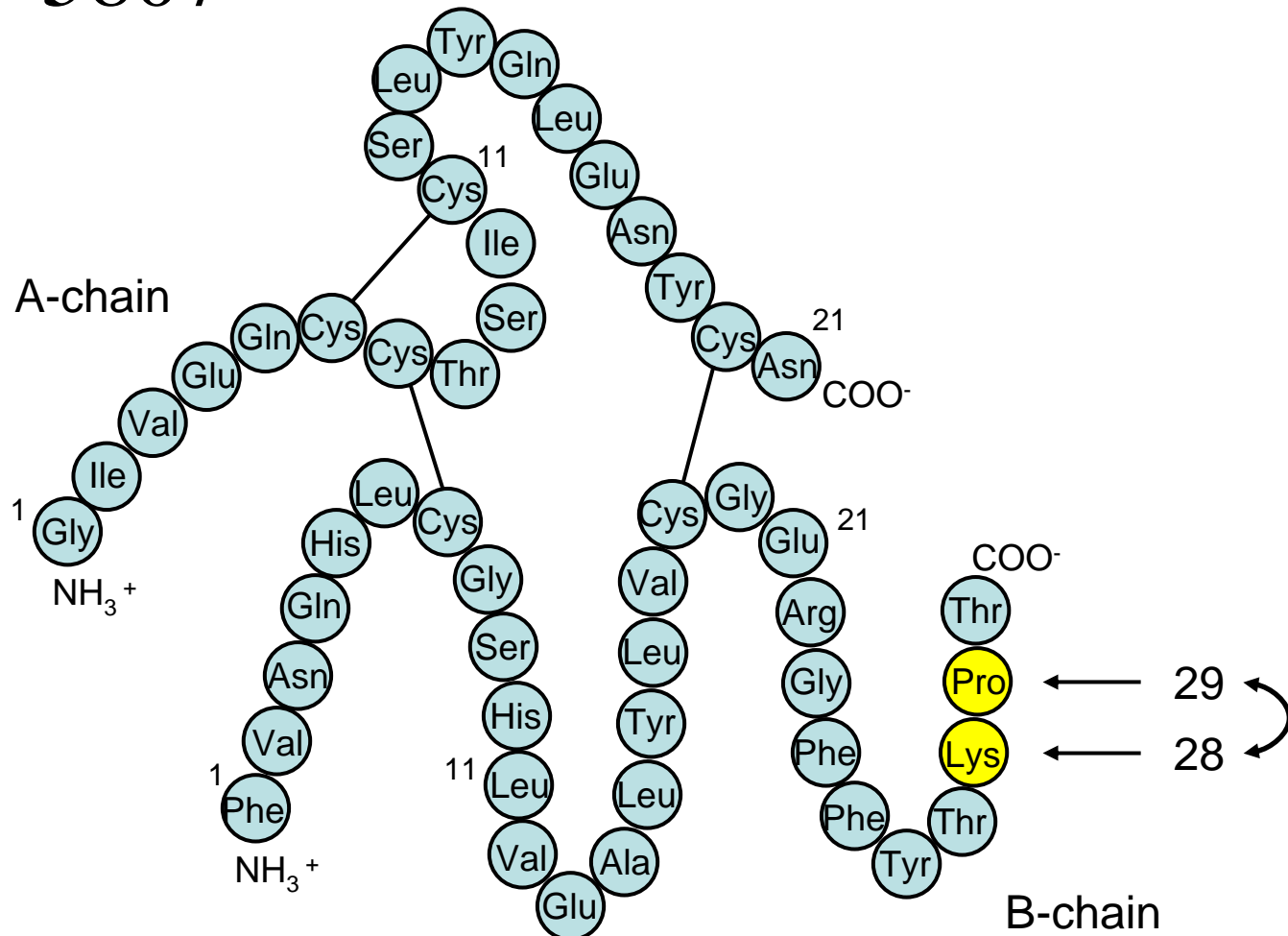
time

45-120 min
lag-phase



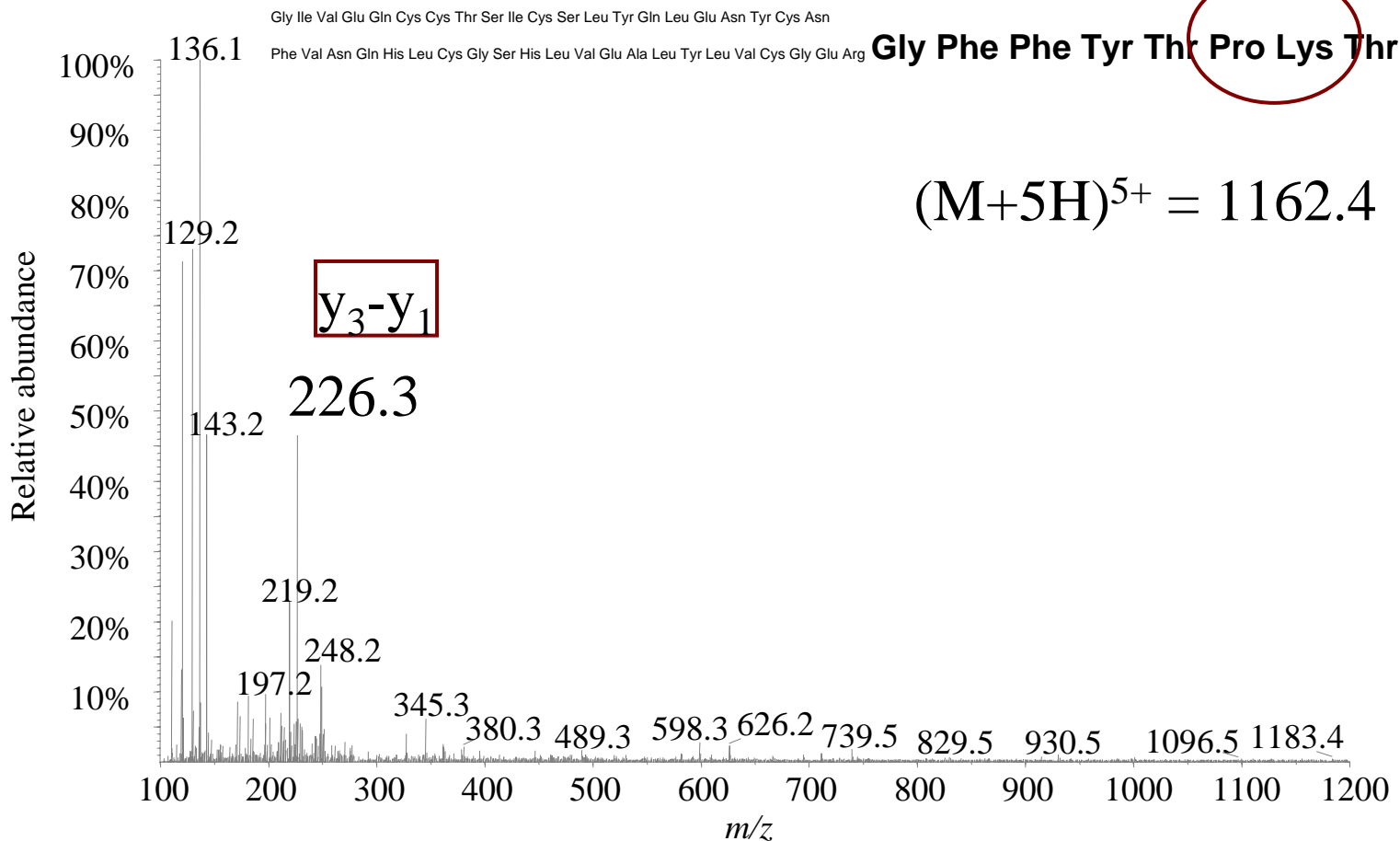
Rapid acting insulins: Humalog LisPro

mol wt = 5807



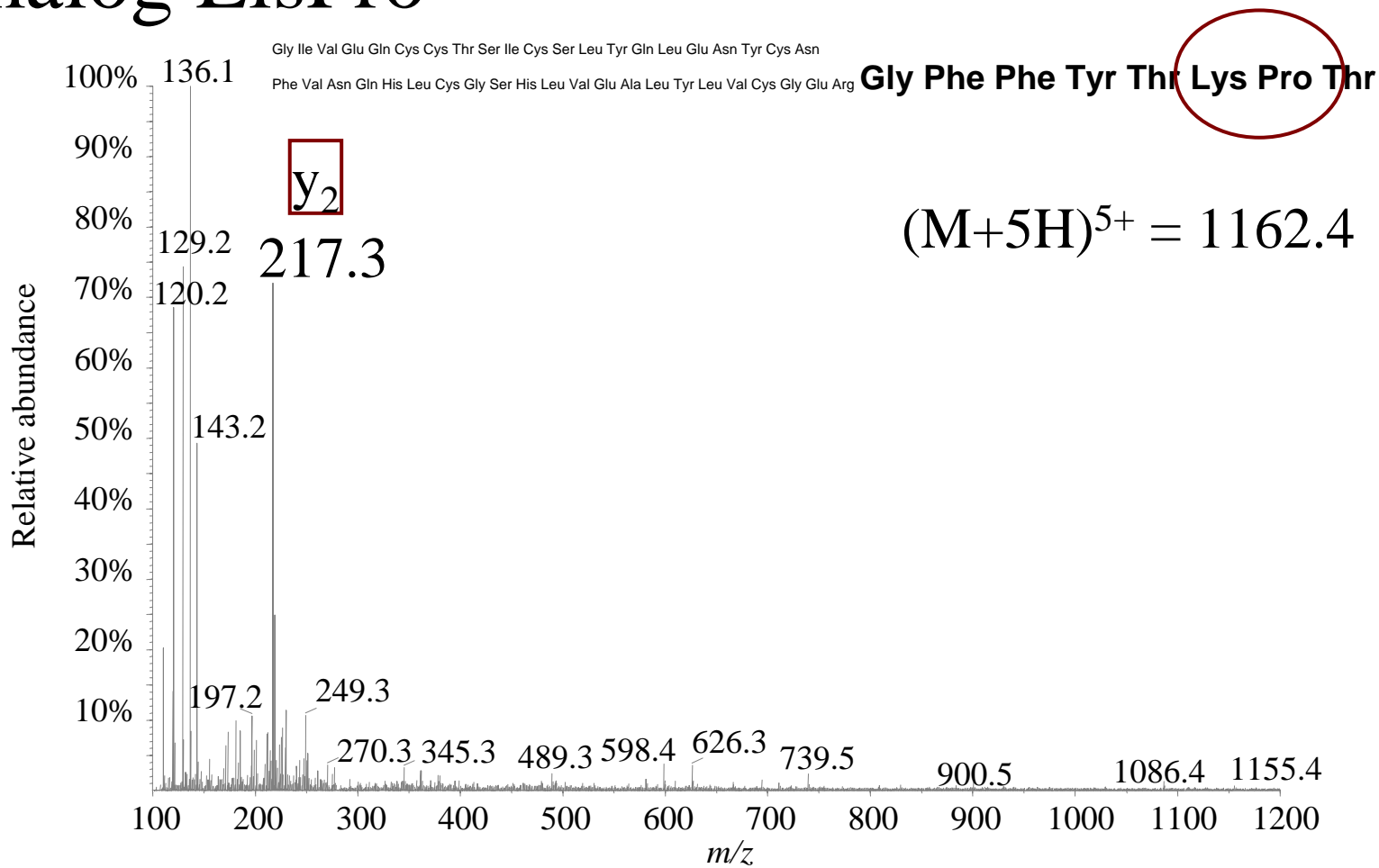


Mass Spectral Information: Human Insulin





Mass Spectral Information: Humalog LisPro





General Information

physiological concentrations of insulins:

0.3 – 2 ng/mL plasma

0.02 – 0.2 ng/mL urine

--→ target concentrations for doping
control purposes



1st SPE

elution of analytes
onto IAC column

immunoaffinity
purification

2nd SPE

elution of analytes
into Eppendorf vial

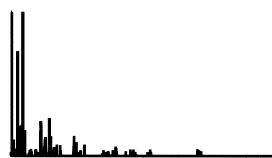
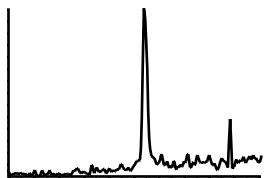


- concentration to dryness
in vacuum centrifuge

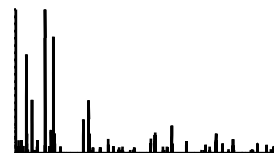
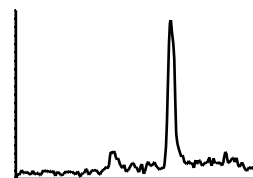
- reconstitution in HPLC buffers

-cleavage into A- and B-chain

-LC-ESI-MS/MS
analysis of intact
insulins

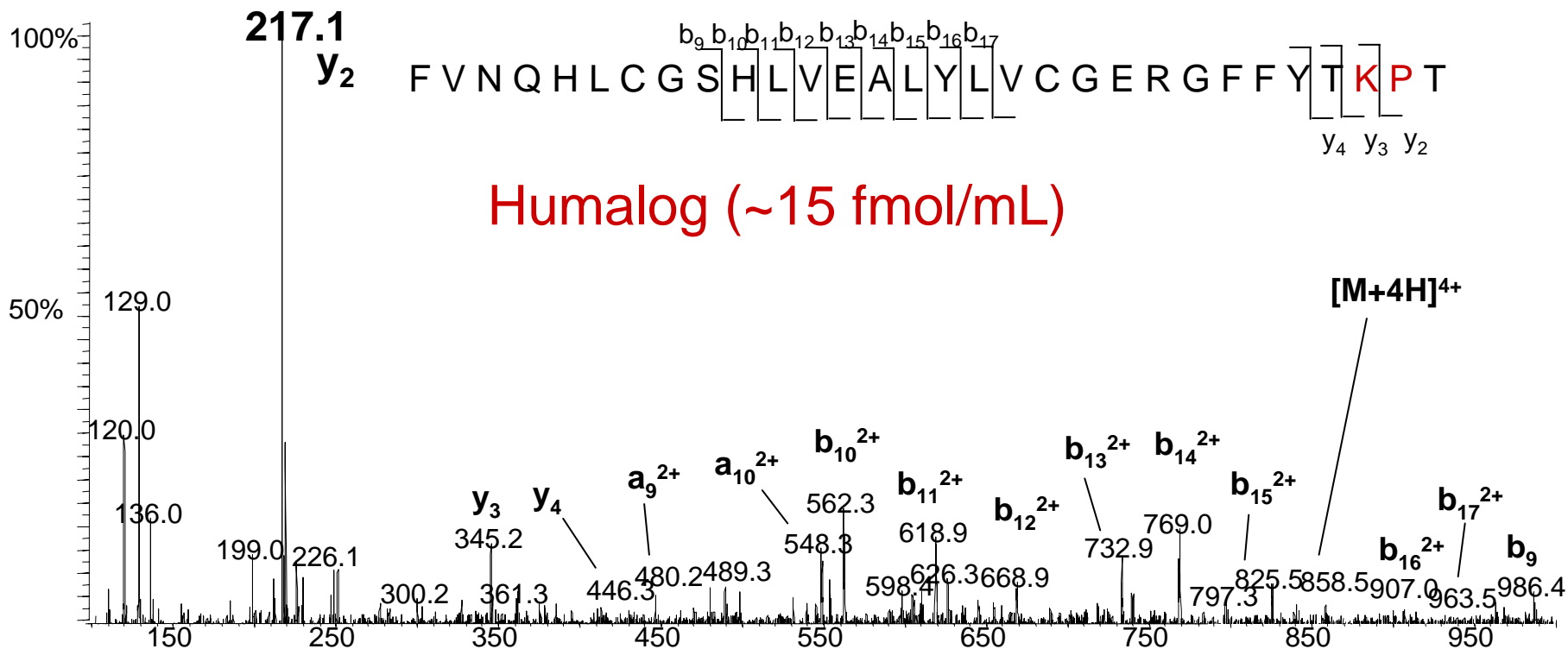


-LC-ESI-MS/MS
analysis of B-
chain





Selected Detection Assays: *Synthetic insulins*





Detection of Insulins in Doping Control Samples in 2005 - 2007

No	compound	discipline
1	Novolog	tennis
2	Novolog	weightlifting
3	Novolog	athletics
4	Novolog	athletics
5	Novolog	wheelchair rugby
6	Novolog	tennis
7	Humalog	hockey
8	Humalog/Lantus	cross-country skiing
9	Apidra	ice hockey
10	Apidra	powerlifting
11	Apidra	powerlifting
12	Apidra	canoeing



Selected Detection Assays:

cross-linked hemoglobins

-Based on bovine hemoglobin

-Inter- and intra-molecularly crosslinked (glutaraldehyde) → no dissociation into subunits

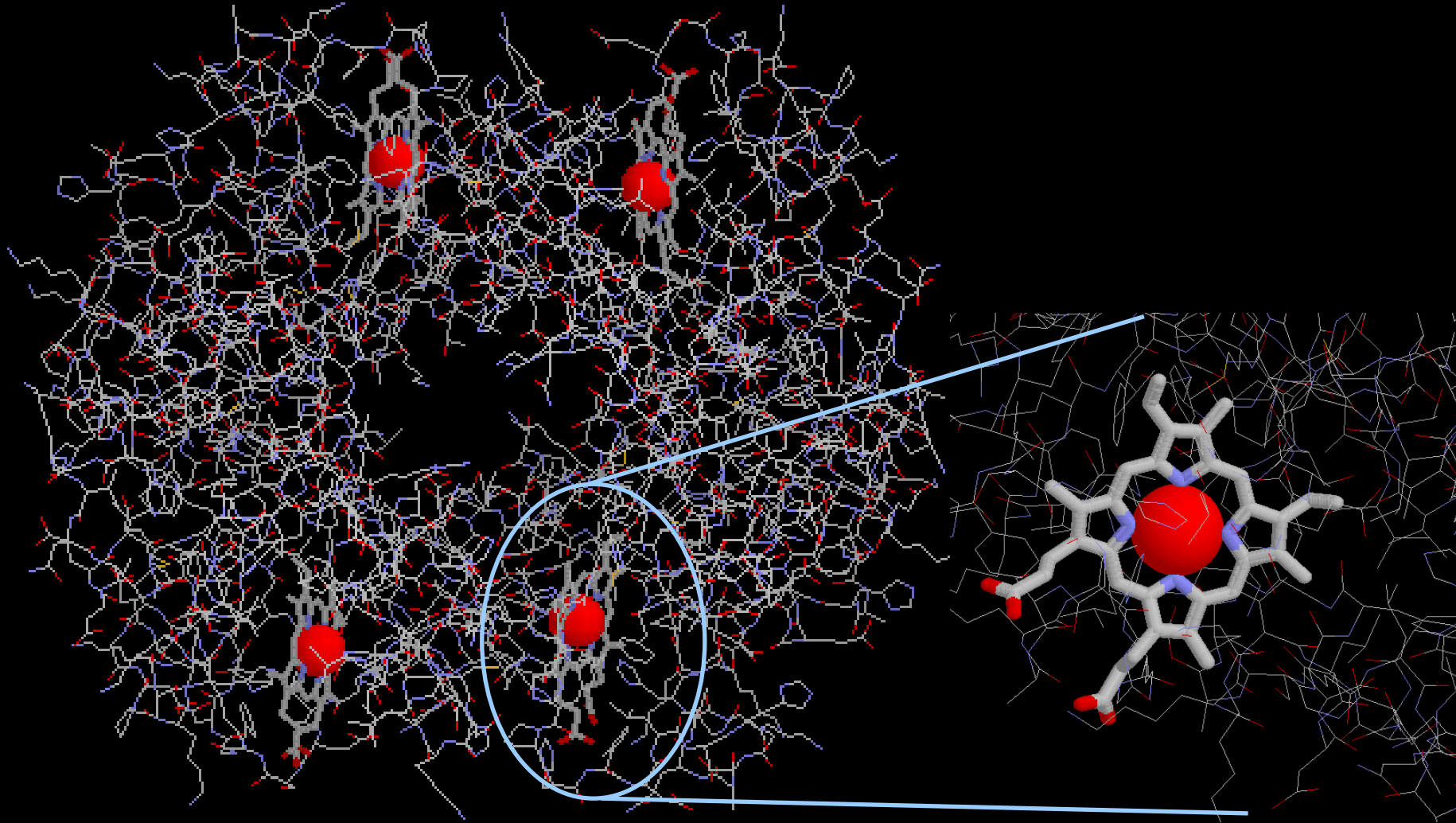
-Average molecular weight of 250 kDa

Amount of application ~ 30g of Hemopure (single bag)

————→ Expected blood concentration: approx. 5mg/mL

Half-life approximately 20h (after infusion of 45g of Hemopure)

Hemoglobin structure





Selected Detection Assays: *cross-linked hemoglobins*

Sequence homology of bovine and human hemoglobin = 85%

Human Hb, alpha-chain (141 AAs)

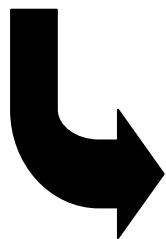
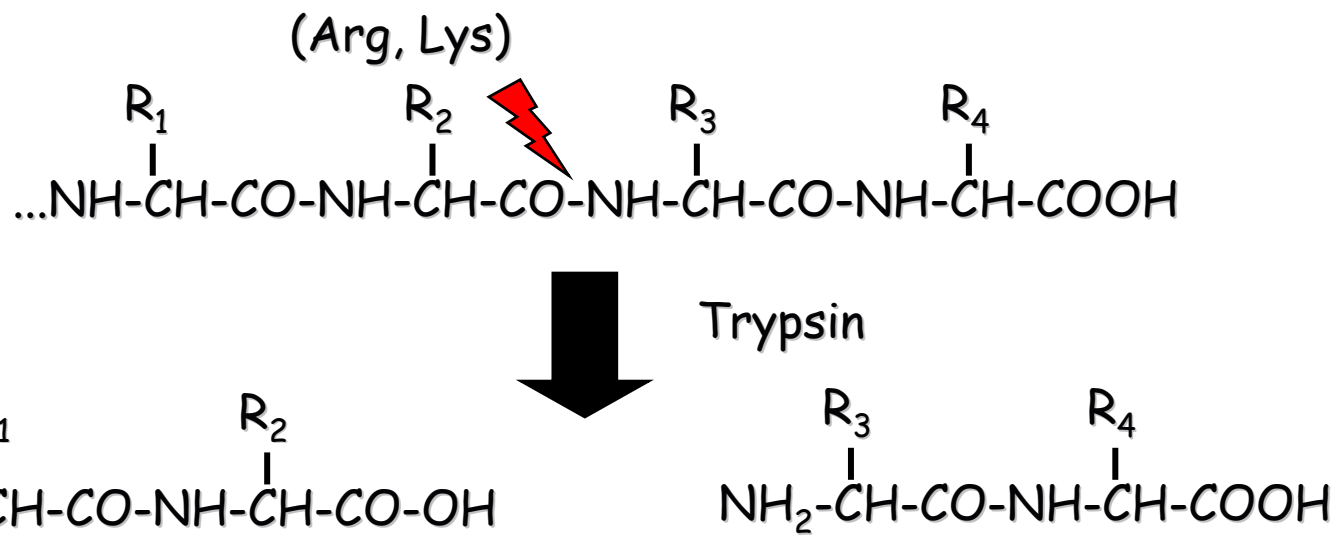
VLSPADKTNV KAAWGKVG^{AH} AGEYGAEALE RMFLSFPTTK TYFPHFDLSH GSAQVKGHGK
KVADALTN^{AV} AHVDDMPNAL SALSDLHAHK LRVPVNF^{KL} LSHCLLV^TLA AHLPAEFTPA
VHASLDKFLA SVSTVLTSKY R

Bovine Hb, alpha-chain (141 AAs)

VLSAADKGNV KAAWGKVG^{GH} AA^{EY}GAEALE RMFLSFPTTK TYFPHFDLSH GSAQVKGHG^A
KVAAALTKAV EHLDDLPGAL SELSDLHAHK LRVPVNF^{KL} LSHSLLV^TLA SHLPSDFTPA
VHASLDKFLA NVSTVLTSKY R



Selected Detection Assays: *cross-linked hemoglobins*



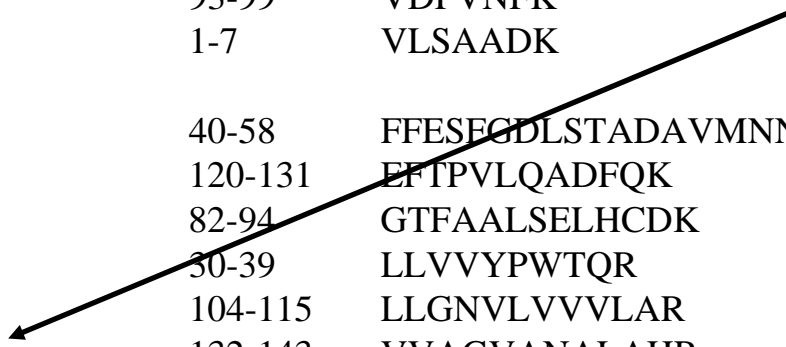
Comparison of derived peptides with theoretical peptide masses using databases (e.g. SwissProt) -> peptide mass fingerprint



Selected Detection Assays: *cross-linked hemoglobins*

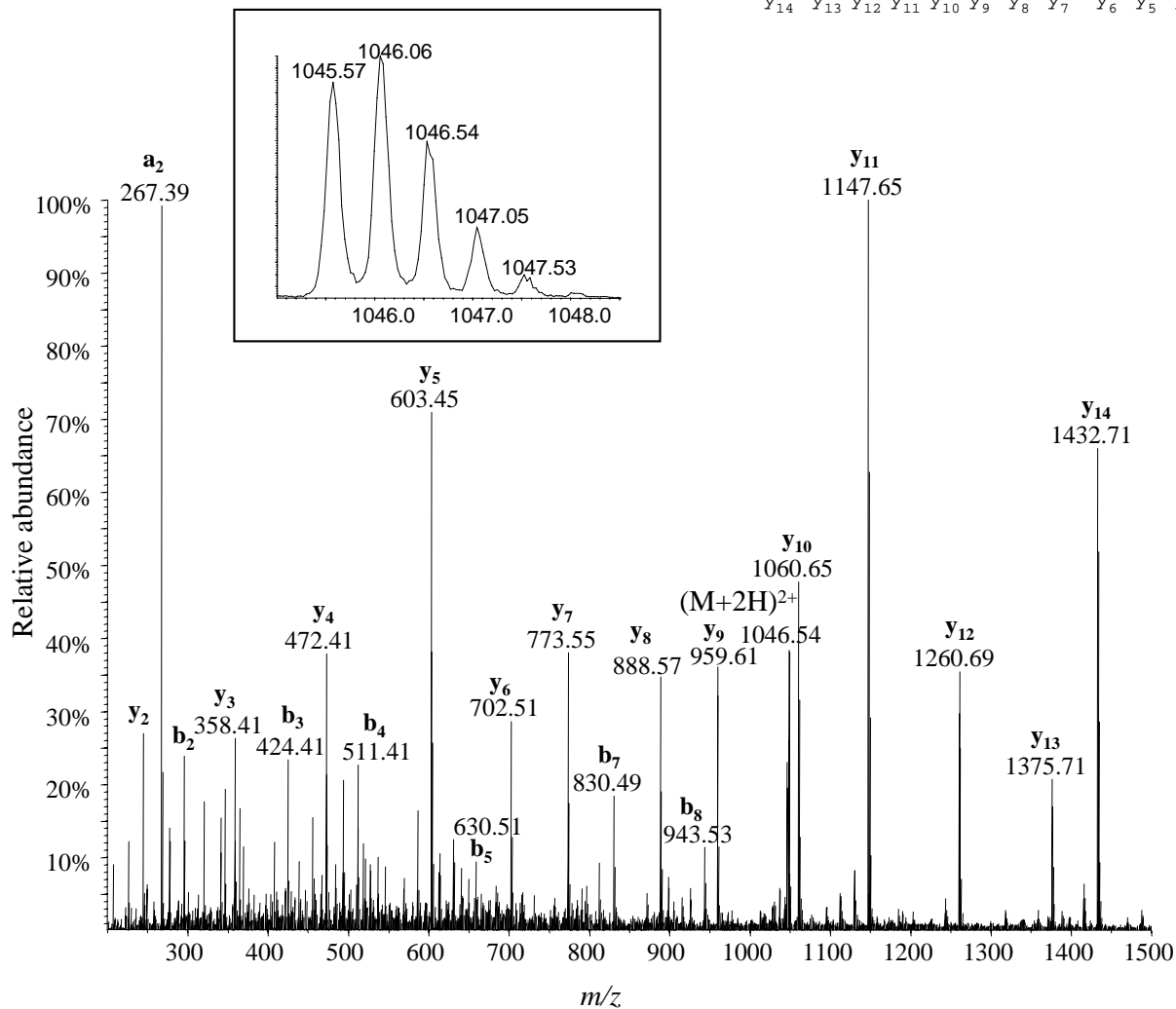
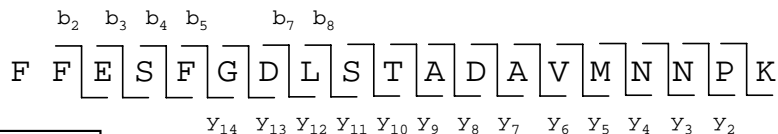
	Mass	position	peptide sequence
alpha-chain			
	2969.6094	100-127	LLSHSLLVTLASHLPSDFTP AVHASLDK
	2367.1938	69-90	AVEHLDDLPGALSELSDLHA HK
	1833.8918	41-56	TYFPFDLSHGSAQVK
	1529.7342	17-31	VGGHAAEYGAELER
	1279.7256	128-139	FLANVSTVLTSK
	1071.5543	32-40	MFLSFPTTK
	818.4406	93-99	VDPVNFK
	703.3984	1-7	VLSAADK
beta-chain			
	2089.9535	40-58	FFESEGDLSTADAVMNNPK
	1422.7263	120-131	EFTPVLQADFQK
	1391.6623	82-94	GTFAALSELHCDK
	1274.7255	30-39	LLVVYPWTQR
	1265.8303	104-115	LLGNVLVVVLAR
	1177.6800	132-143	VVAGVANALahr
	1101.5534	19-29	VDEVGGEALGR
	1098.5578	95-103	LHVDPENFK
	1097.5295	66-75	VLDSFSNGMK

Unique peptides
derived from bovine
Hb





MS/MS of bovine Hb peptide





Selected Detection Assays:

increase of hematocrit

**(since 1997 threshold levels established by
different federations: ♂ 50, ♀ 47)**



49.5 % water

1.09 % lipids, carbohydrates, electrolytes

4.4 % proteins

42.8 % erythrocytes

0.07 % leukocytes

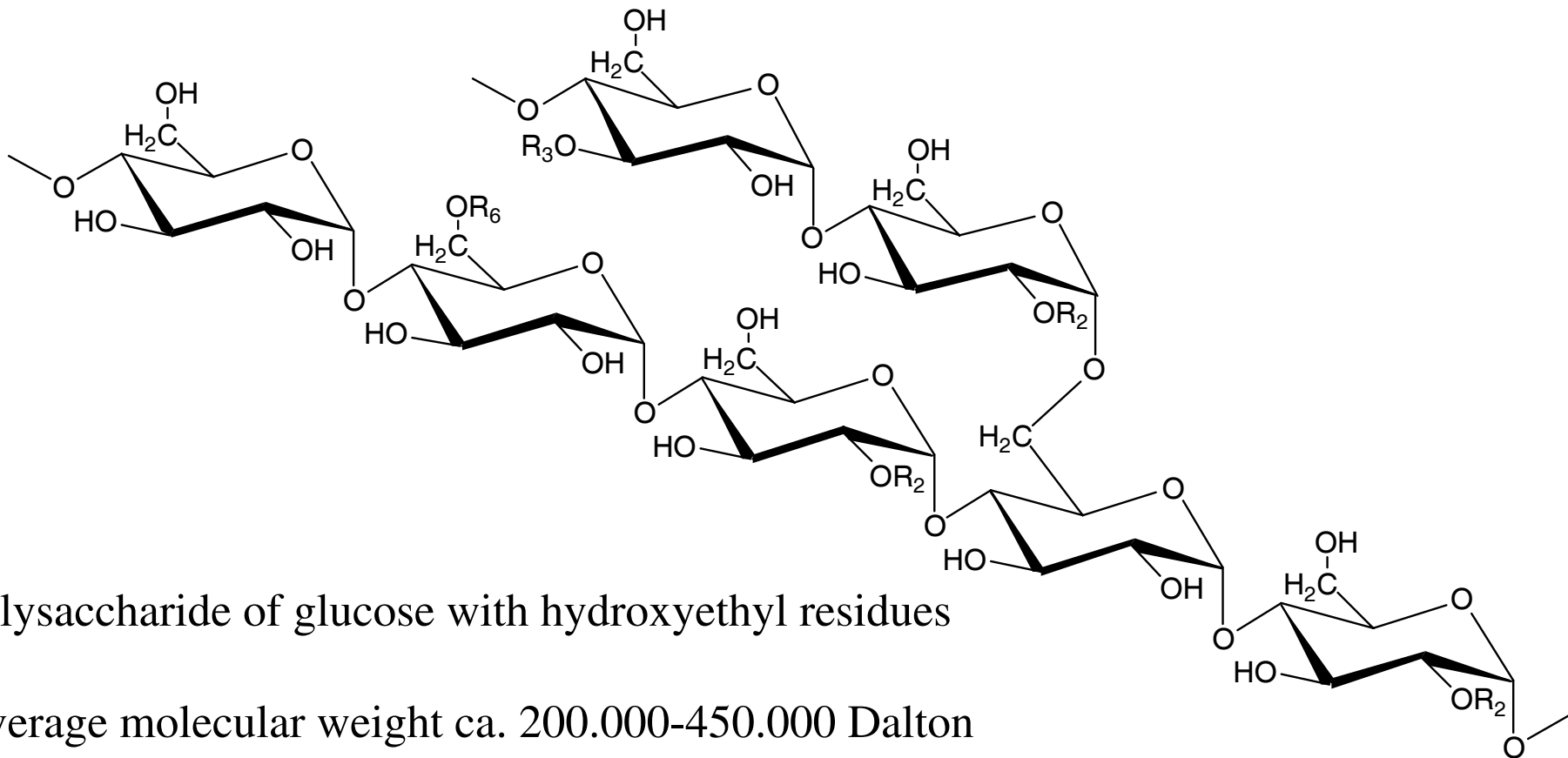
2.14 % thrombocytes

blood

plasma

Selected Detection Assays:

Hydroxyethyl starch (HES)



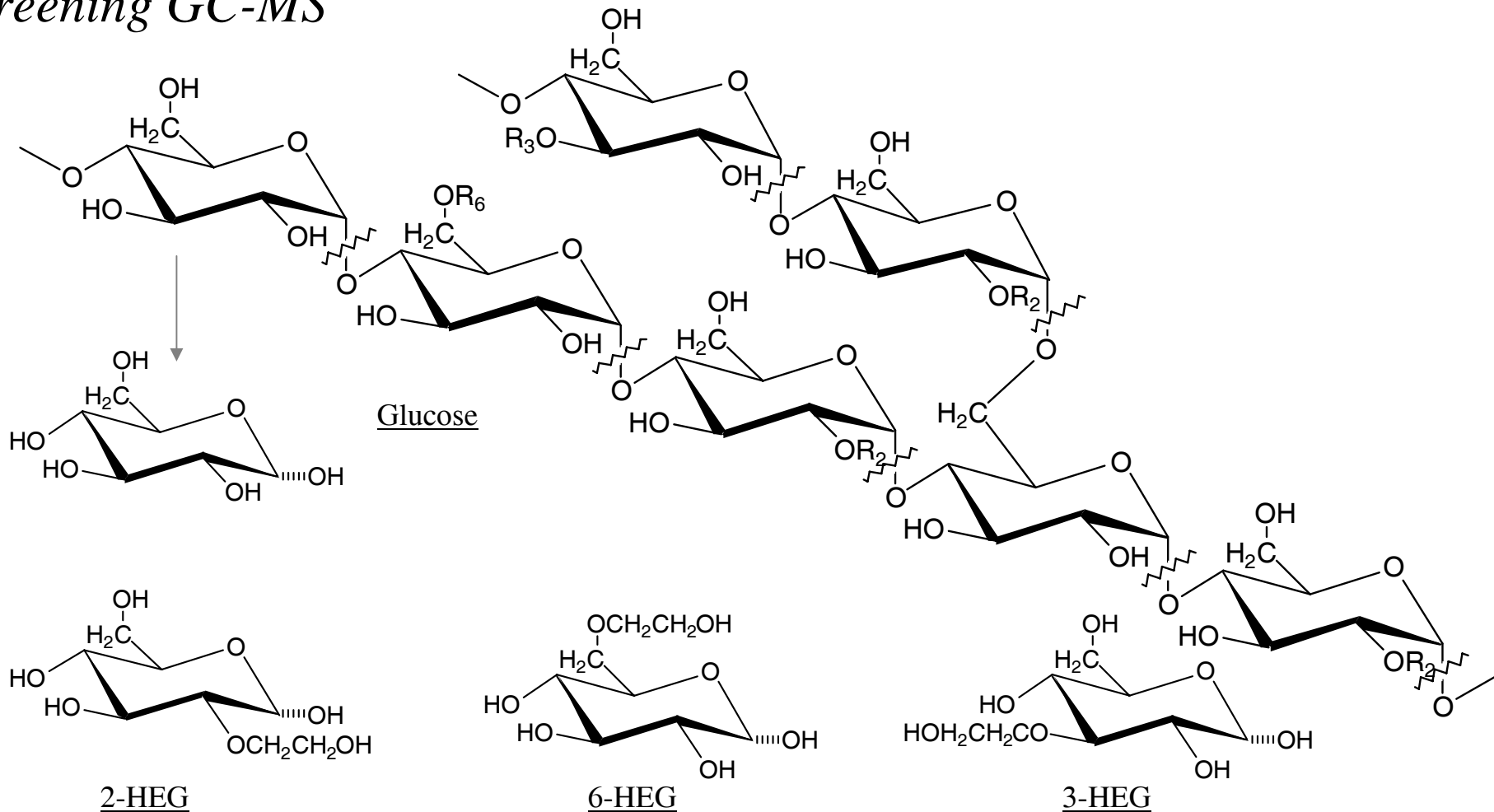
-Polysaccharide of glucose with hydroxyethyl residues

-Average molecular weight ca. 200.000-450.000 Dalton
(accounts for ca. 10.000-27.500 monosaccharides)



Selected Detection Assays: *HES*

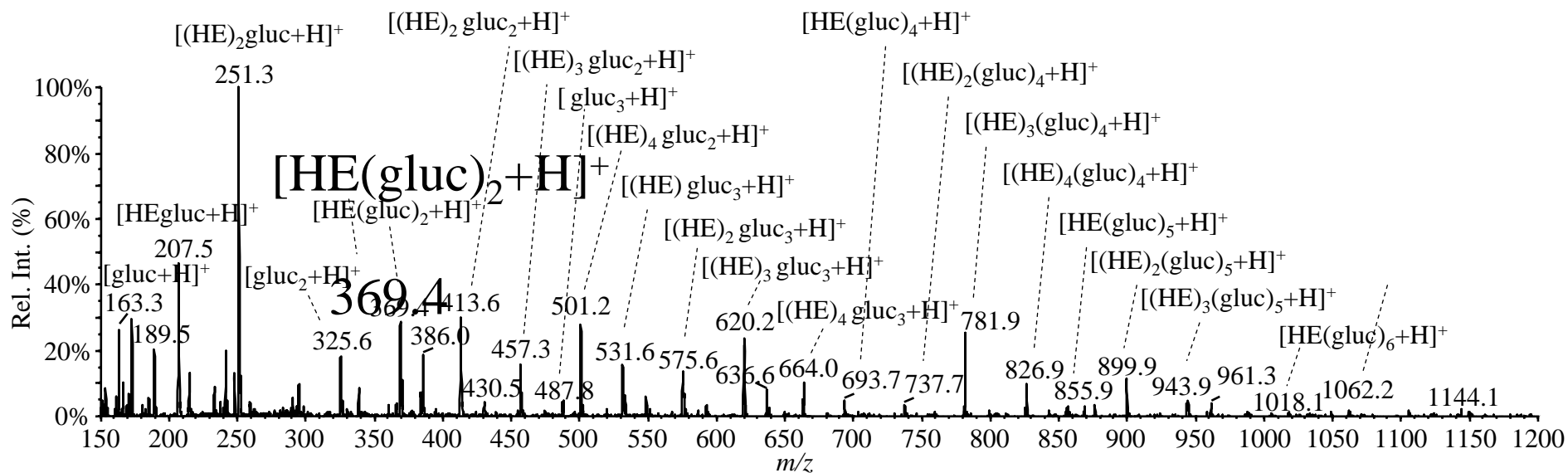
Screening GC-MS





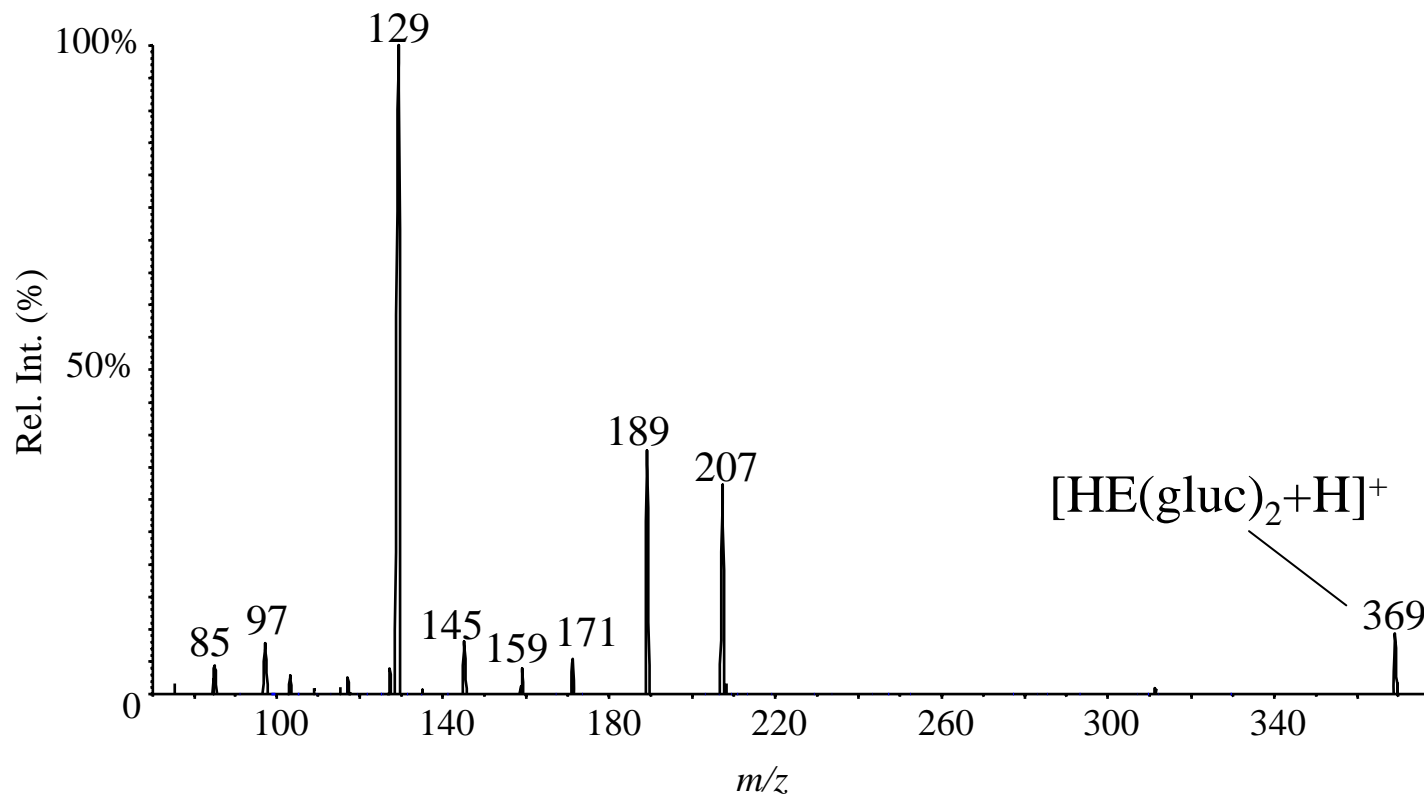
Selected Detection Assays: *HES*

ESI-full MS spectrum of HES





Selected Detection Assays: *HES*



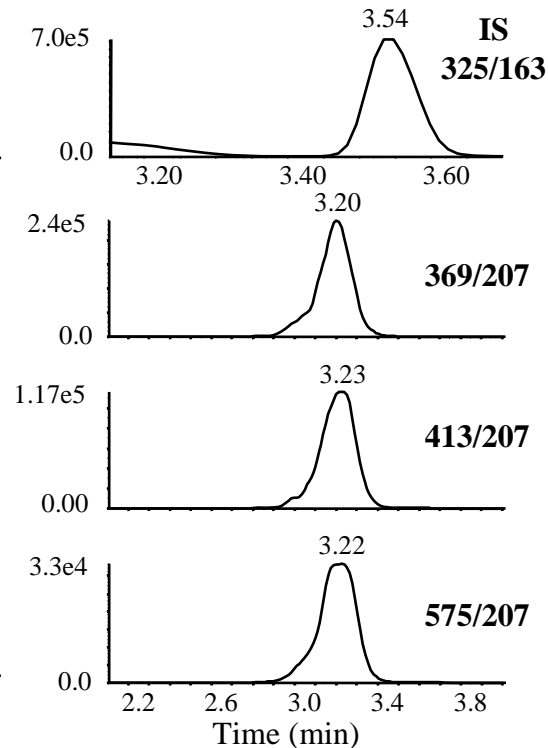
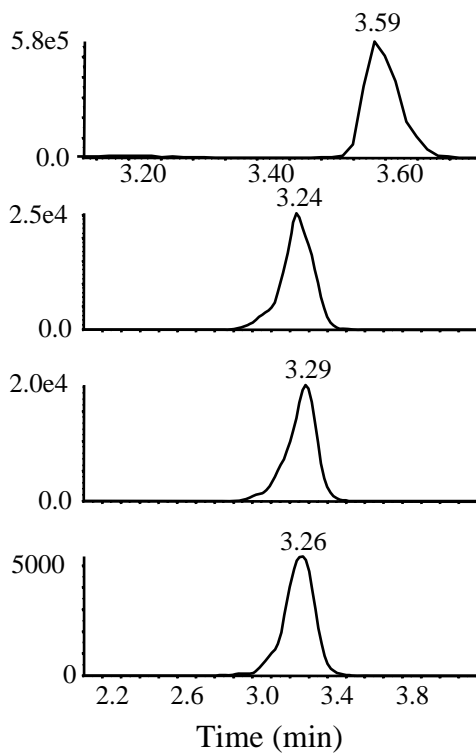
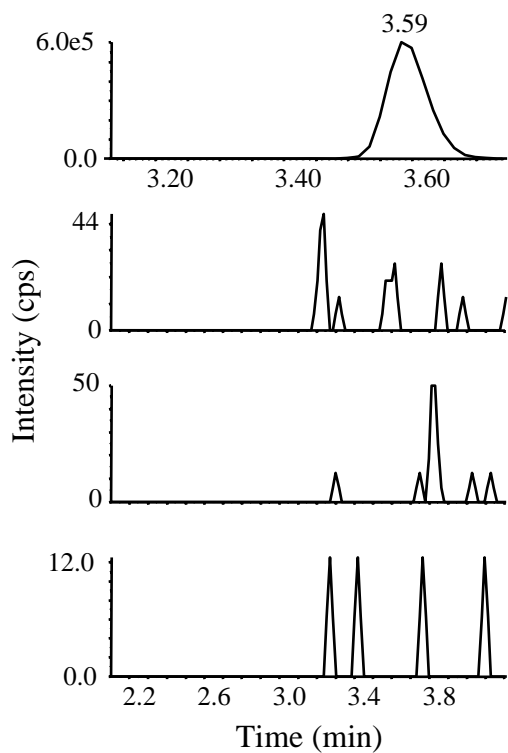


Selected Detection Assays: *HES*

Blank HES

QC HES

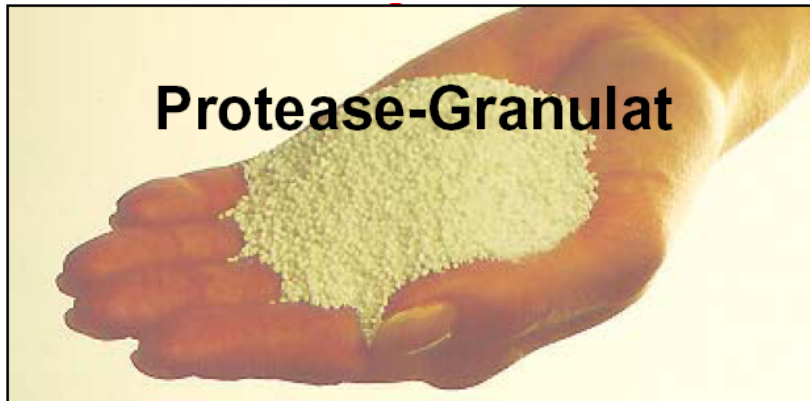
Post administration sample
HES





Selected Detection Assays:

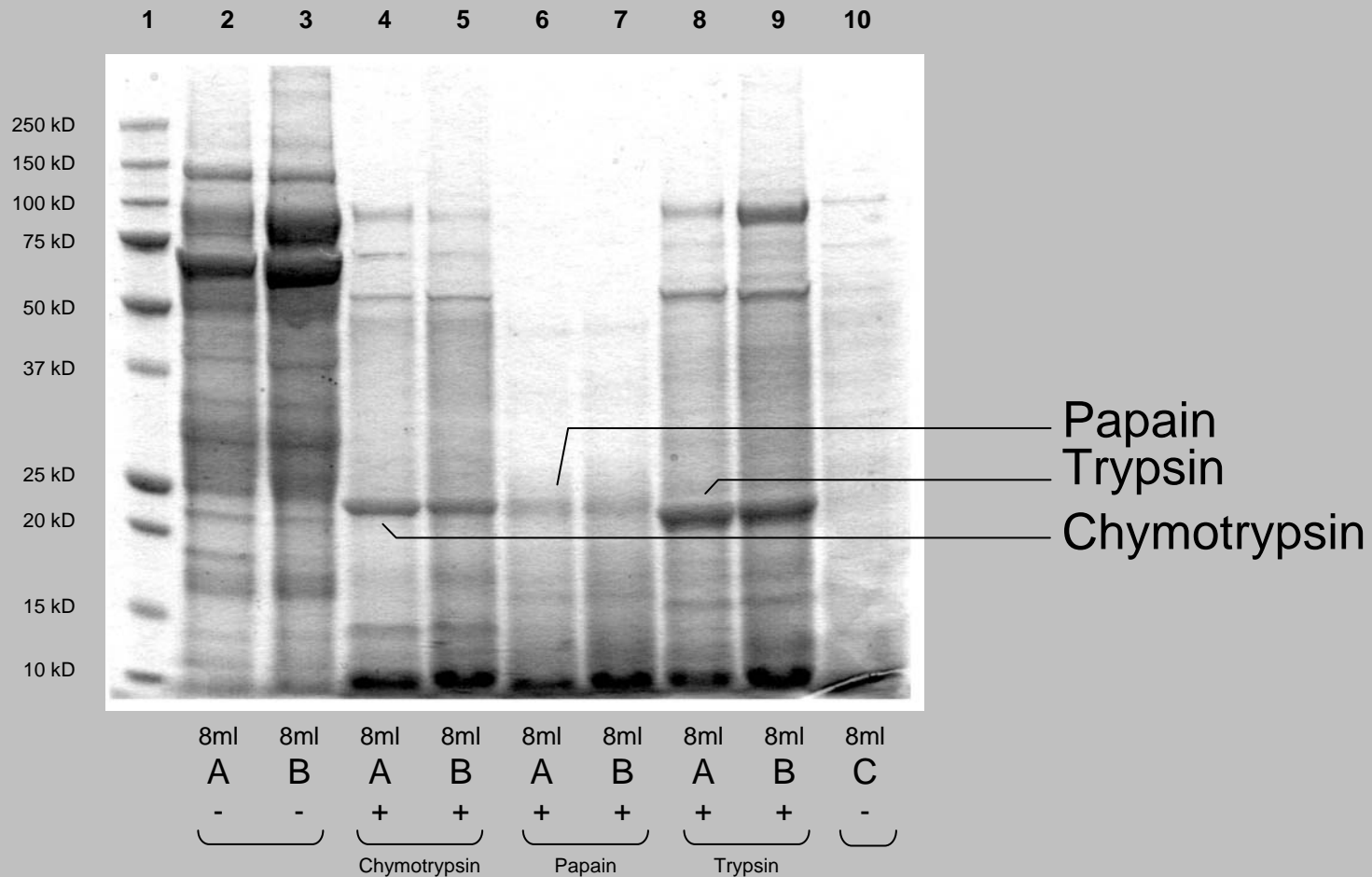
Manipulation





Selected Detection Assays:

Manipulation





Issues: Designer steroids



→ modified structure (with unknown molecular weight)
is not detected



Issue: Designer Steroids

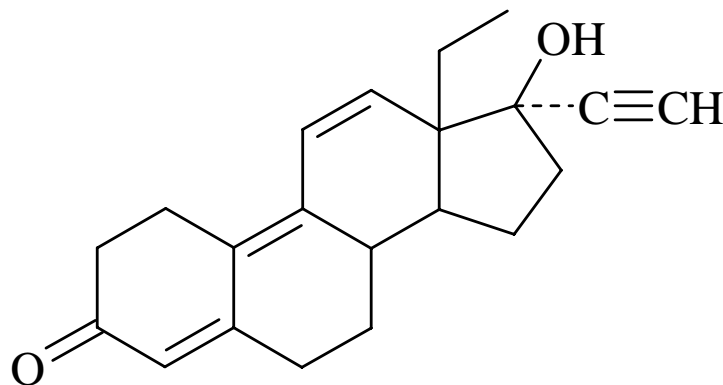
The case of „Tetrahydrogestrinone“ (THG)

-spring 2003: the US Anti-Doping Agency (USADA) is provided with an empty syringe with a letter stating that the former content was used for doping purposes by elite athletes

Issue: Designer Steroids

The case of „Tetrahydrogestrinone“ (THG)

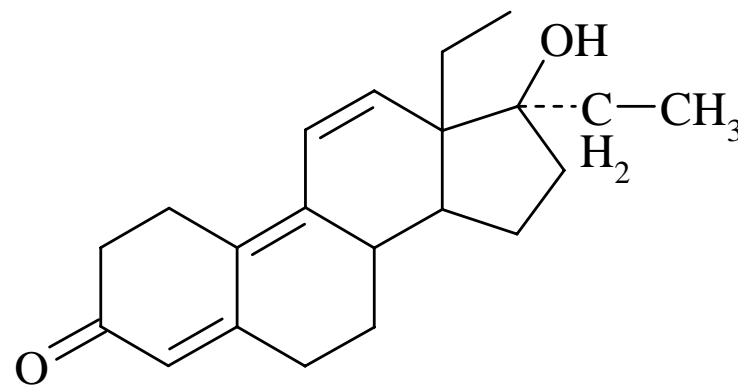
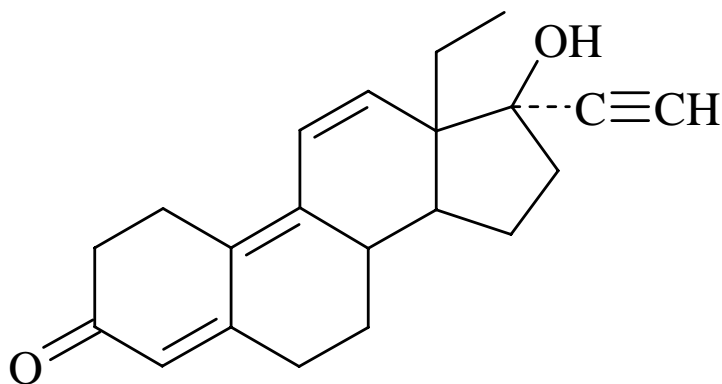
-Sample is transferred to the US doping control laboratory in Los Angeles; residues were identified as related compound to gestrinone.



Gestrinone (treatment of endometriosis – no anabolic activity)

Issue: Designer Steroids

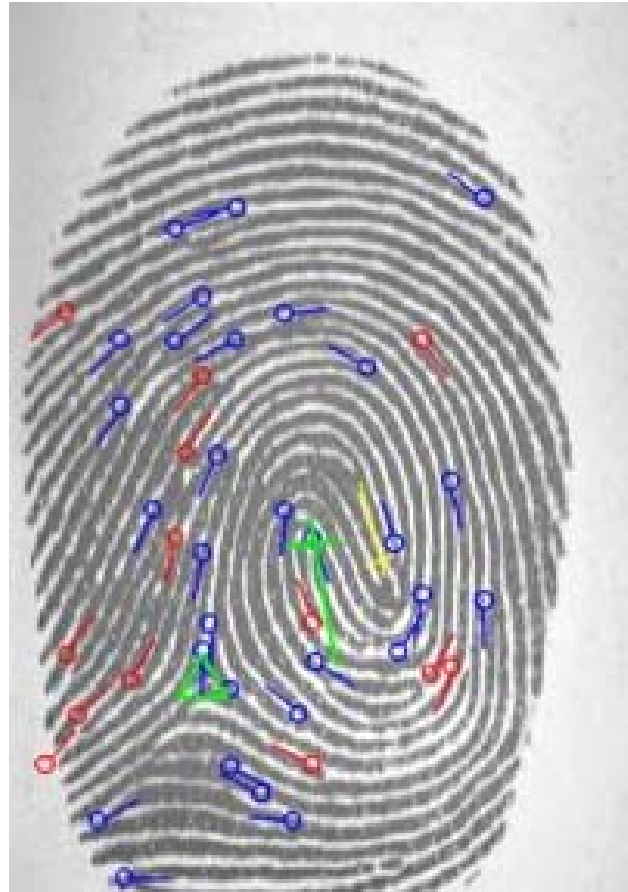
The case of „Tetrahydrogestrinone“ (THG)



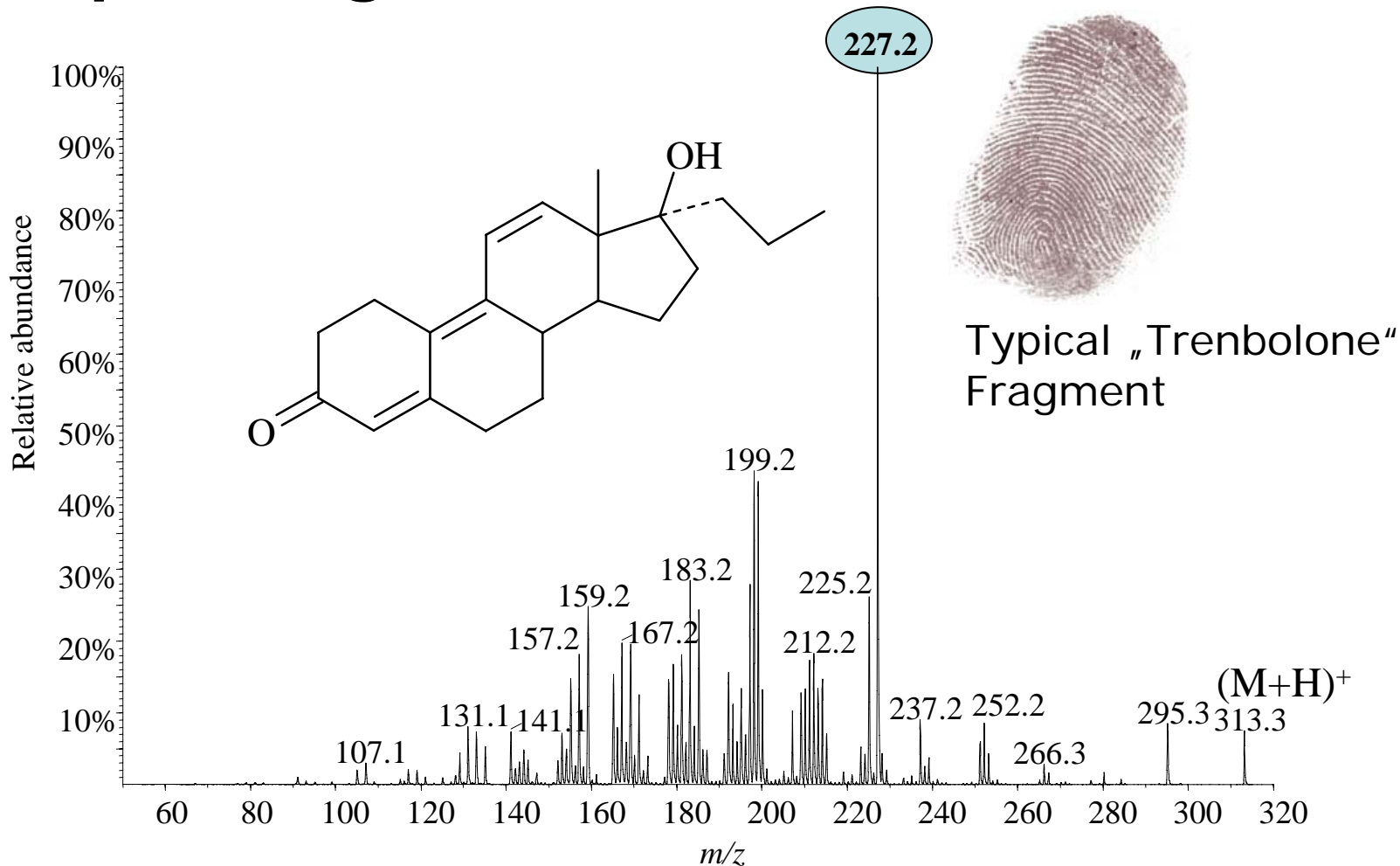
Tetrahydrogestrinone (THG)



Characteristic fragments corresponding to conserved nuclei



Characteristic fragments corresponding to conserved nuclei





Greek weightlifters banned from Beijing Olympics

June 18, 2008 10:00am

Greek Olympic weightlifting coach Christos Iakovou was all smiles during the Athens Olympics in 2004 but had little reason to be grinning yesterday. He was suspended after 11 Greek weightlifters tested positive for banned substances.

ELEVEN Greek weightlifters have been suspended for two years after testing positive for a banned steroid.

The International Weightlifting Federation said that Greece will still be able to send a team of four weightlifters - three men and a woman - to the Beijing Olympics.

The IWF said the decision was made by its executive board last Friday.

Eleven of the Greek team's 14 weightlifters tested positive for the steroid methyltrienolone during a March out-of-competition test in Athens, and the results were announced in April. The athletes have not been named.

The IWF said the Greek weightlifting federation was also sanctioned, but gave no details.

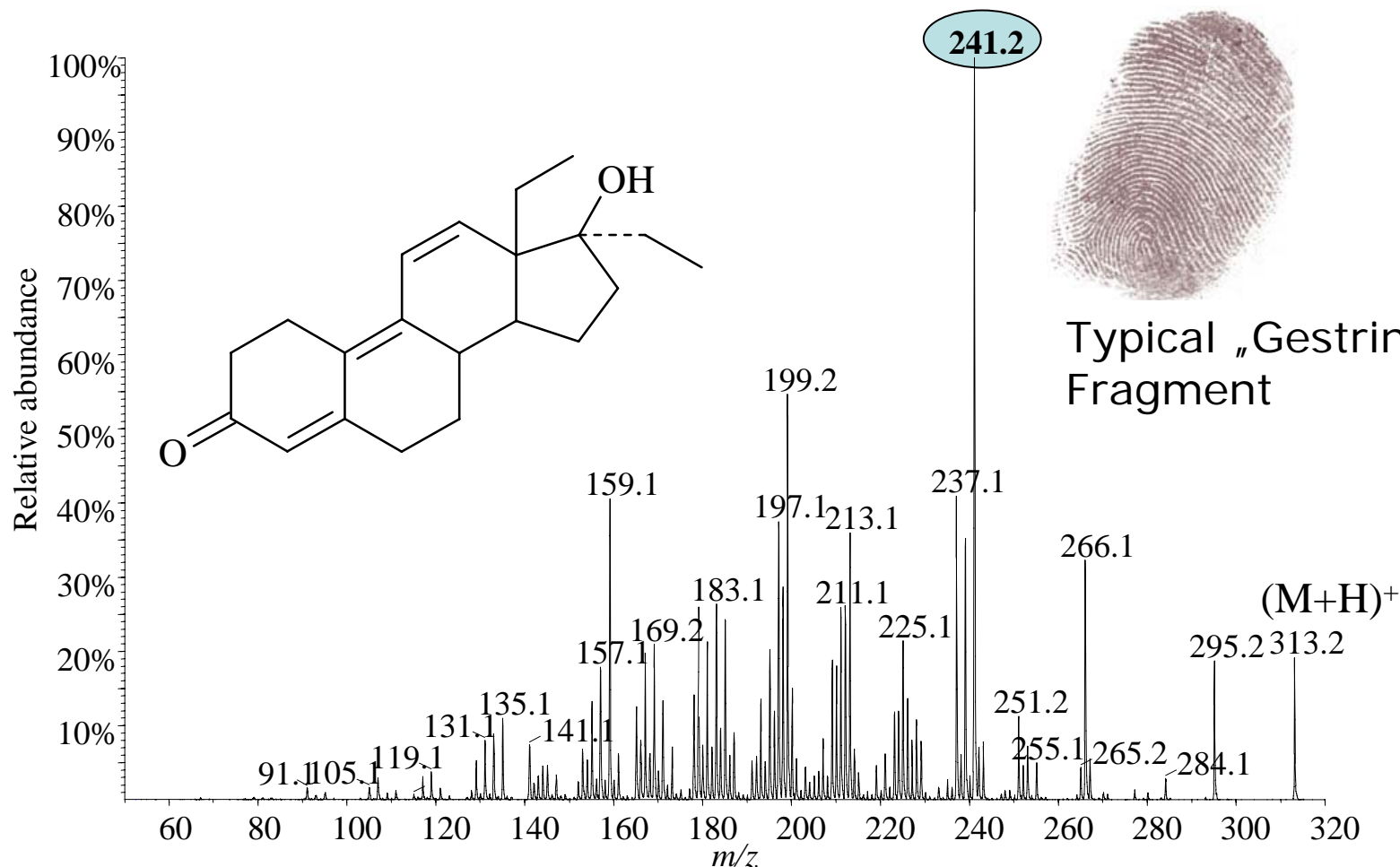
A Greek federation official said the IWF fined the federation euro 250,000 (\$387,000). He was speaking on condition of anonymity as he was not authorized to comment on the affair.

The official said the four Greek athletes for Beijing would be selected "in the next few days."

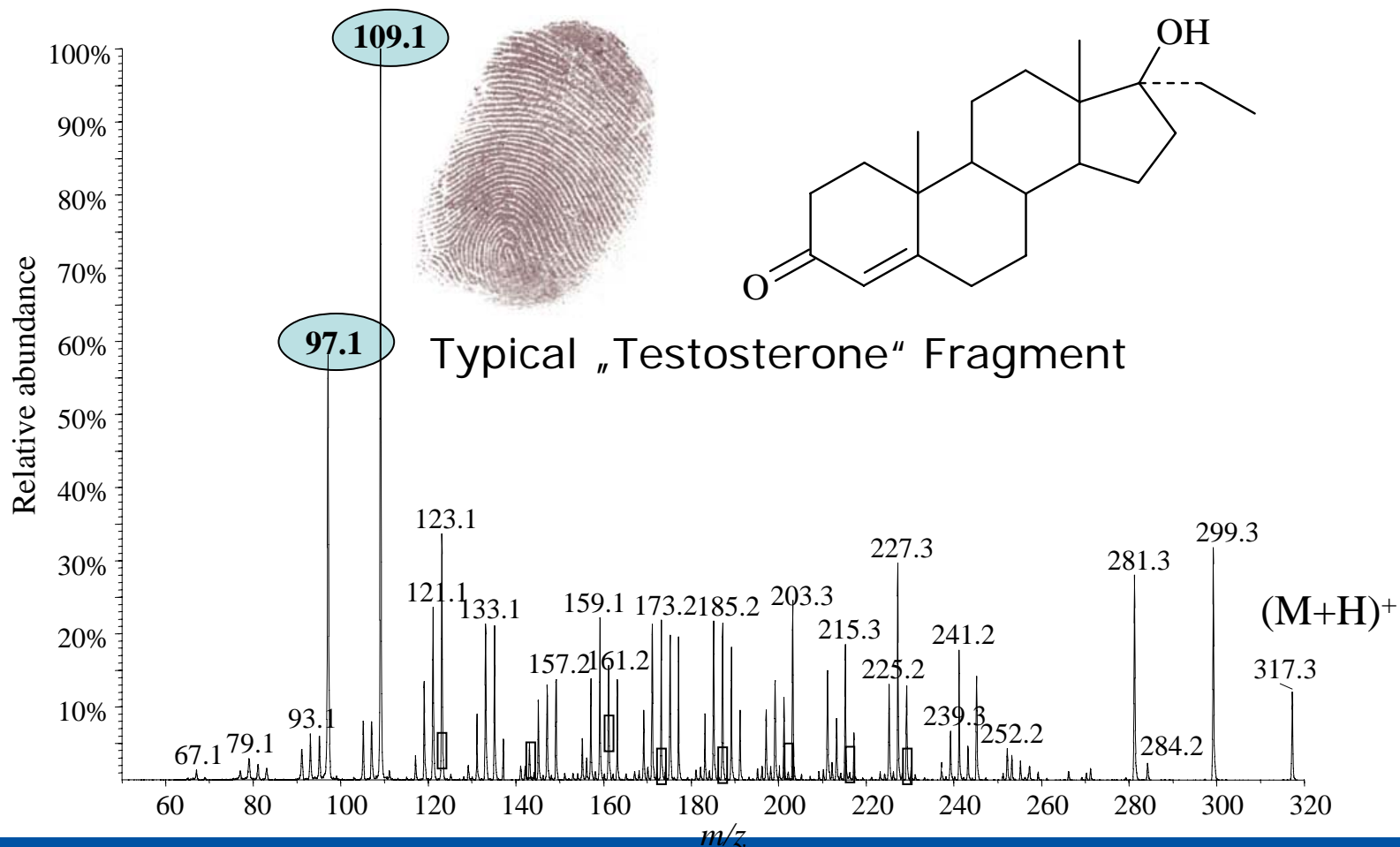
He said the weightlifters would "definitely" include Nikos Kourtidis, 22, one of the three members of the national team who did not test positive in March.



Characteristic fragments corresponding to conserved nuclei



Characteristic fragments corresponding to conserved nuclei





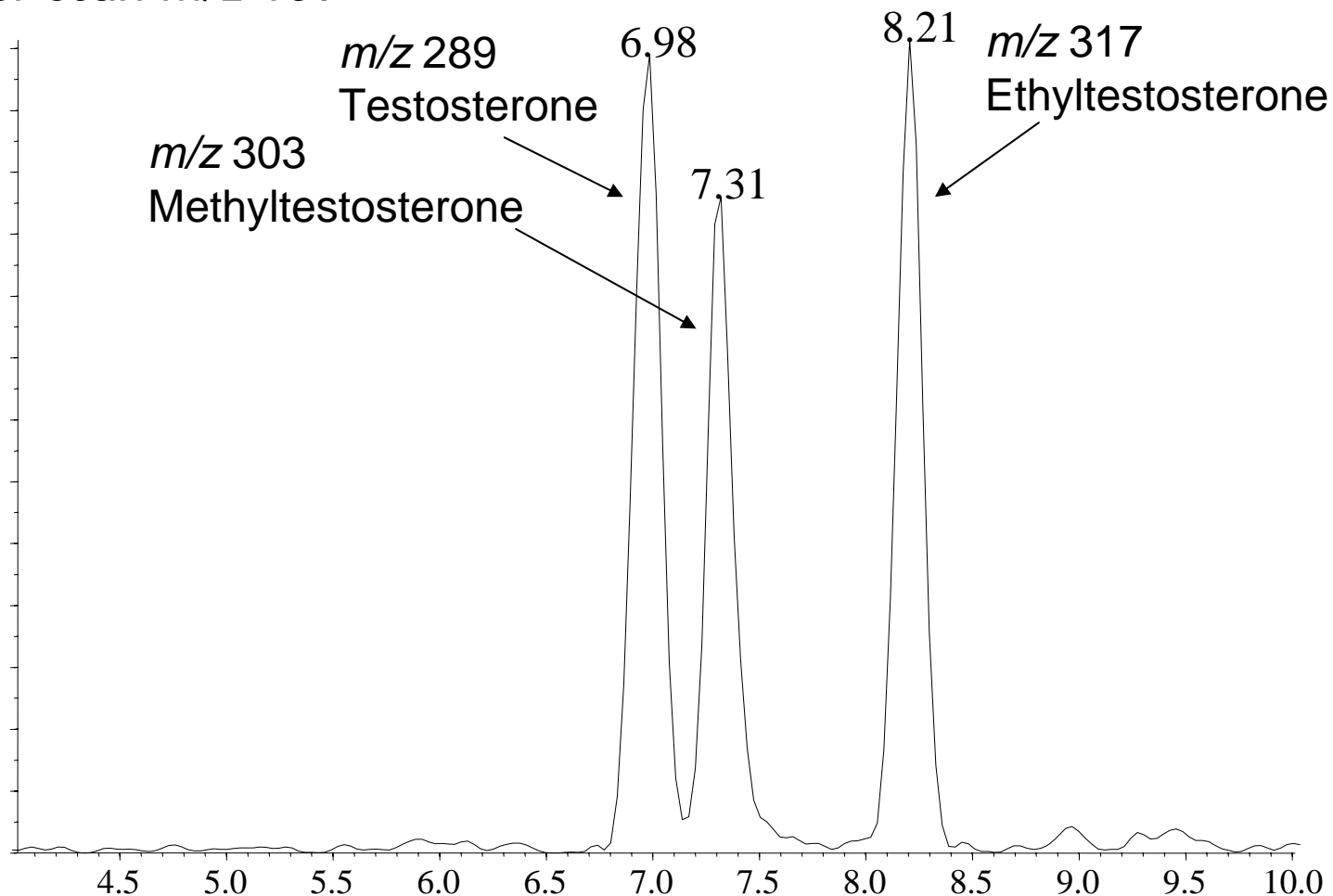
Characteristic fragments corresponding to conserved nuclei

<i>m/z</i> 241	Gestrinone Analogs
<i>m/z</i> 227	Trenbolone Analogs
<i>m/z</i> 187	1-Testosterone Analogs
<i>m/z</i> 109	Testosterone/Nortestosterone Analogs
<i>m/z</i> 97	Testosterone Analogs



Precursor Scan Screenings

Precursor scan m/z 109





Steroid doping

Reduced production of testosterone - atrophy of testes

Steroid screening and steroid profiling uncovers doping practices

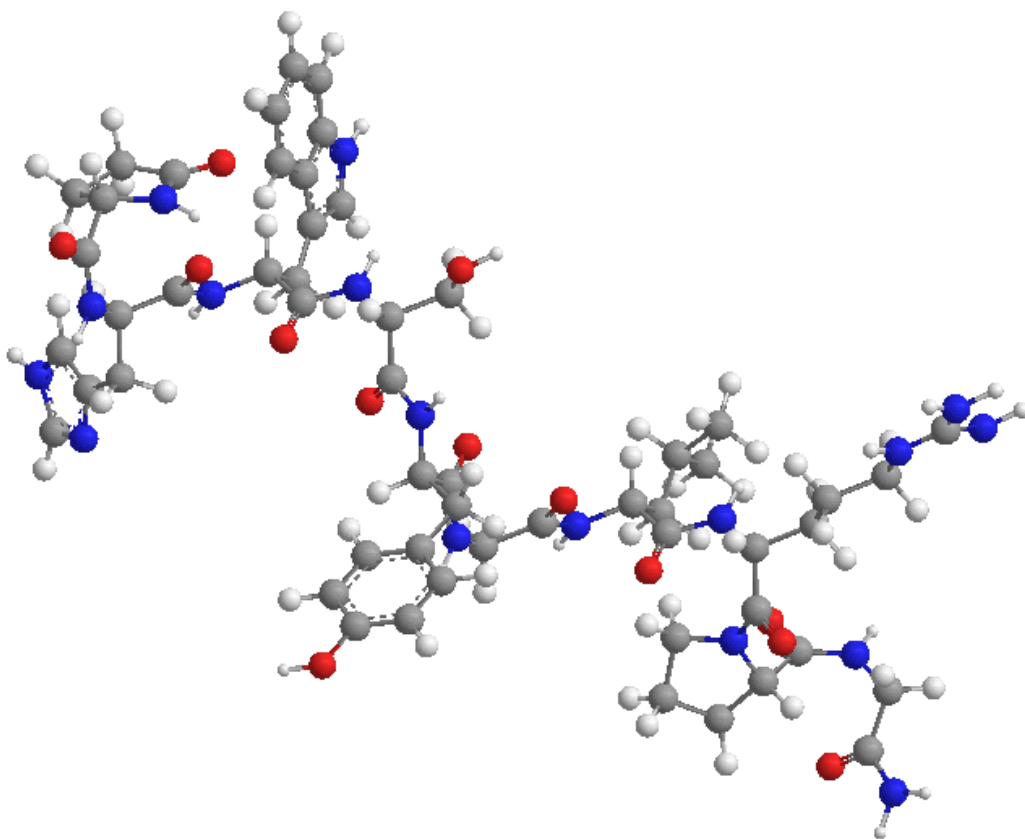
- Use of synthetic testosterone

- Isotope ratio mass spectrometry (IRMS) as detection tool



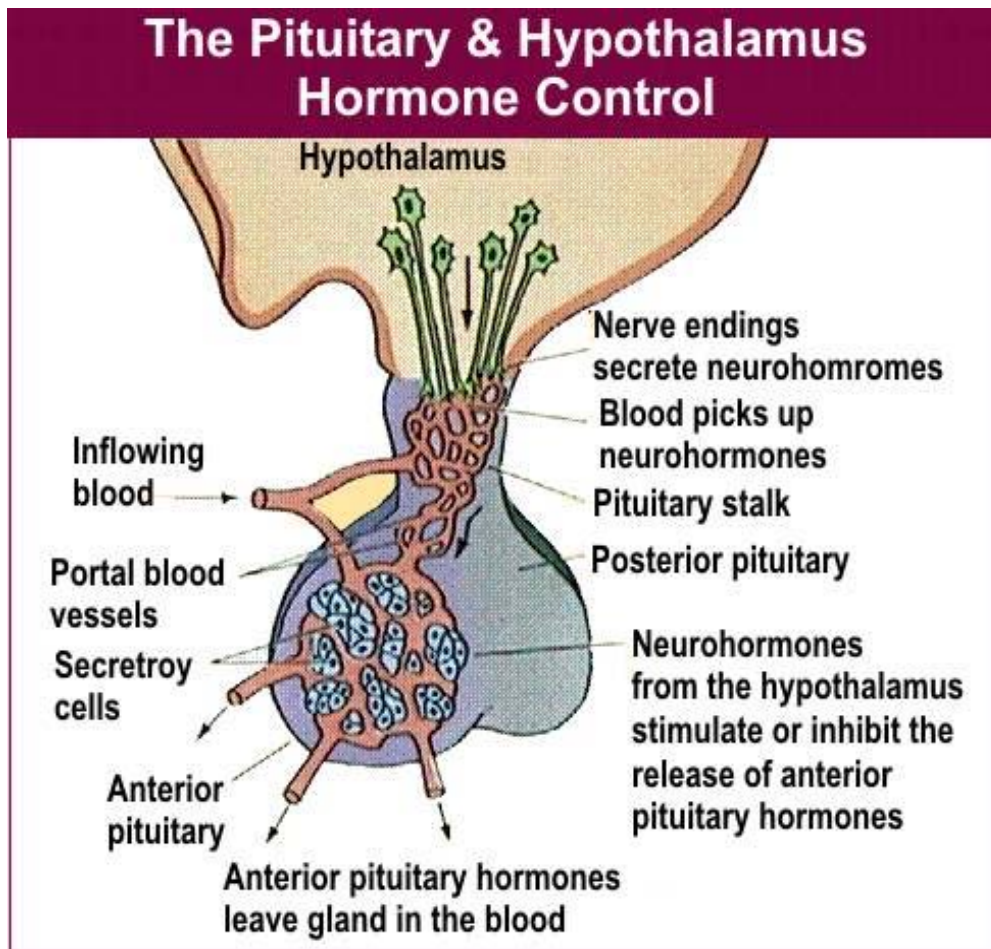
Selected Detection Assays: *Gonadorelin (LH-RH)*

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂



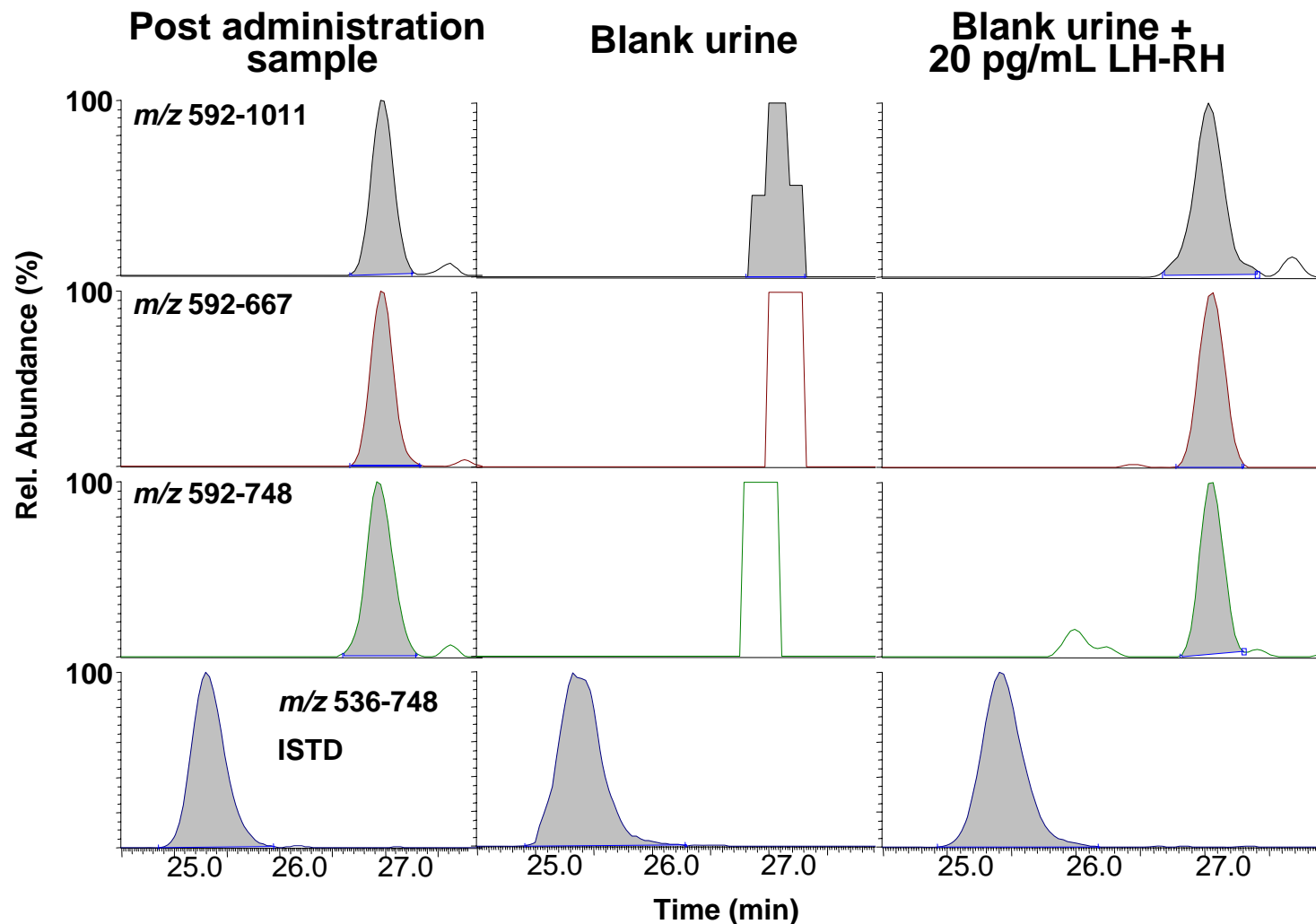


Selected Detection Assays: *Gonadorelin (LH-RH)*



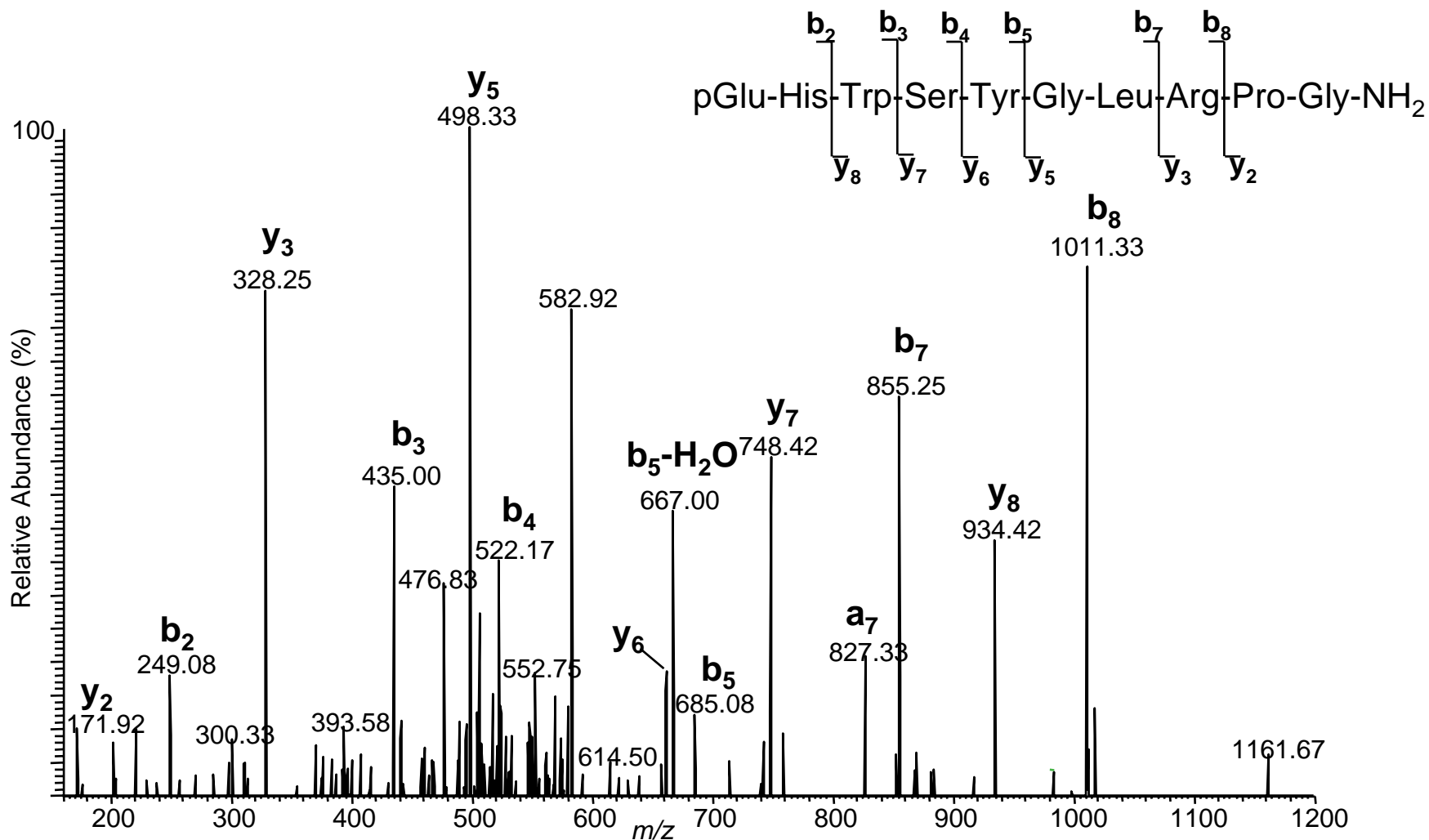


Selected Detection Assays: *LH-RH*





Selected Detection Assays: *LH-RH*





Substances and Methods Prohibited at All Times (In- and Out-Of-Competition)

S1. Anabolic Agents

Anabolic agents are prohibited

1. Anabolic Androgenic Steroids (AAS)

a. Exogenous* AAS, including:

...boldenone, ...methyltestosterone, ...nandrolone, ...etc

b. Endogenous** AAS:

...androstenediol, ...testosterone, ...etc

Other Anabolic Agents, including but not limited to:

**Clenbuterol, selective androgen receptor modulators (SARMs),
tibolone, zeranol, zilpaterol**



...other anabolic agents

Novel class of drugs:

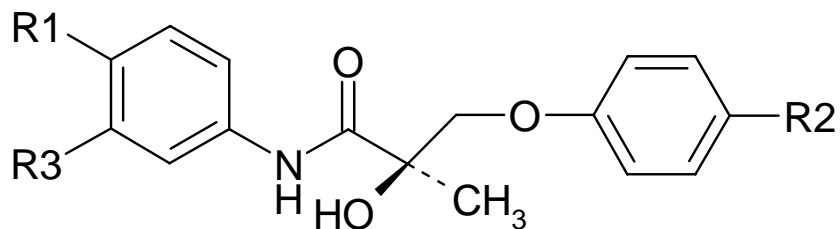
**Selective androgen receptor modulators
(SARMs)**

Categorized into

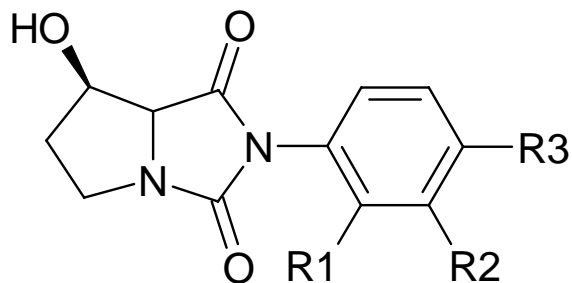
- aryl-propionamide
- bicyclic hydantoin
- quinoline
- tetrahydroquinoline
- (benzimidazole)



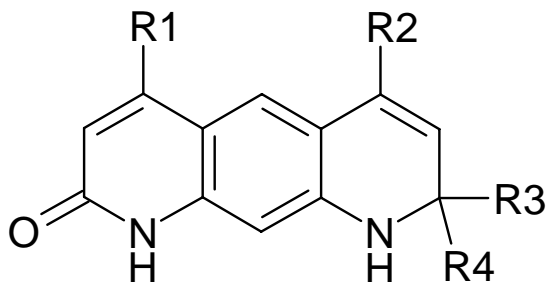
...SARMs structures



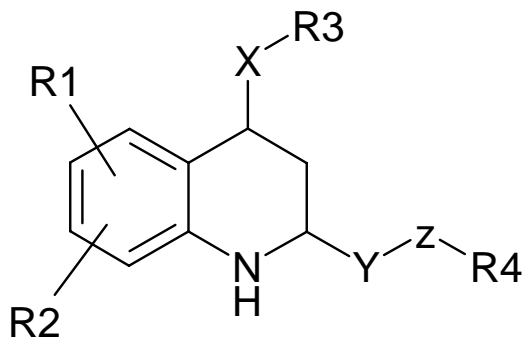
aryl-propionamide



bicyclic hydantoin



quinoline



tetrahydroquinoline



...SARMs benefits

- Full agonists in anabolic organs (e.g. muscle, bone, etc.)
- Not substrate for 5α -reductase
- possess tissue selective activities – hydantoins reported to be muscle selective (animal experiments)



...SARMs benefits

Potentially useful for prevention or treatment of

-muscle wasting

-osteoporosis

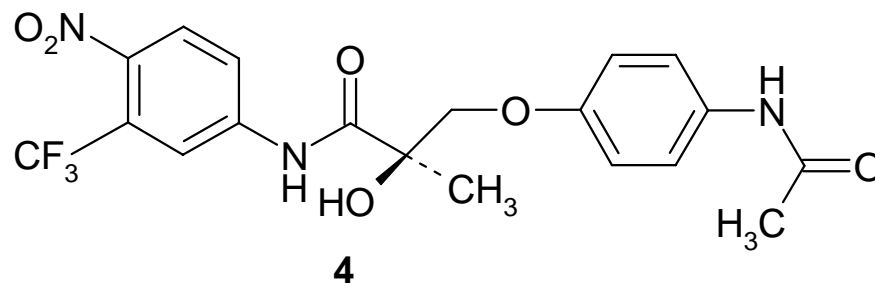
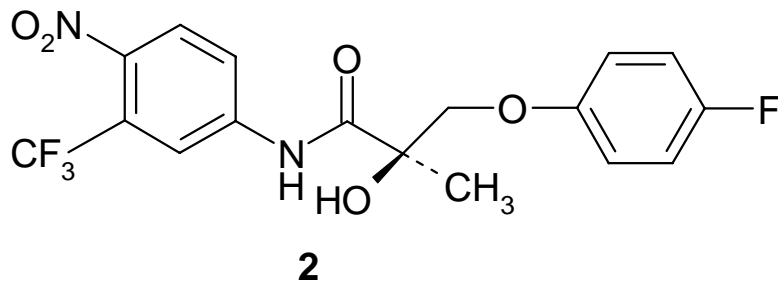
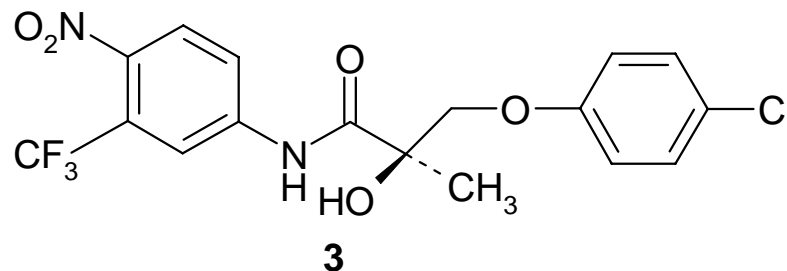
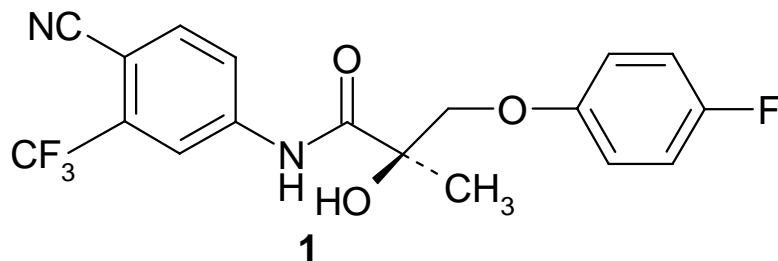
-frailty

-male contraception

Basically without androgenic side effects – high potential for misuse in sports

...Synthesis of SARMs

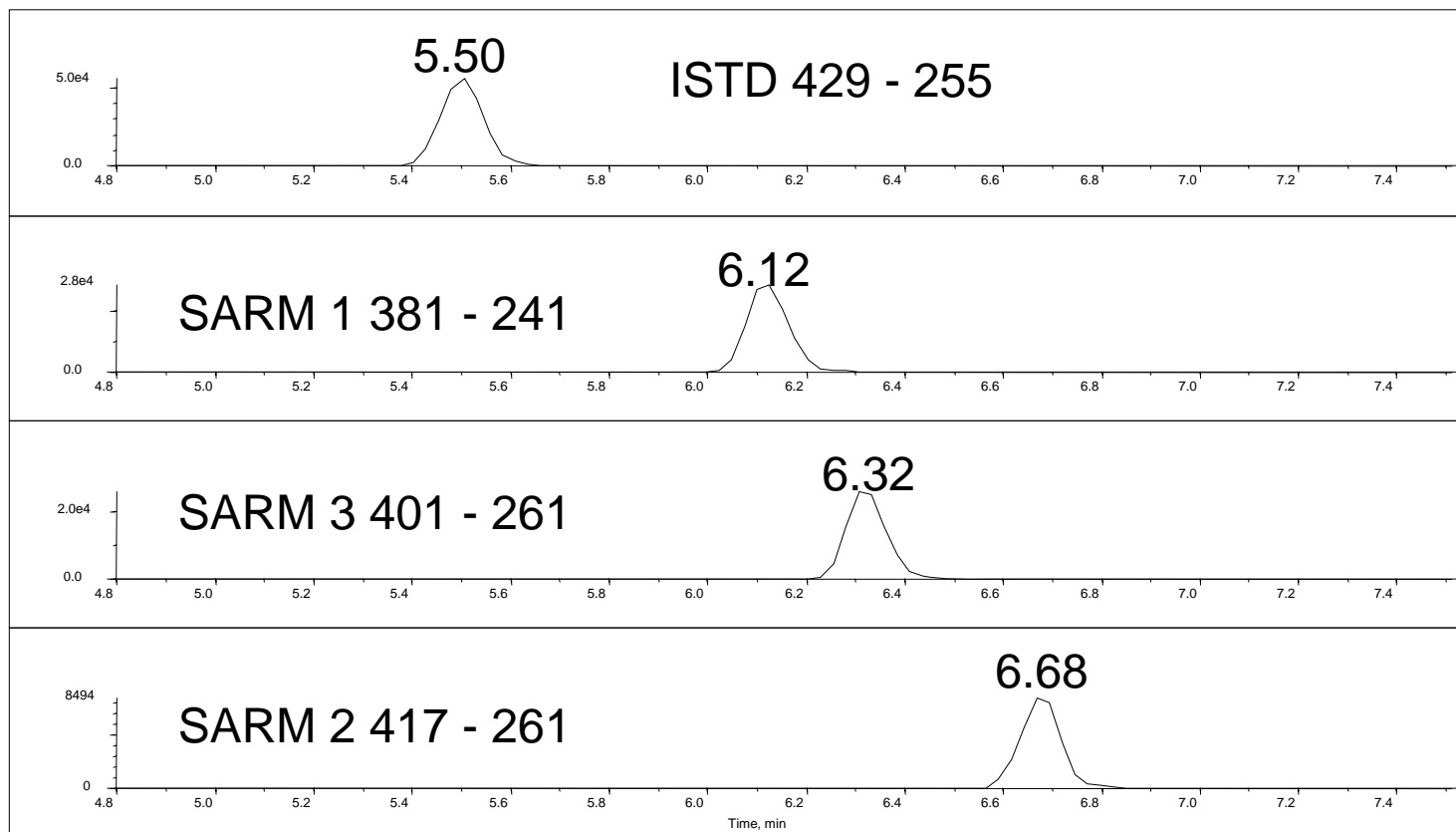
Four model aryl-propionamide derived SARMs





Analytical assay

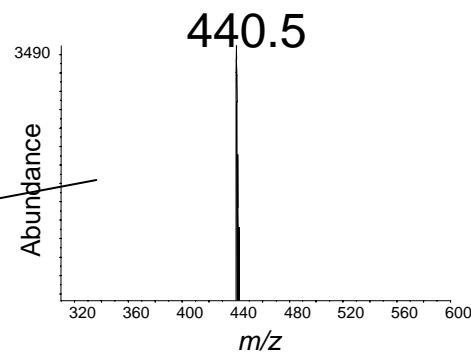
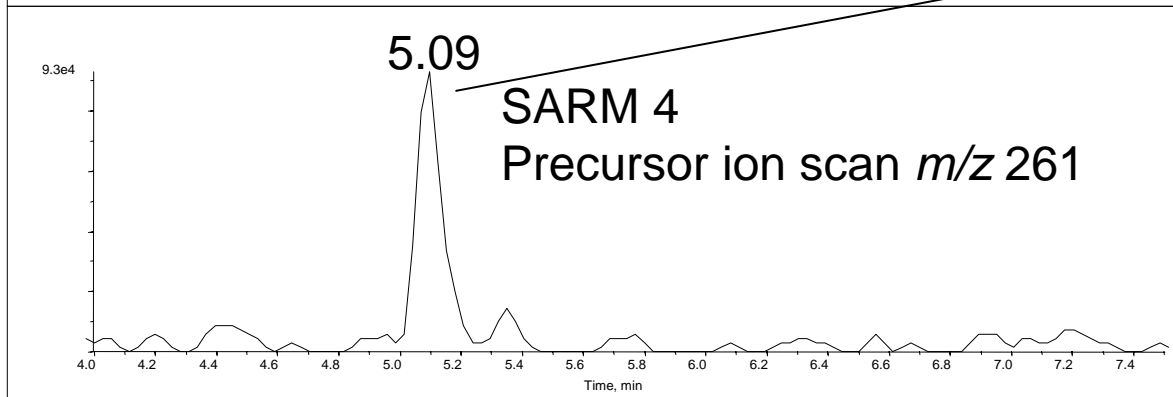
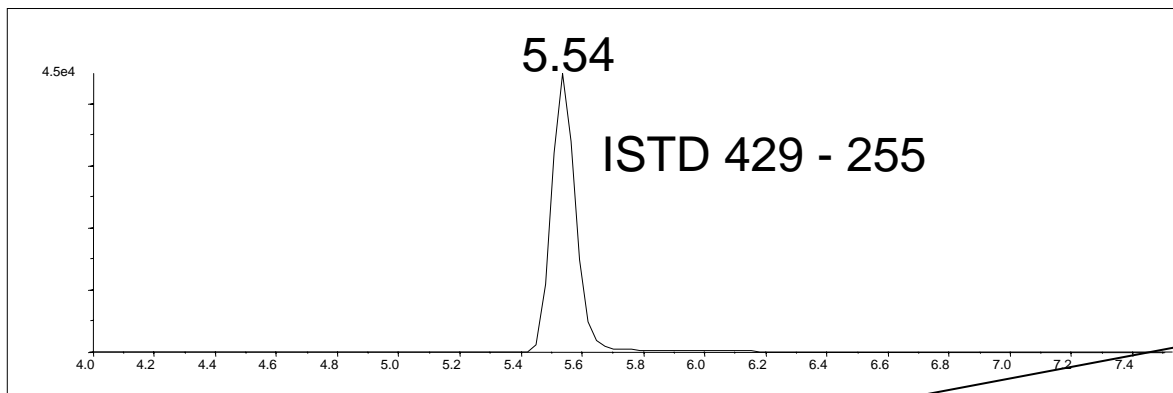
Urine specimen spiked to 1ng/mL





Precursor Scan Screening m/z 261

Urine specimen spiked to 50 ng/mL of SARM 4

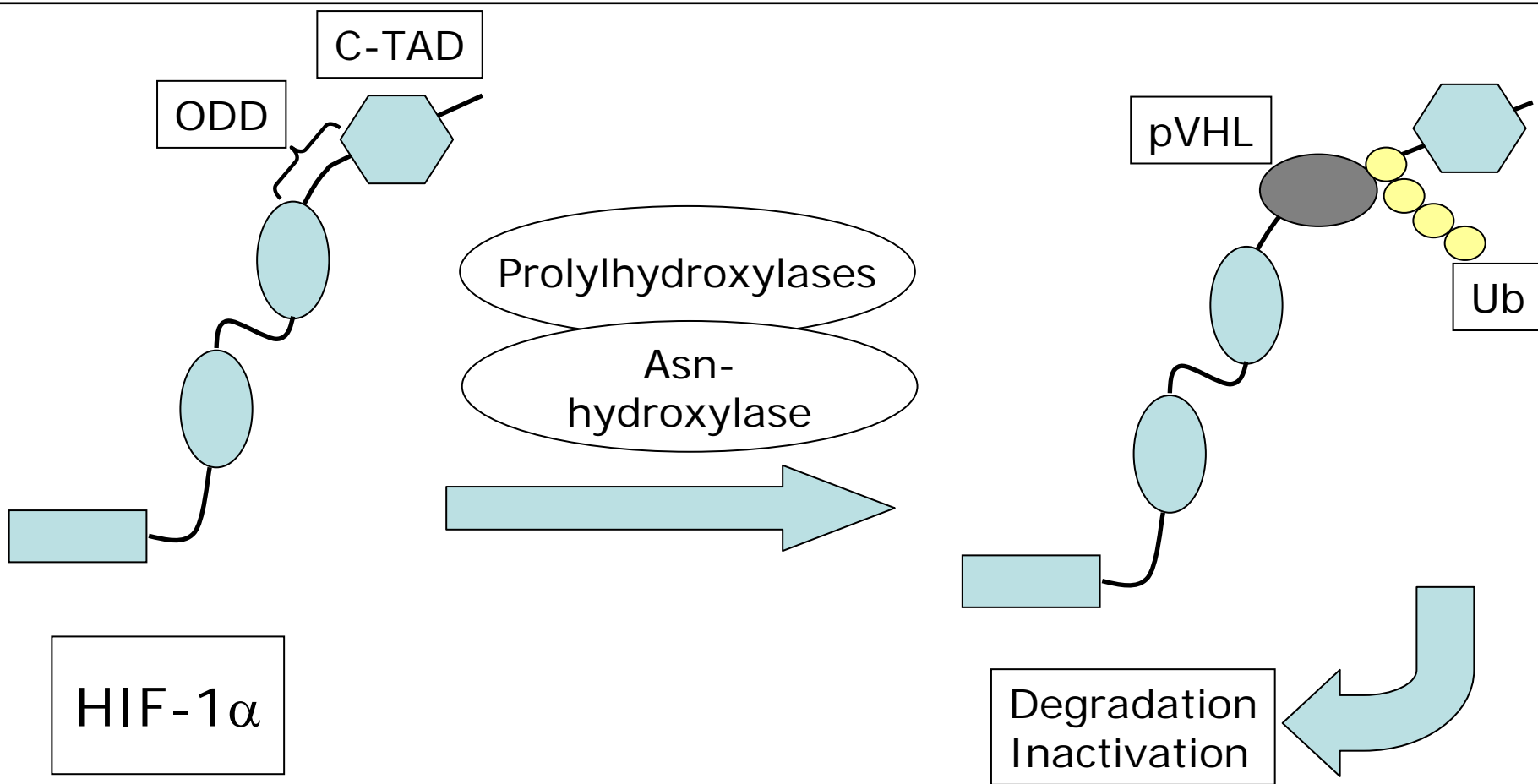




...other "secret" compounds?

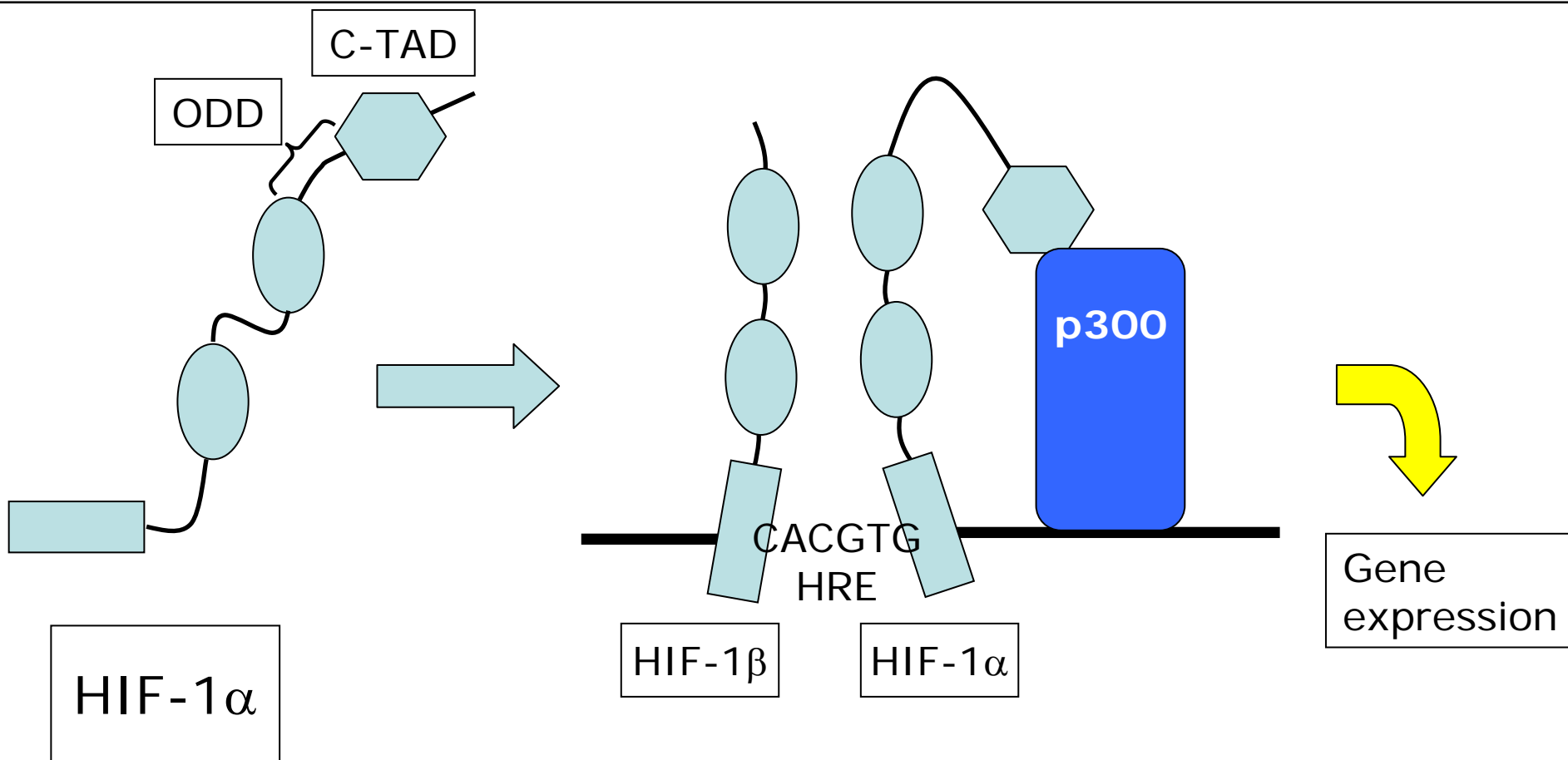
...HIF-Stabilizers

Normoxia



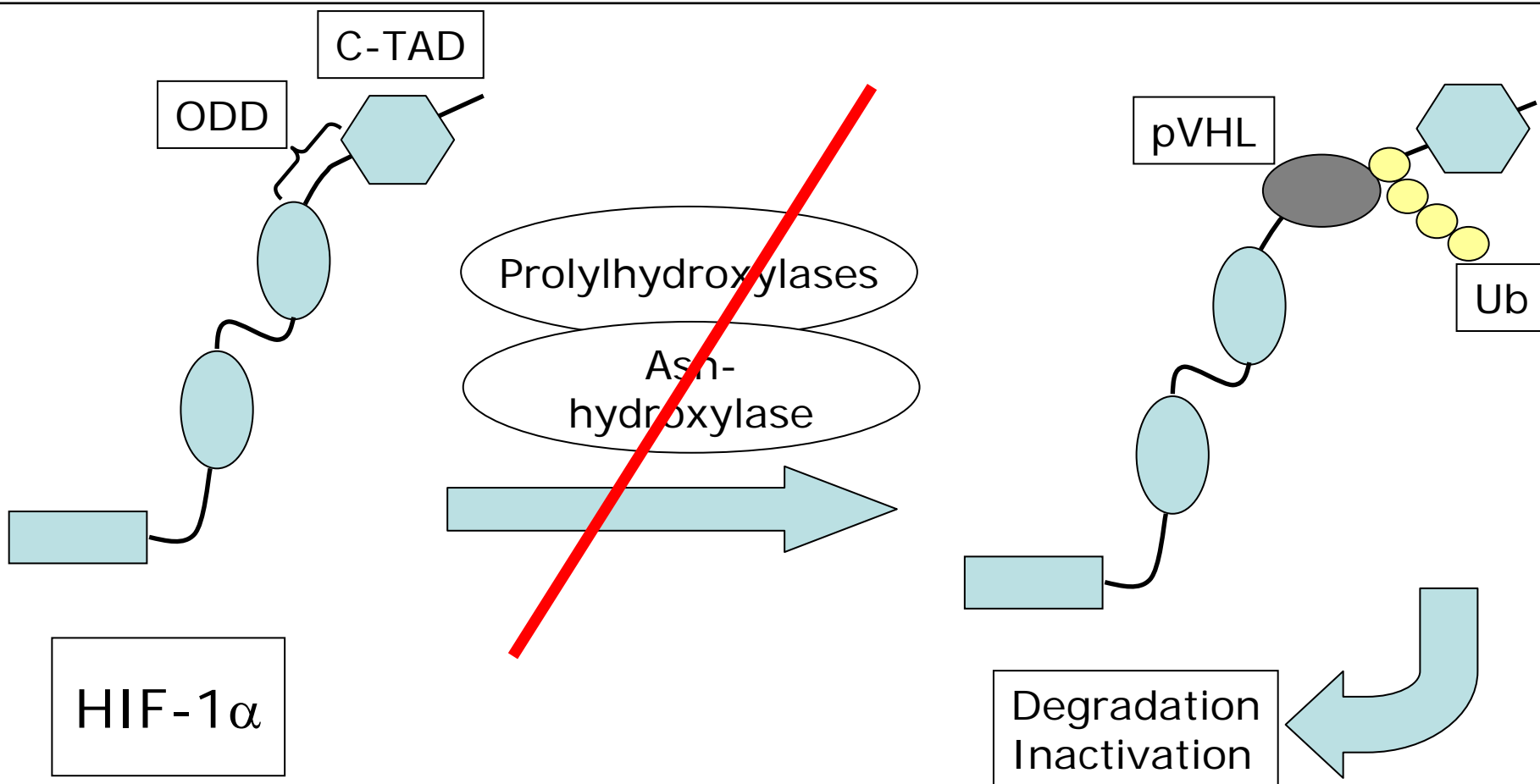
...HIF-Stabilizers

Hypoxia



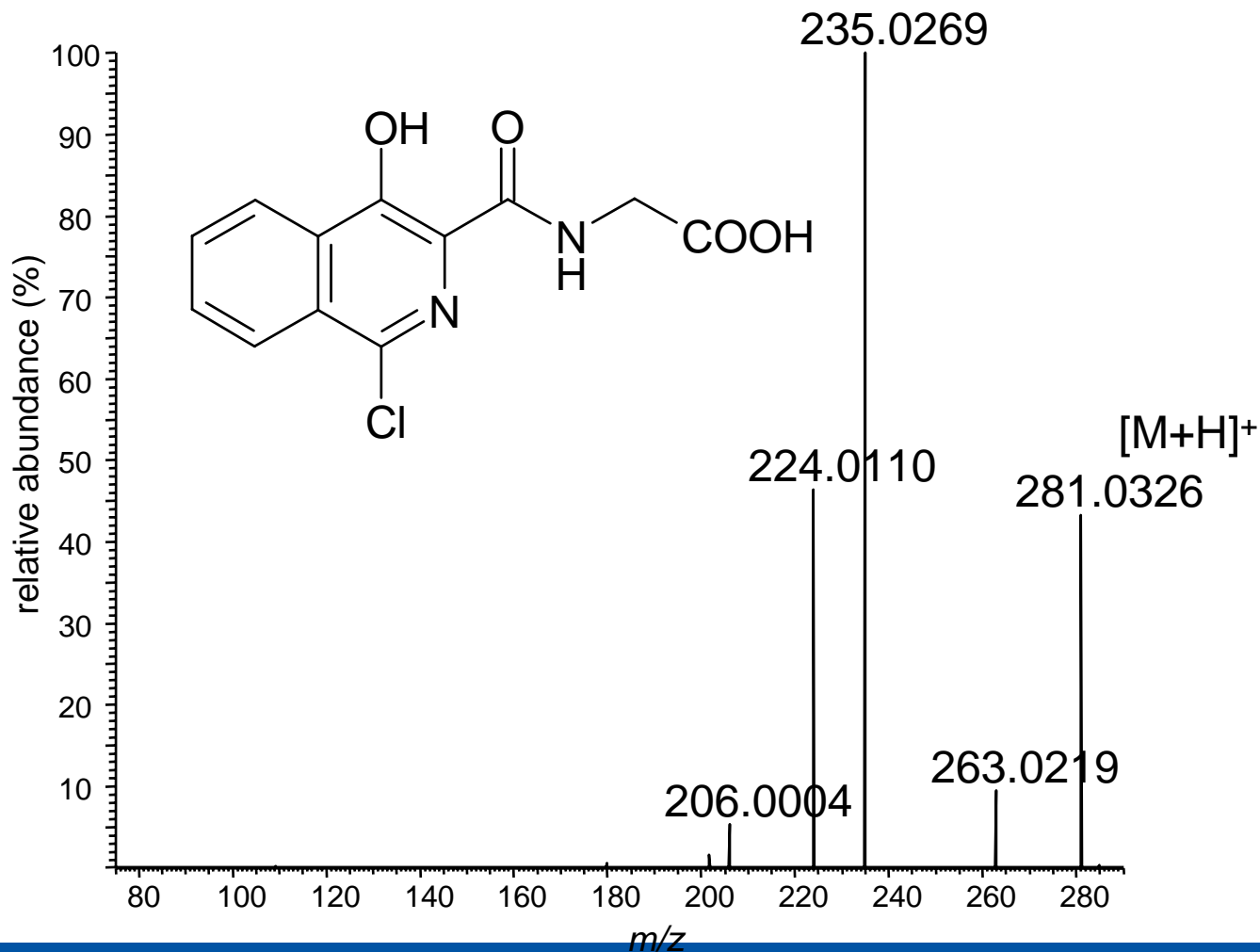
...HIF-Stabilizers

Normoxia – HIF-stabilizer



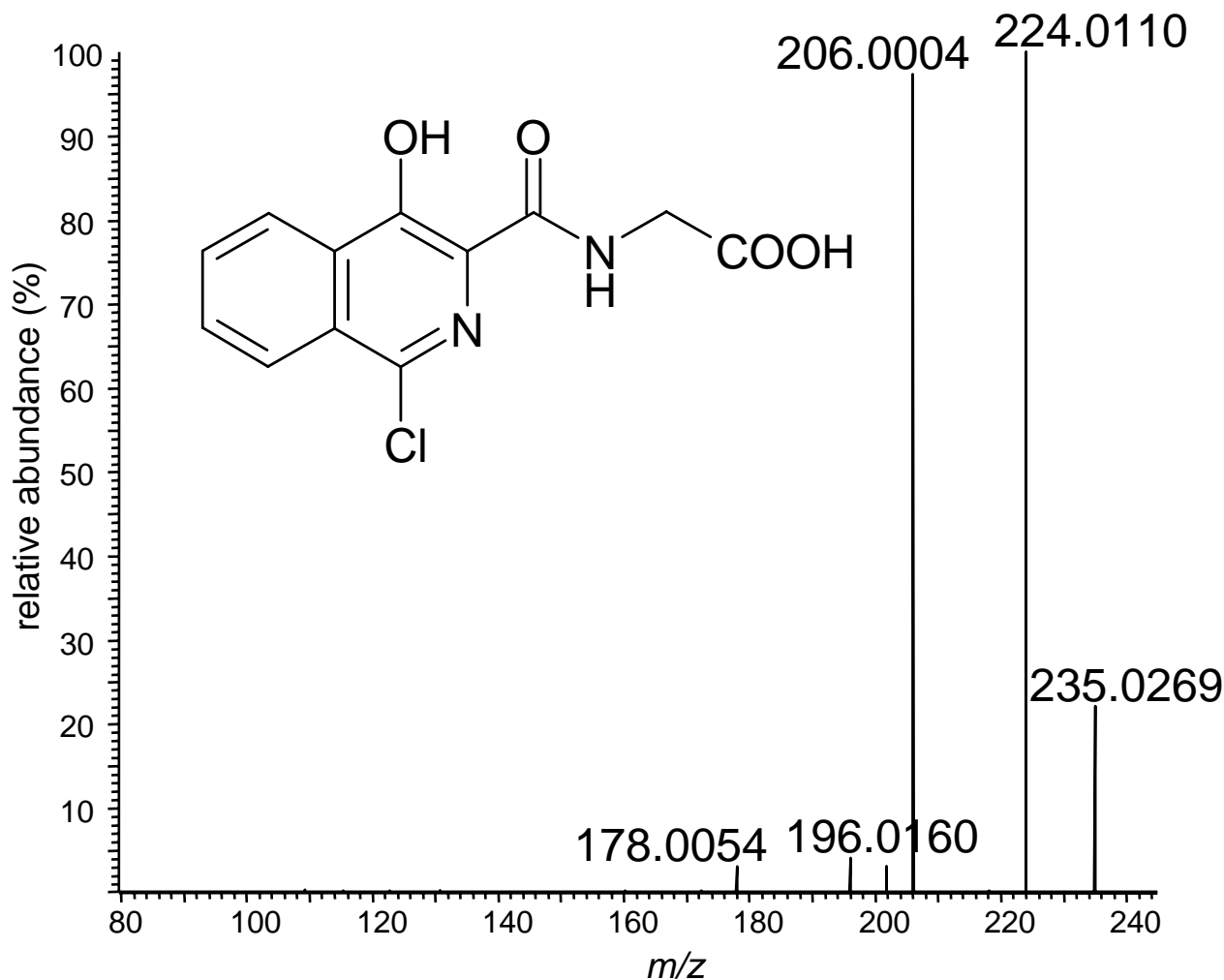


...HIF-Stabilizers





...Characterization

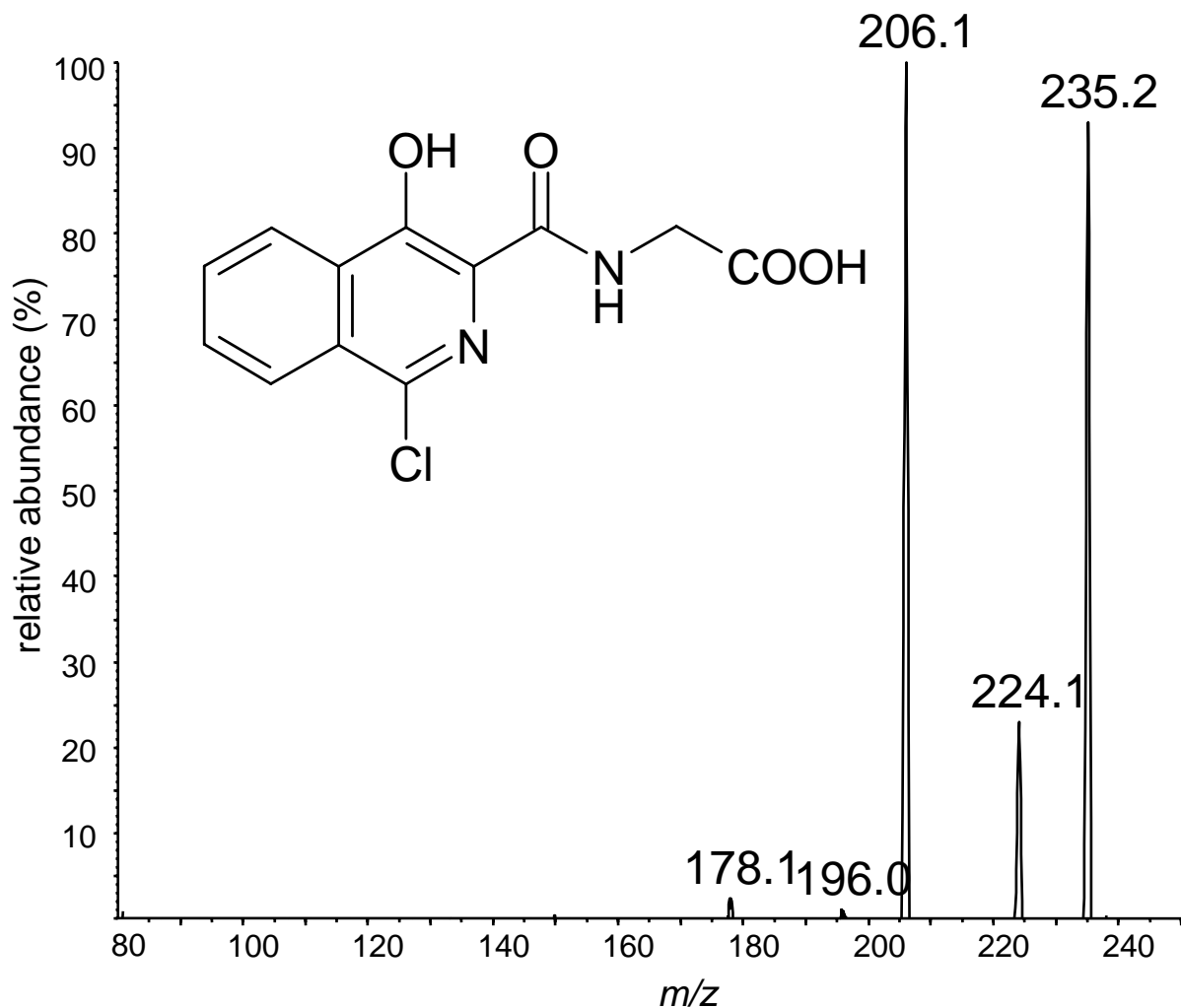


MS³
281 – 235

**Loss of
HCN (-27)
addition of
1/2 O₂ (+16)**



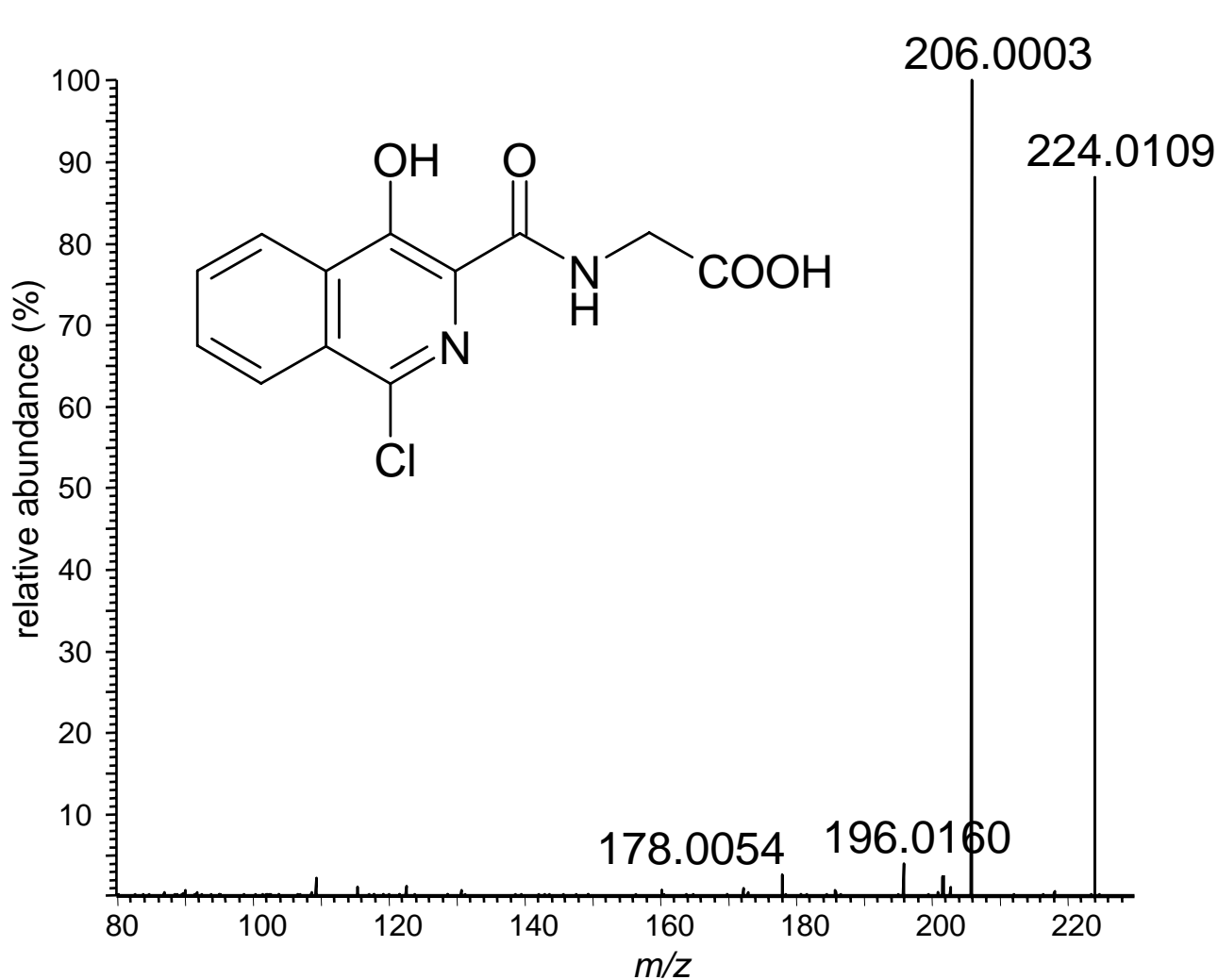
...Characterization



MS³
281 – 235
triple quad

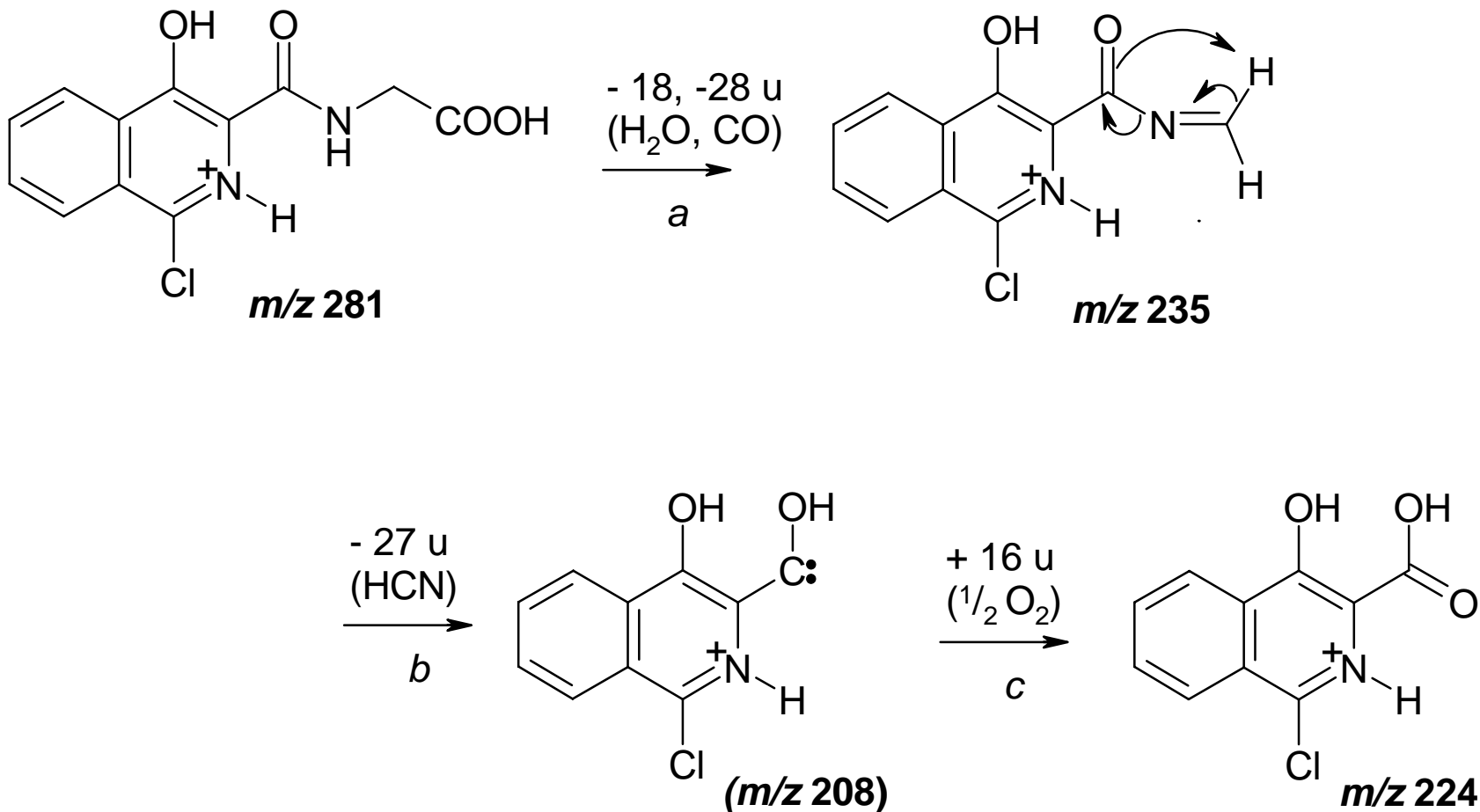


...Characterization



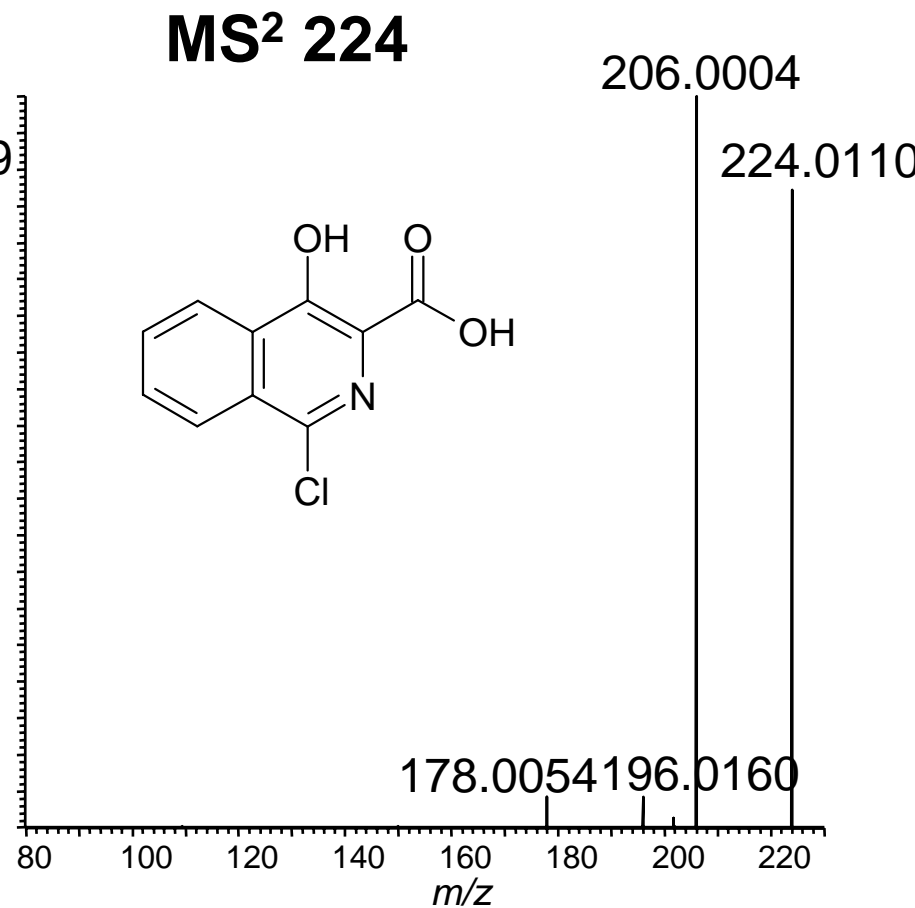
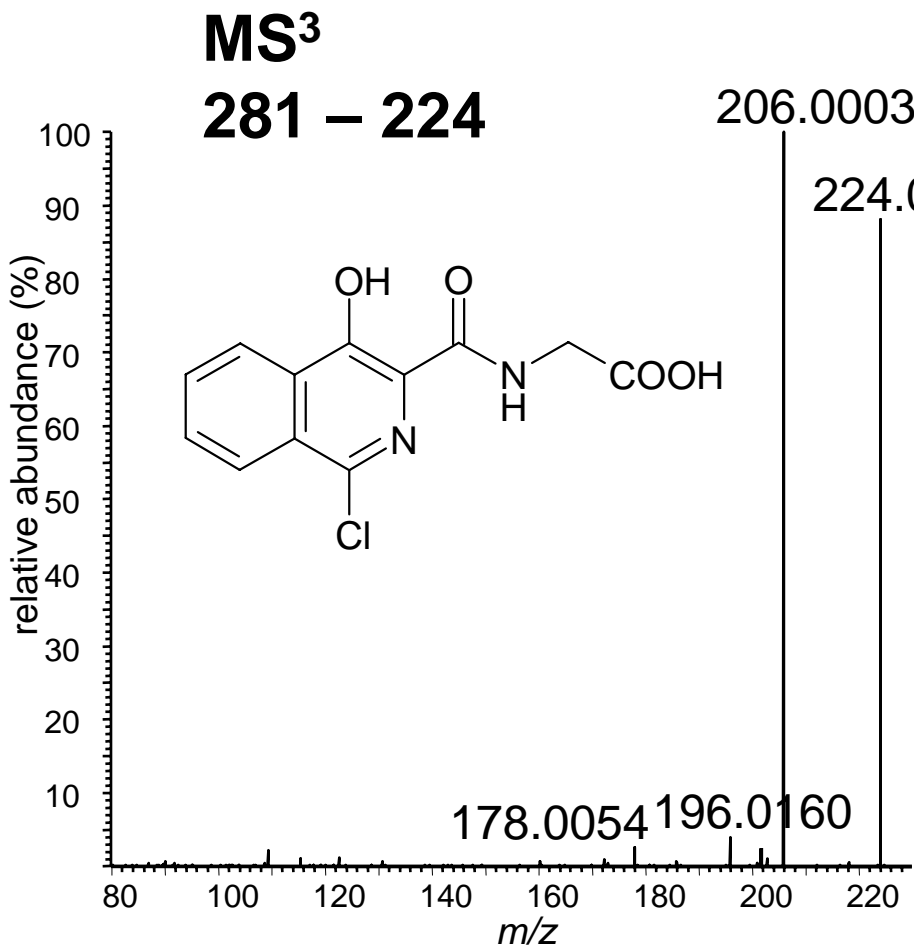
MS³
281 – 224

...Characterization



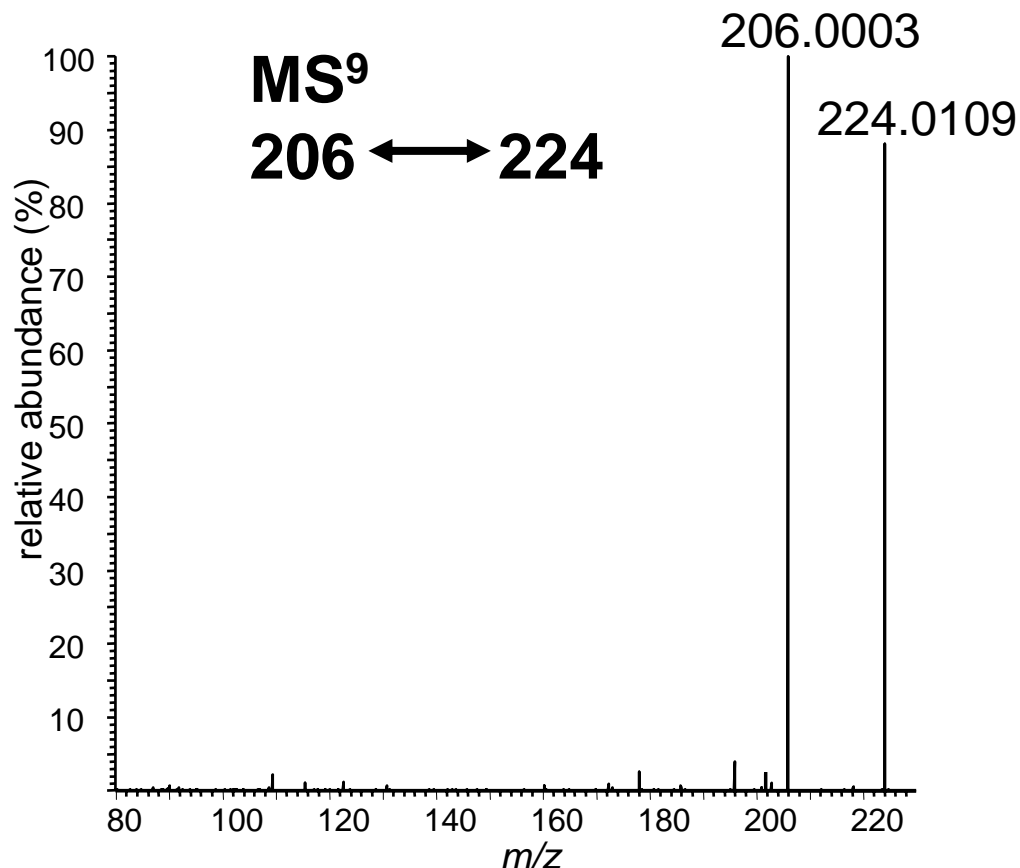


...Characterization





...Characterization



„Ping-Pong“ Experiment
dissociation of *m/z* 224 to
206 and regeneration of *m/z*
224 in the iontrap under
storage conditions

Thank you

