Liquid Chromatography - Mass Spectrometry

What it is and How to do it

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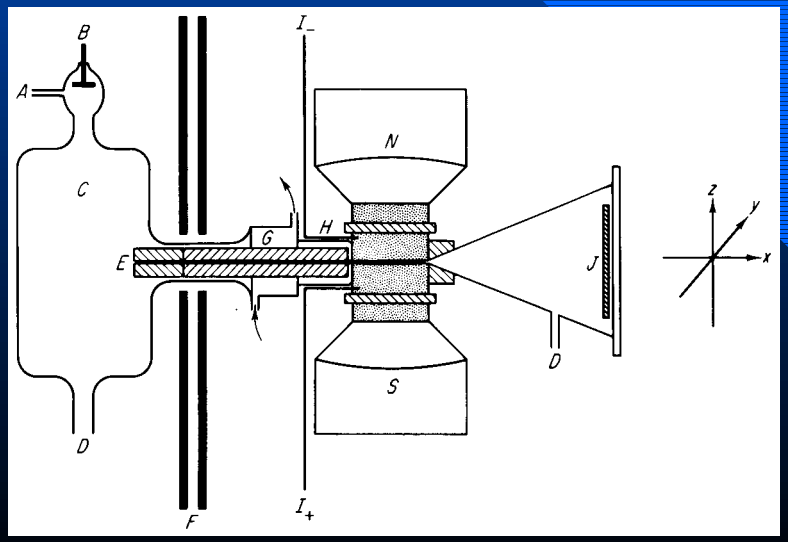
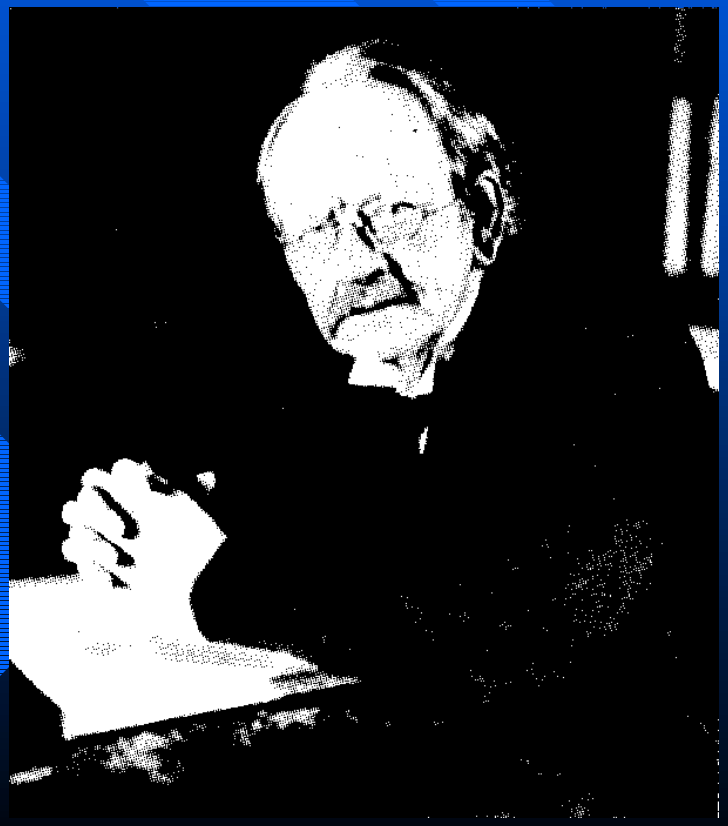
ANALYZE ME

Everything You Need to Know About Mass Spectrometry

(in 10 minutes or less)

- J.J. Thomson (1910)
  - parabola mass spectrograph
  - positive and negative ions
  - isotopes of neon





Mass Spectrometric Alphabet Soup

EI	FI	MSMS	ITMS
CI	ECIN	LD	FTMS
FAB	NICI	MALDI	RIMS
FIB	PPINICI	TOF	LAMMA
FD	GCMS	QQQ	PDMS
APCI	LCMS	EBEB	ICPMS
ESP	ICPMS	BEEB	FTMS
TSP	PB	EBQQ	ROOMS

Some Concepts to Clarify

- relative molecular mass
- chemical vs monoisotopic molecular mass
- what the mass spectrometer really measures
- isotope patterns
- accurate mass vs. high resolution
- accurate mass measurements
- one does not prove a structure: one can only disprove it!

Atomic Weight
vs
Relative Atomic Mass

- Weight measures the influence of an external gravitational field on a quantity of matter
- Mass is a measure of the amount of matter, independent of any gravitational field
- mass of an atom of $^{16}O = 1.65979 \times 10^{-24}$ grams
- ^{12}C is the current standard
- the dalton is the accepted atomic mass unit

The Oxygen Standard

- the a.m.u. was originally defined to be 1/16 the atomic mass of oxygen
- oxygen discovered (1929) to include two minor isotopes – ^{17}O (0.04%) and ^{18}O (0.2%)
- chemists' scale based on wet chemical measure of oxygen's mass, "in error" because of the presence of the minor isotopes
- physicists' scale focused on intrinsic mass of the ^{16}O isotope
- scales differed by 0.0044 amu
- IUPAC standardization efforts began in 1956

The Carbon Standard

- IUPAC adopted the ^{12}C standard in 1960
- amu = 1/12 of the mass of a ^{12}C atom
- defined in reference to a specific isotope rather than on an element (including all of its naturally occurring isotopes)
- simple conversion of all chemists' and physicists' tabulations to the ^{12}C standard

	IA	IIA	IIIB	IVB	VB	VIB	VIIB	VIII			IB	IIB	IIIA	IVA	VA	VIA	VIIA	O
1	+1 1 H 1.0079																	2 He 4.003
2	+1 3 Li 6.941	+2 4 Be 9.012											+3 5 B 10.81	+4 6 +2 C 12.011	+5 7 +3 N −3 14.007	8 O −2 15.999	9 F −1 18.998	10 Ne 20.18
3	+1 11 Na 22.99	+2 12 Mg 24.30											+3 13 Al 26.98	+4 14 +2 Si 28.08	+5 15 +3 P −3 30.97	+6 16 +4 S −2 32.06	+7 +5 +3 17 Cl +1 −1 35.45	18 Ar 39.95
4	+1 19 K 39.10	+2 20 Ca 40.08	21 Sc 44.96	22 Ti 47.90	23 V 50.94	+6 +3 24 Cr 52.00	+5 +4 +2 25 Mn 54.94	+3 +2 26 Fe 55.85	+3 +2 27 Co 58.93	+3 +2 28 Ni 58.71	+2 +1 29 Cu 63.55	+2 30 Zn 65.38	+3 31 Ga 69.72	+4 +2 32 Ge 72.59	+5 +3 33 As 74.92	+6 +4 34 Se −2 78.96	+7 +5 +3 +1 −1 35 Br 79.90	36 Kr 83.80
5	+1 37 Rb 85.47	+2 38 Sr 87.62	39 Y 88.91	40 Zr 91.22	41 Nb 92.91	42 Mo 95.94	43 Tc 98.91	44 Ru 101.07	45 Rh 102.91	46 Pd 106.4	+1 47 Ag 107.87	+2 48 Cd 112.40	+3 49 In 114.82	+4 +2 50 Sn 118.69	+5 +3 51 Sb 121.75	+6 +4 52 Te −2 127.60	+7 +5 +3 +1 −1 53 I 126.90	54 Xe 131.30
6	+1 55 Cs 132.91	+2 56 Ba 137.34	57 La 138.91	72 Hf 178.49	73 Ta 180.95	74 W 183.85	75 Re 186.2	76 Os 190.2	77 Ir 192.22	78 Pt 195.09	79 Au 196.97	+2 +1 80 Hg 200.6	+3 +1 81 Tl 204.4	+4 +2 82 Pb 207.2	+5 +3 83 Bi 209.0	+6 +4 84 Po (210)	85 At (210)	86 Rn (222)
7	+1 87 Fr (223)	+2 88 Ra 226.0	89 Ac (227)	104 Ku* (264)	105 Ha* (260)	106 " (263)												

Lanthanum Series	58 Ce 140.12	59 Pr 140.1	60 Nd 144.24	61 Pm (147)	62 Sm 150.4	63 Eu 151.96	64 Gd 157.2	65 Tb 158.93	66 Dy 162.50	67 Ho 164.93	68 Er 167.26	69 Tm 168.93	70 Yb 173.04	71 Lu 174.97
Actinium Series	90 Th 232.0	91 Pa 231.0	92 U 238.0	93 Np 237.0	94 Pu (242)	95 Am (243)	96 Cm (247)	97 Bk (247)	98 Cf (247)	99 Es (254)	100 Fm (253)	101 Md (256)	102 No (254)	103 Lr (257)

I conceive, therefore, that when we say the atomic weight of, for instance, calcium is 40, we really express the fact that, while the majority of calcium atoms have an actual atomic weight of 40, there are not a few of which are represented by 39 or 41, a less number by 38 or 42, and so on.

Is it not possible… that these heavier and lighter atoms may have been… sorted out by a process resembling chemical fractionation? This sorting out may have taken place… while atomic matter was condensing...., but also… in geological ages by successive solutions and reprecipitations of the various earths. … I do not think it beyond the power of chemistry to test this feasibility.

W. Crookes (1886)
address to the British Association,
Birmingham

Monoisotopic vs. Chemical Relative Molecular Mass

- relative molecular mass (r.m.m.), in daltons
- chemical (average) -- using periodic table atomic masses (weighted averages)
- monoisotopic -- using the lowest mass stable isotope (an arguable rule)
- nominal (integral) mass -- no decimal places
- accurate (exact) mass -- four decimal places
- mass defect (sufficiency or deficiency)
- which one to use?

Monoisotopic accurate masses of selected elements

- ^{12}C – 12.00000
- ^{13}C – 13.00336
- ^{1}H – 1.007825
- ^{14}N – 14.00307
- ^{16}O – 15.99491
- ^{18}O – 17.99916
- ^{19}F – 18.9984
- ^{32}S – 31.9721
- ^{34}S – 33.96787
- ^{35}Cl – 34.9689
- ^{37}Cl – 36.9659

- H^0 – 1.007825
- H^+ – 1.007276

- Sources of masses and abundances:
  - P. DeBievre, I.L. Barnes (1985). **Int'l J. Mass Spectrometry & Ion Processes** 65,211-30.
  - **CRC Handbook**
  - O.A. Mamer & A. Lesimple (2004). **JASMS** 15,626
  - your friendly local mass spectroscopist

An Example:

hexatriacontane, $C_{36}H_{74}$

- C 36 x 12.0000 = 432.0000 | 12.011 = 432.396
- H 74 x 1.0078 = 74.5772 | 1.008 = 74.592

506.5772 | 506.988

- 65 hydrogen atoms contribute 0.5 da of excess mass

36 x 12 = 432
74 x 1 = 74

506

An Example:

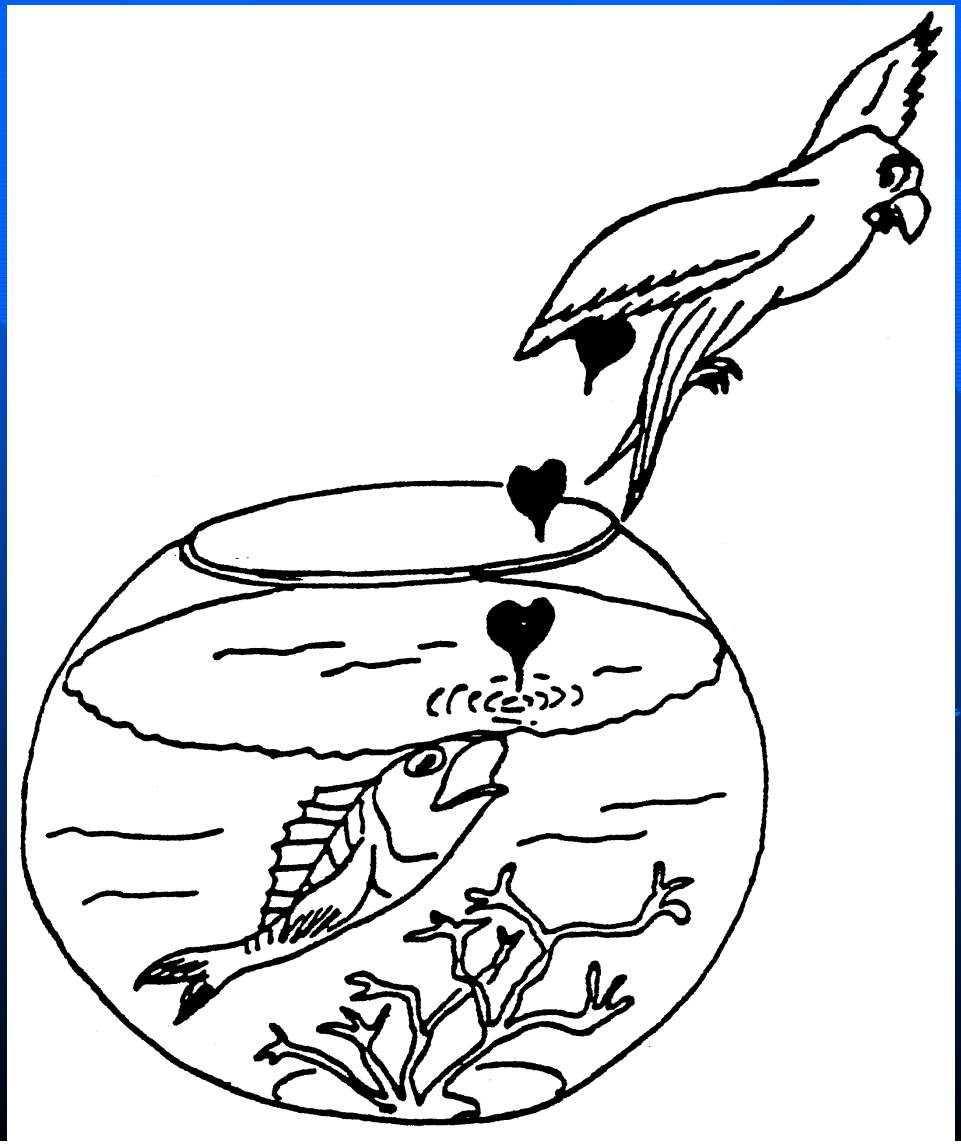
$C_{14}H_{12}NOFCl_2$

C	14 x 12.0000 = 168.0000	12.001 = 168.154
H	12 x 1.0078 = 12.0936	1.008 = 12.096
N	1 x 14.0031= 14.0031	14.007 = 14.007
O	1 x 15.9949 = 15.9949	15.999 = 15.999
F	1 x 18.9984 = 18.9984	18.998 = 18.998
^{35}Cl	2 x 34.9689 = 69.9378	35.45 = 70.900
	299.0278	300.154

High Resolution vs Accurate Mass Measurement

- high resolution -- ability to distinguish different elemental compositions
- accurate mass measurement -- precise relative mass measurement (to 4 decimal places or more)
- high resolution nominal mass measurement
- low resolution accurate mass measurement
- see K. Biemann (1990) Methods in Enzymology 193,295-305

LC-MS -- a difficult courtship



P.J. Arpino (1982). **Trends in Analytical Chemistry** 1,154.

Comparison of Gas Loads

- Packed column GC -- 3-5 mL/min
- Capillary column GC -- 0.5 - 1.5 mL/min
- Conventional HPLC -- 1-3 mL/min
  - » hexane -- 180 - 540 mL/min
  - » chloroform -- 280 - 840 mL/min
  - » methanol -- 350 - 1650 mL/min
  - » water -- 1250 - 3720 mL/min

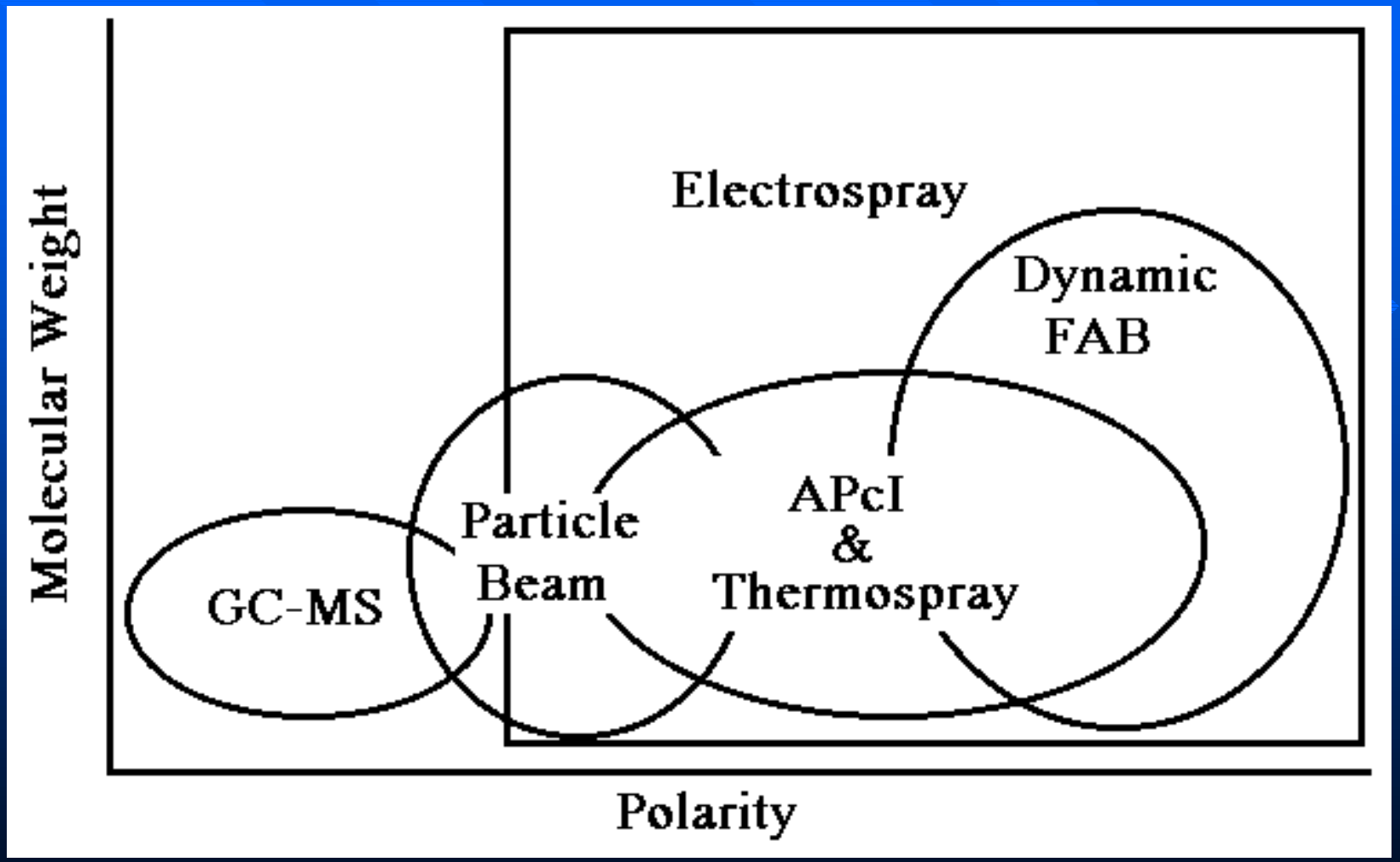
LC-MS Historical Perspective

- DLI (direct liquid introduction)
  - Tal'rose *et al.* (1968) **Russian J Phys Chem** 42,1658-64.
  - Baldwin & McLafferty (1973) **Org Mass Spectrom** 7,1111-12.
- Moving belt interface
  - Scott *et al.* (1974) **J Chromatog** 99,395-405.
  - McFadden *et al.* (1976) **J Chromatog** 122,389-96.
- API and APcI
  - Horning *et al.* (1974) **J Chromatog Sci** 12,725-9.

LC-MS Historical Perspective

- Particle Beam (MAGIC)
  - Willoughby & Browner (1984) **Anal Chem** 56,2625-31.
- Continuous flow FAB
  - Caprioli, Fan & Cottrell (1986) **Anal Chem** 58,2949-54.
- Thermospray
  - Blakley, Carmody & Vestal (1980) **Anal Chem** 52,1636-41.
- Electrospray
  - Yamashita & Fenn (1984) **J Phys Chem** 88,4452-9.

The LC-MS problem domain



"No instrument is functioning so well
that it cannot be disassembled,
altered, reassembled
and *perhaps* improved."

M.L. Vestal
Salt Lake City
circa 1981

APCI & Electrospray

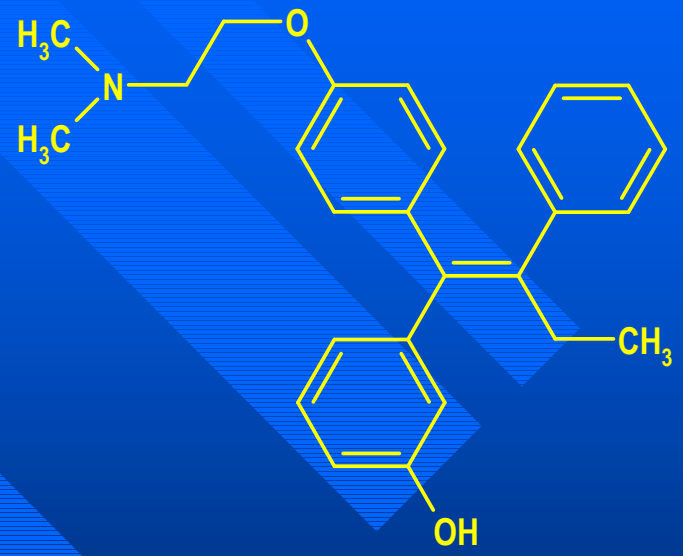
- API = Atmospheric Pressure Ionization
  - APCI = Atmospheric Pressure Chemical Ionization
  - ESP = Electrospray
- Soft ionization (minimal fragmentation)
- Applicable to polar, water-soluble molecules (i.e. pharmaceuticals)
- ESP - most notable direct application to peptides (pharmaceuticals of the future).

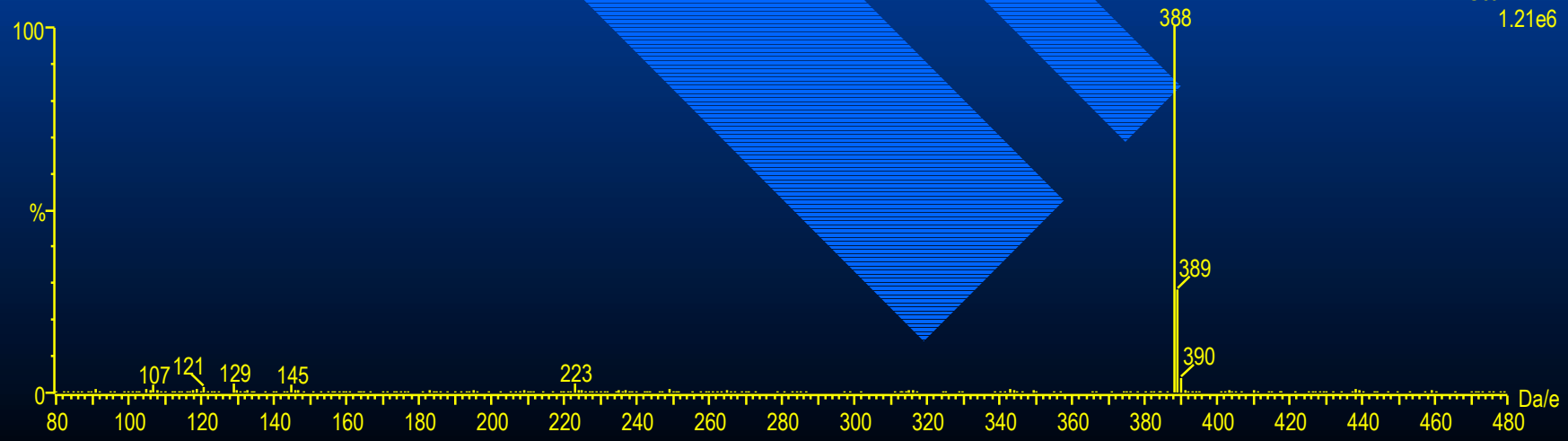
Tandem Mass Spectrometry (MS-MS)

- Relative molecular mass is important, but insufficient to identify a structure
- Structure elucidation and confirmation
- Necessary to fragment the molecular ions produced by APCI & ESP
- Products, parents, constant neutral loss by collision-induced decompositon (CID)
- Ion-genetic relationships -- true MS-MS
- “Up Front CID” -- poor man’s MS-MS

Droloxifene

- $C_{26}H_{29}NO_2$ r.m.m. 387
- Breast cancer
- Osteoporosis

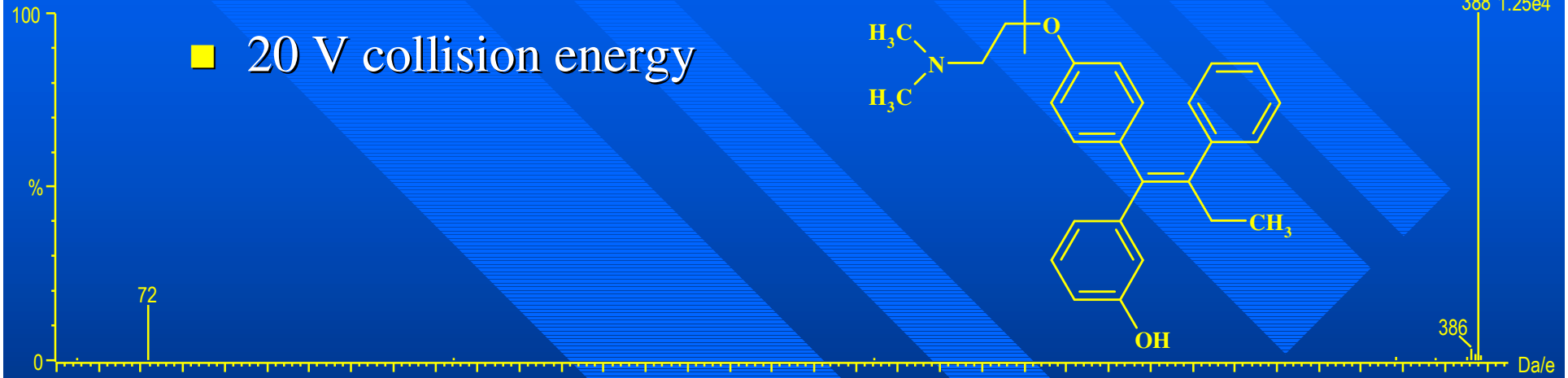




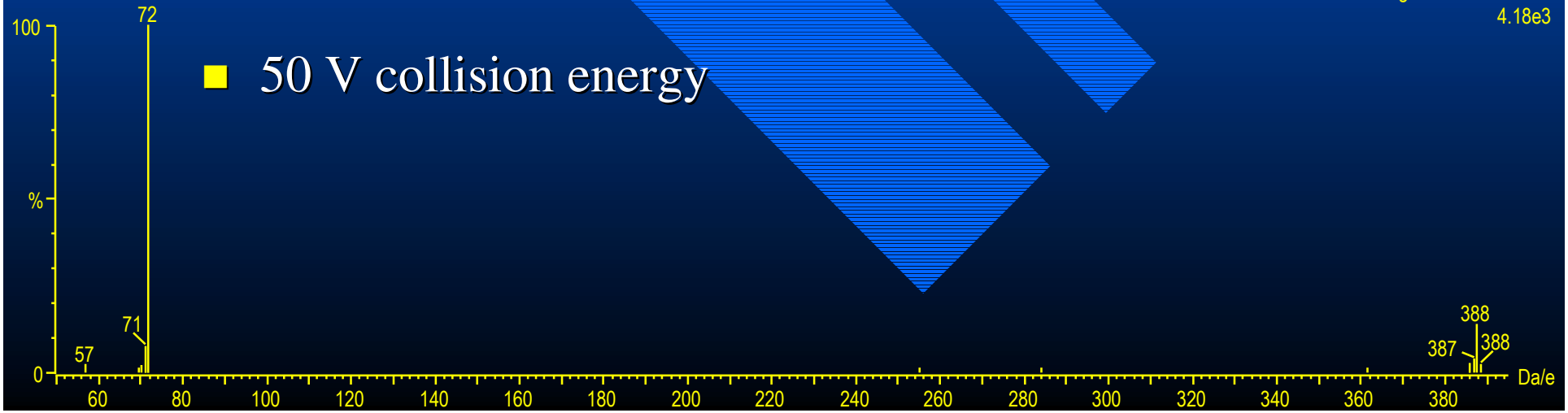
MS-MS of Droloxifene:

Products of m/z 388 $[M+H]^+$

- 20 V collision energy

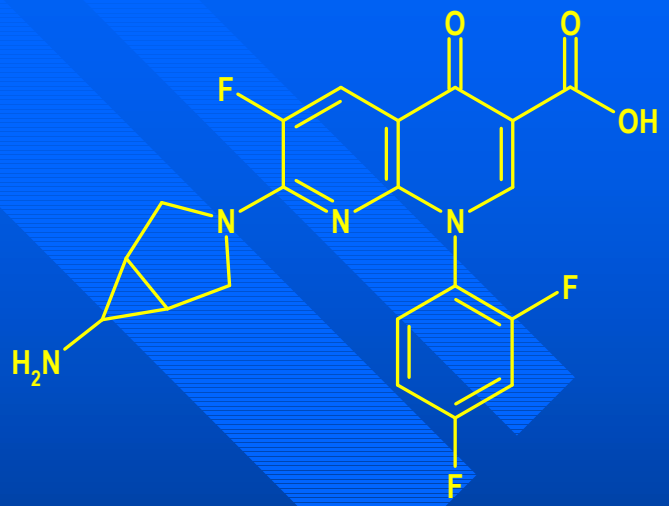


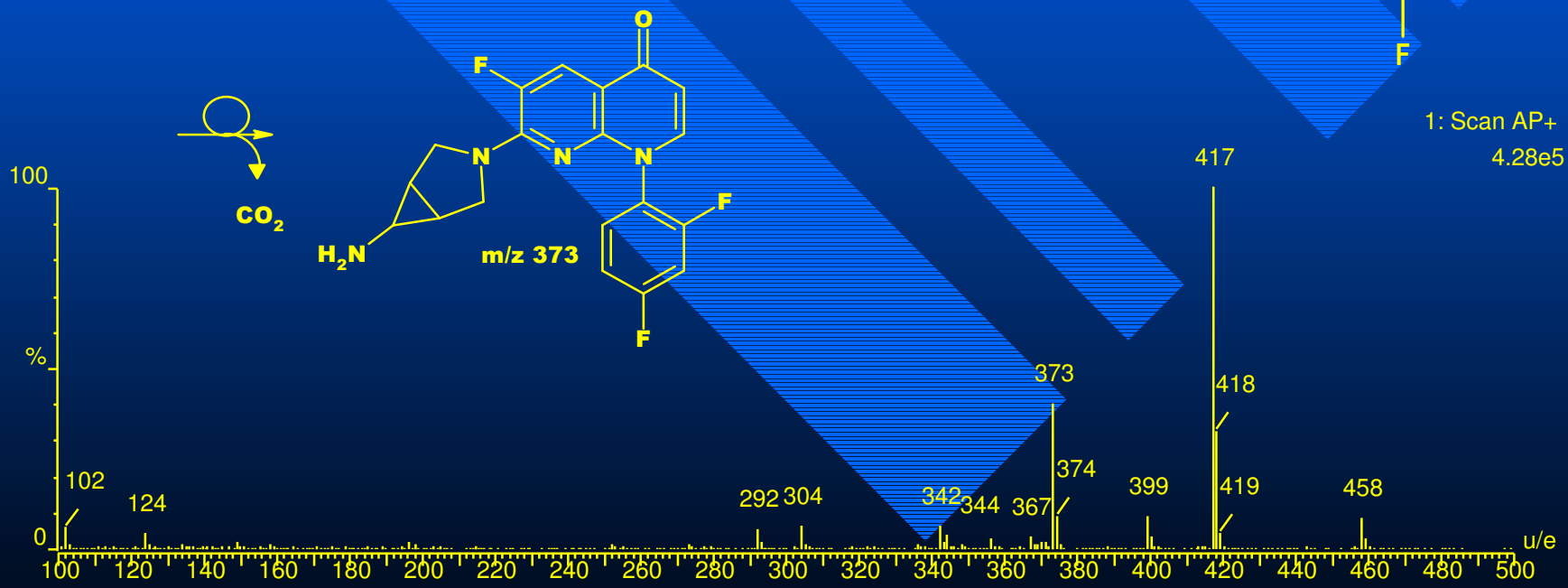
- 50 V collision energy



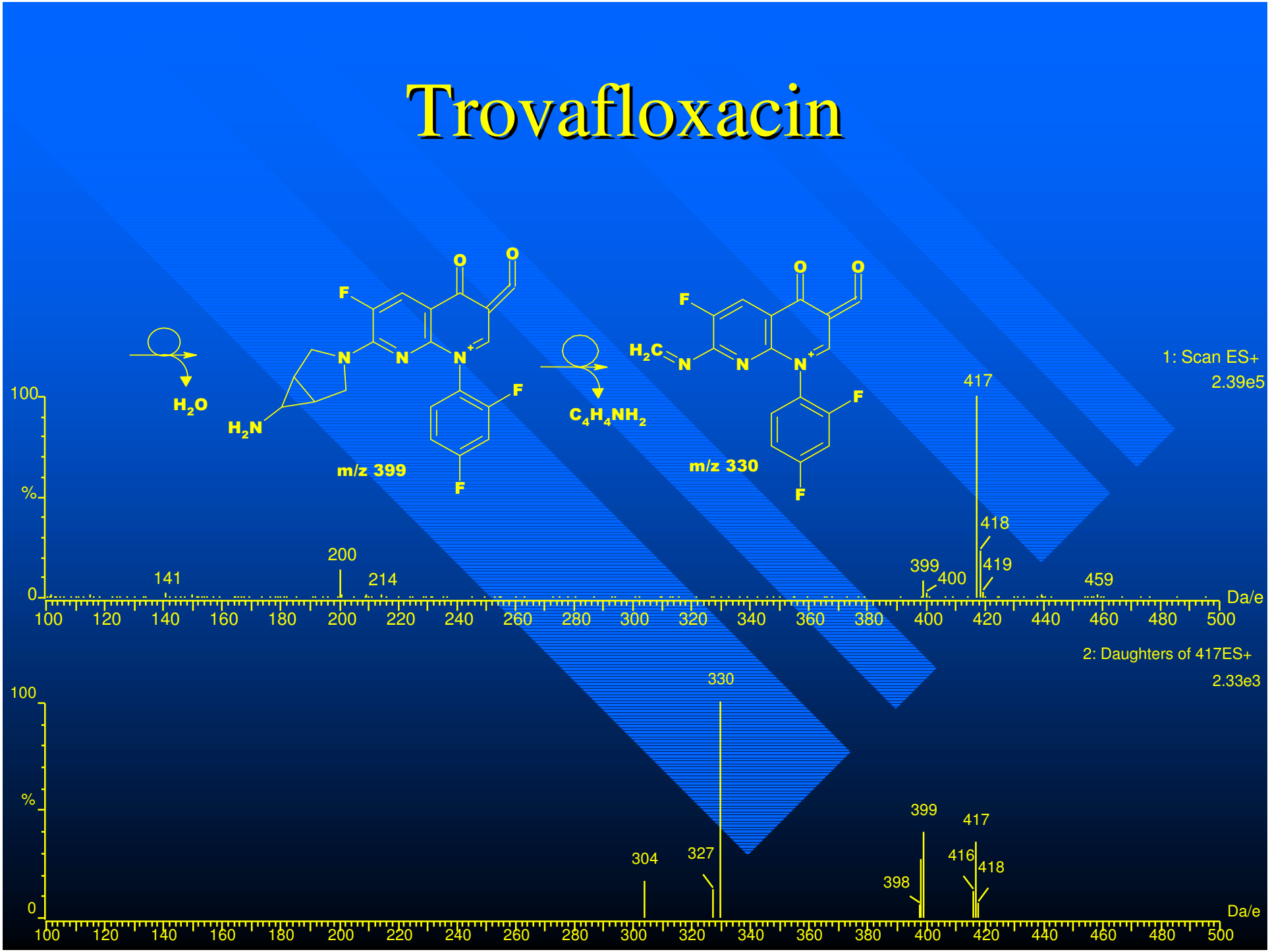
Trovafloxacin

- "Quinolone" antibiotic
- $C_{20}H_{15}N_4O_3F_3$ r.m.m. 416





Trovafloxacin

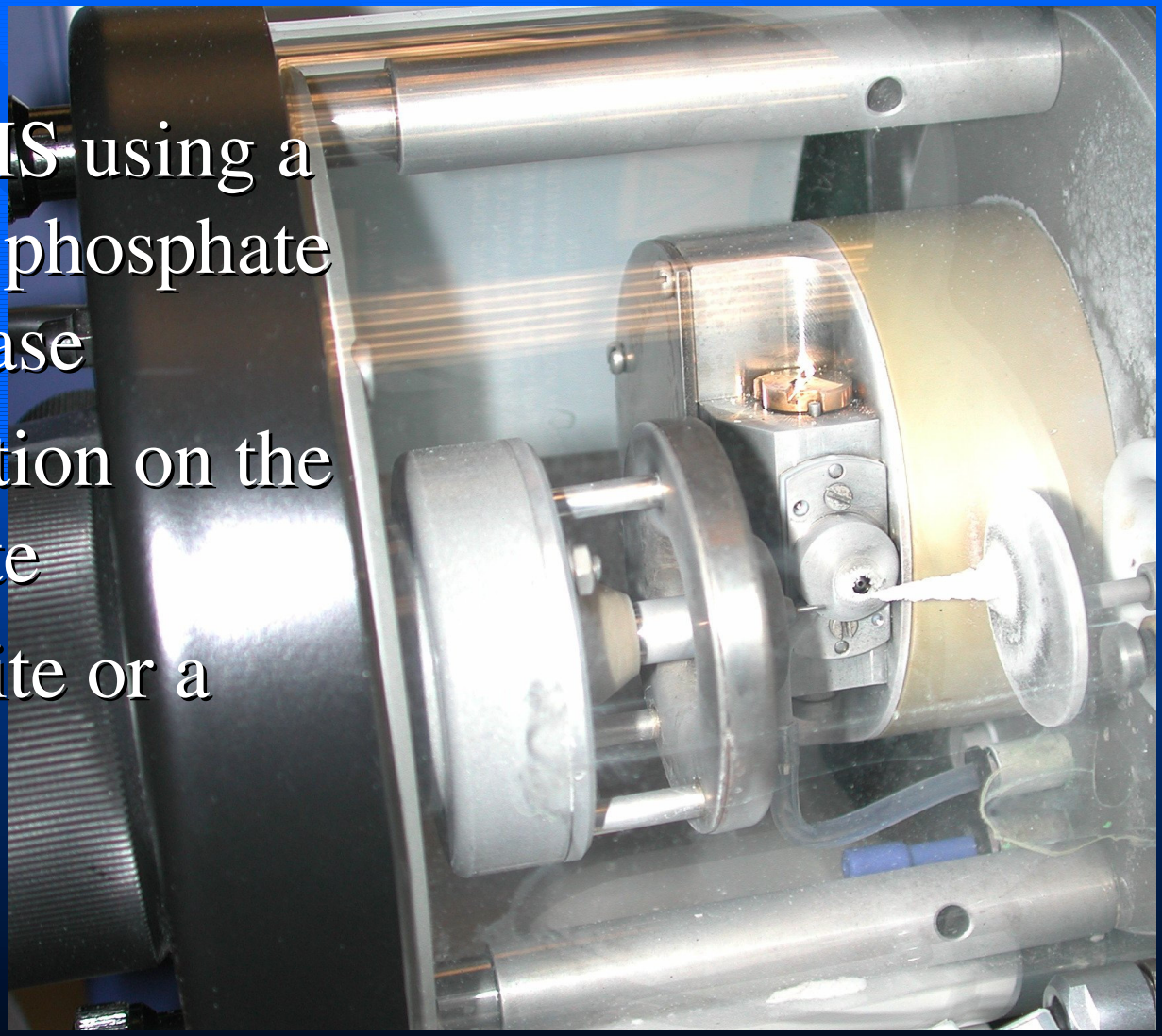


HPLC Considerations for LC-MS: Compatible Mobile Phases

- Volatile buffer salts!
  - NH_4 formate, NH_4 acetate (pH ~4 to 9)
  - 0.1% (v/v) trifluoroacetic acid (down to pH 2) or ammonium trifluoroacetate
- Some volatile buffers don't work well
  - TFA suppression and the TFA fix
  - Triethylamine suppression
- Substitution of nonvolatile buffer salts

No Phosphates!!!!!!

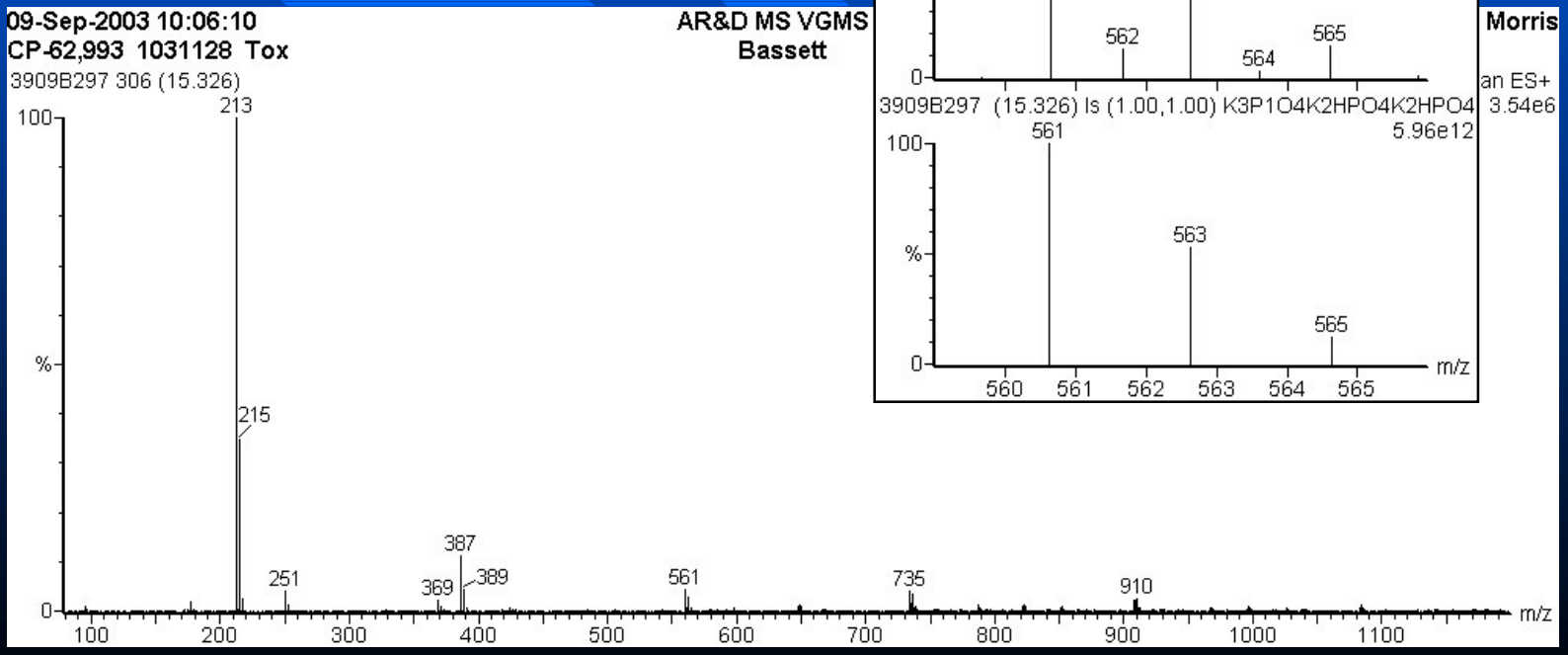
- ESP LC-MS using a potassium phosphate mobile phase
- Accumulation on the striker plate
- A stalagmite or a stalactite?



Phosphates in the mass spectrometer

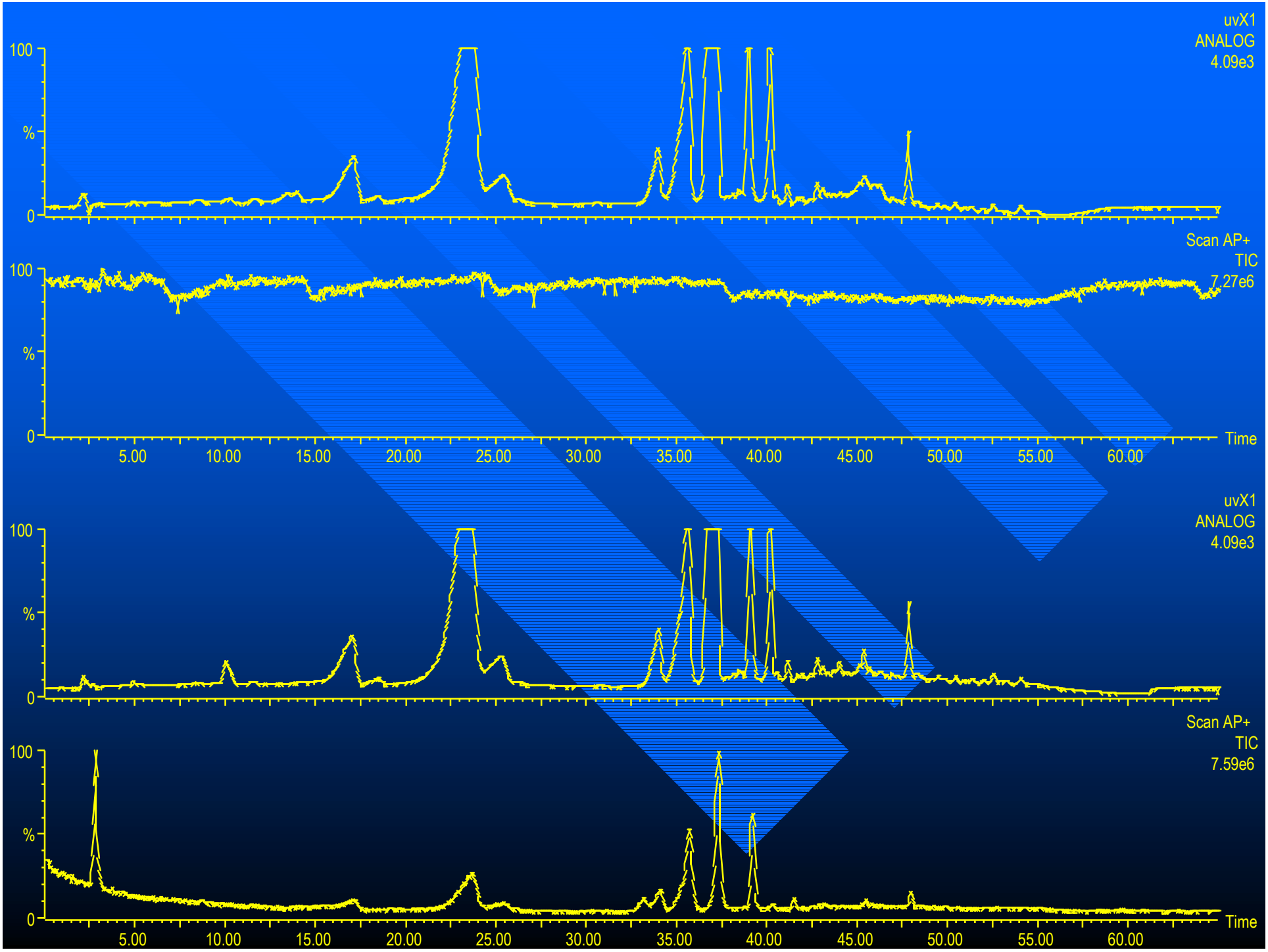
- H_3PO_4 – 98
- KH_2PO_4 – 136
- K_2HPO_4 – 174
- K_3PO_4 – 212

- ^{39}K – 93.3%
- ^{41}K – 6.7%



Problems with TEA

- triethylamine (TEA) a common mobile phase additive for peak shape conditioning
- a “volatile” buffer salt
- However, it quenches the ion signal!!!


uvX1
ANALOG
4.09e3
100
%
0
Scan AP+
TIC
7.27e6
100
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Time
uvX1
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35.00
40.00
45.00
50.00
55.00
60.00
Time

HPLC Considerations for LC-MS: Miscellaneous Points

- Put a UV-Vis detector in line!
- Common organic components are OK
- Gradients are OK
- Small bore columns are OK

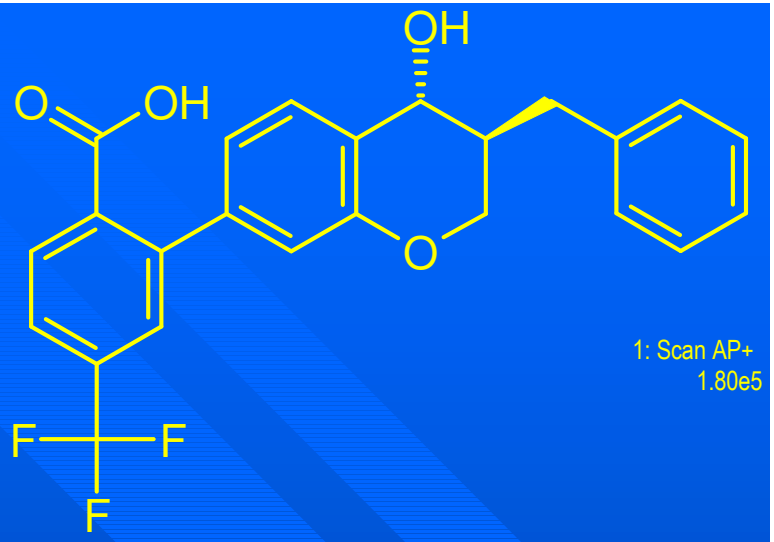
UV-Active Components that Don't Respond

- "Volatile" small molecules
  - methyl and propyl parabens
  - benzoic acid
  - simple aromatic amines
  - simple aromatic aldehydes

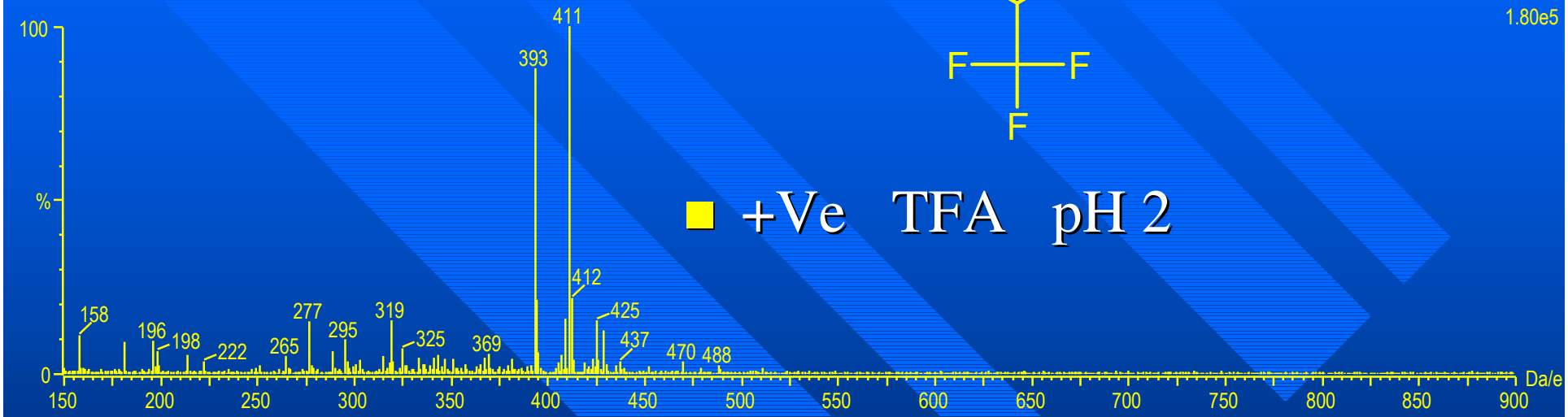
UV-Silent Components

- The mass spectrometer is a more universal detector?
- Compounds with no UV chromophore
  - azithromycin
  - other azalide antibiotics
- UV-silent excipients in drug product formulations

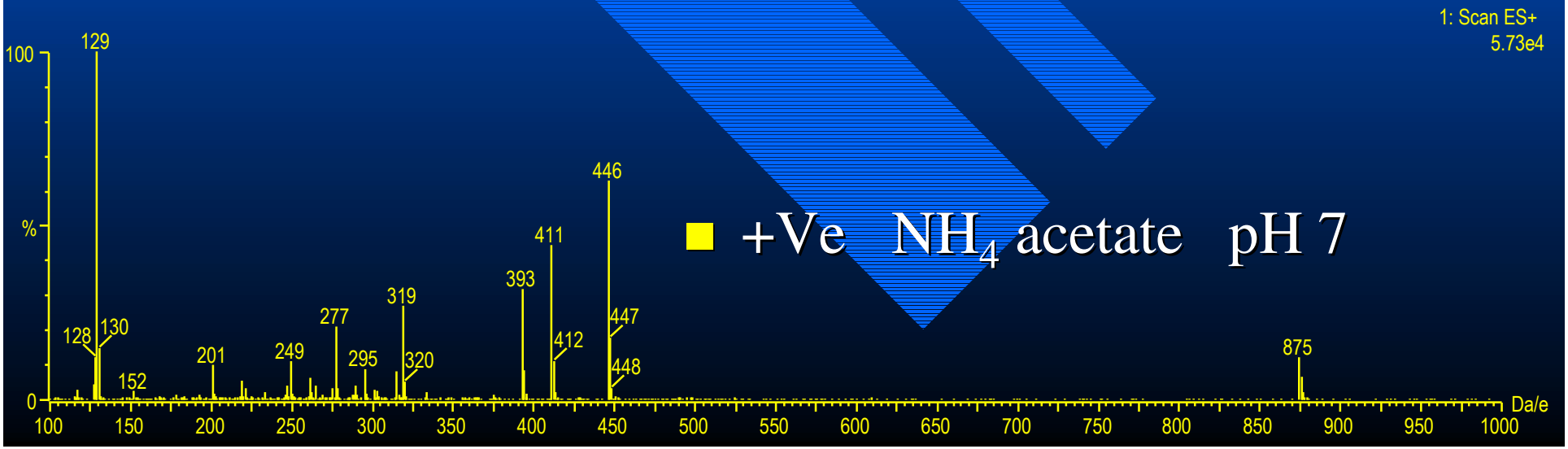
LTB4 antagonist: API spectra



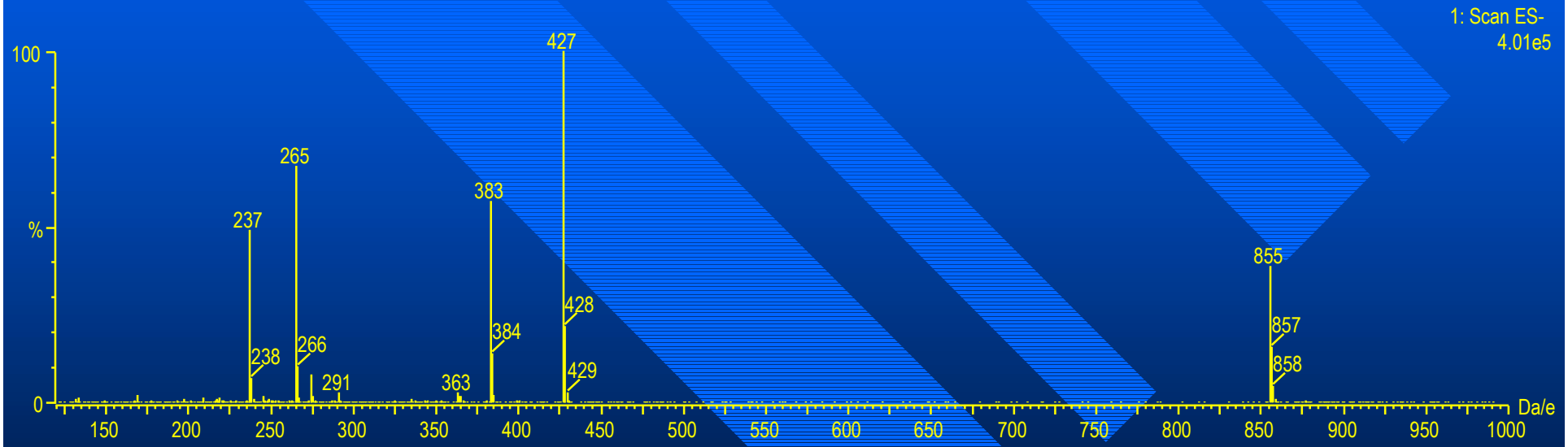
- +Ve TFA pH 2



- +Ve NH_4 acetate pH 7

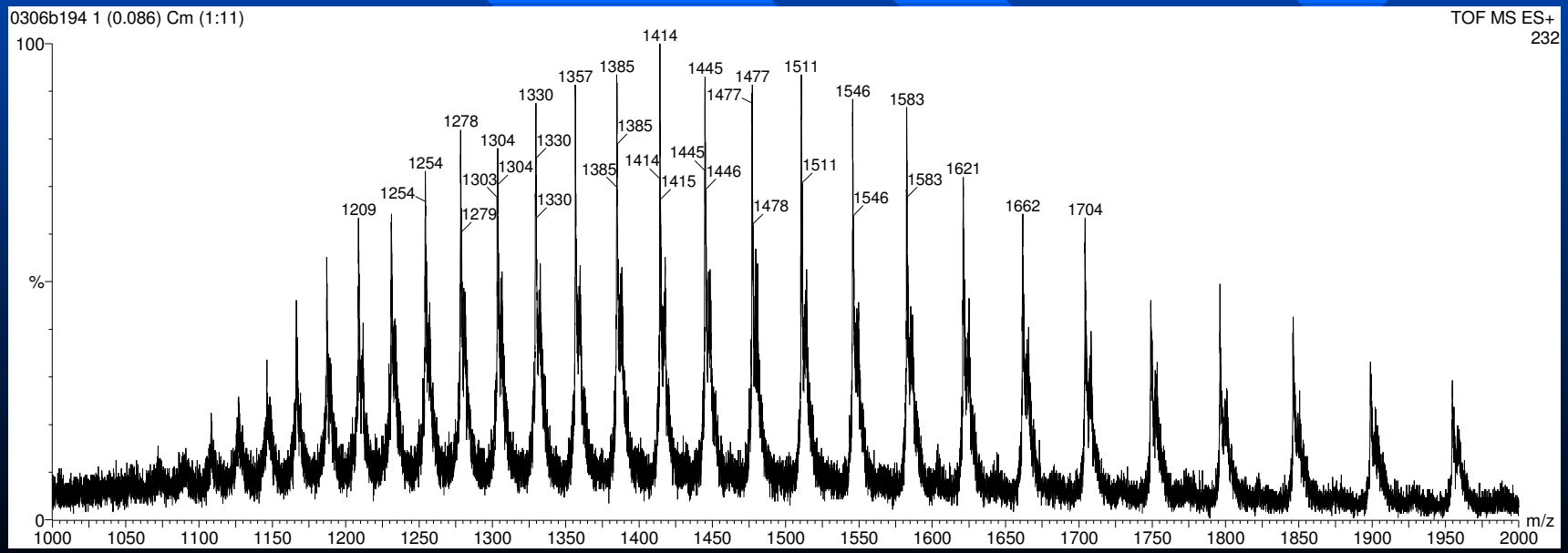


- -Ve NH_4 acetate pH 7



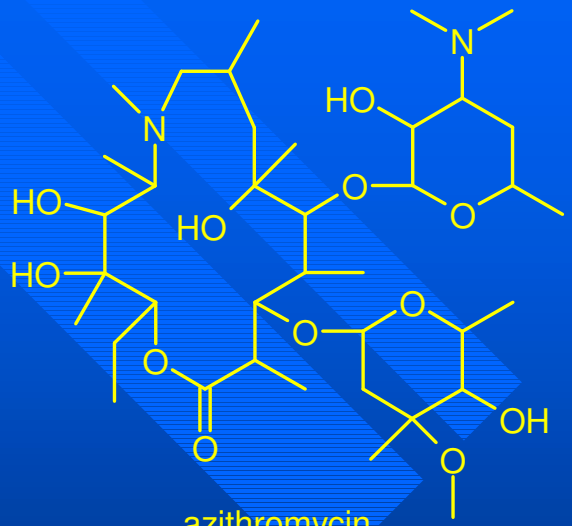
Multiply Charged Spectrum of a Protein

- bovine serum albumin
- measured ave mol mass of 66,424 daltons
- two additional proteins at higher masses

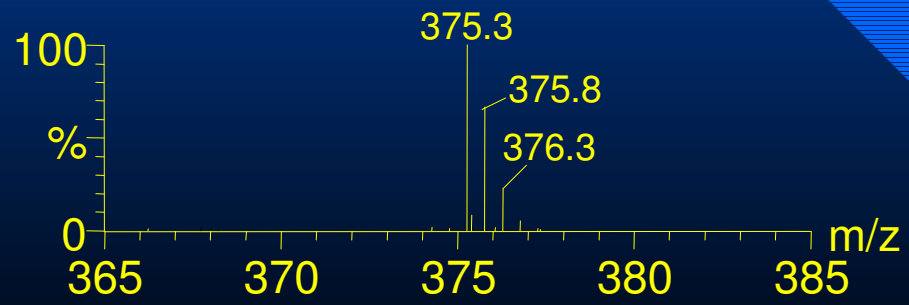


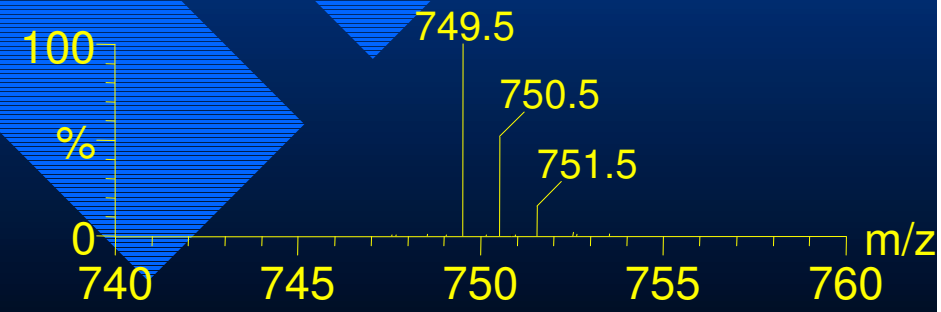
Doubly Charged Molecular Ions

- macrolide antibiotics
  - erythromycin
  - azithromycin (FAB, too)
  - other macrolides



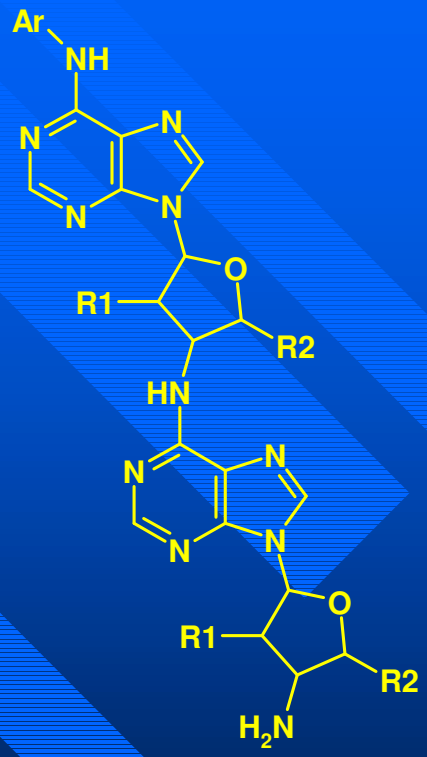
azithromycin
$C_{38}H_{72}N_2O_{12}$
r.m.m. 748.5085

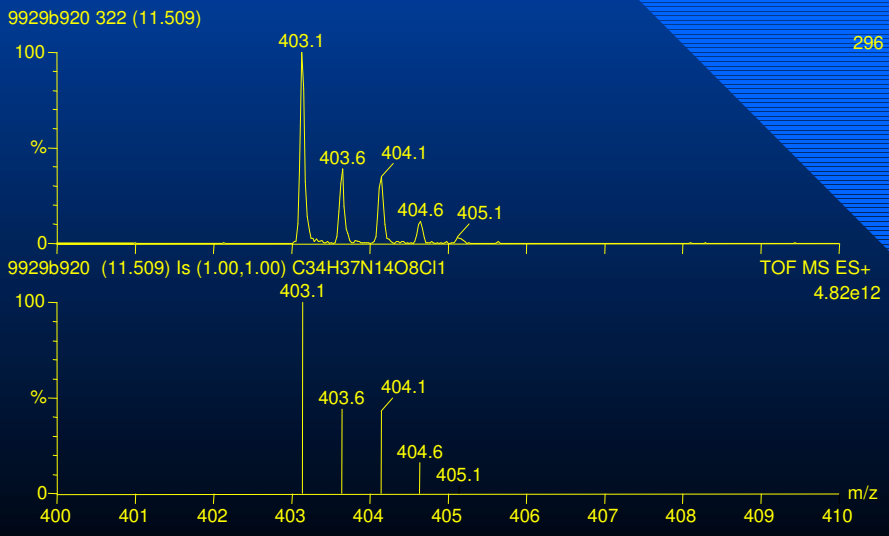


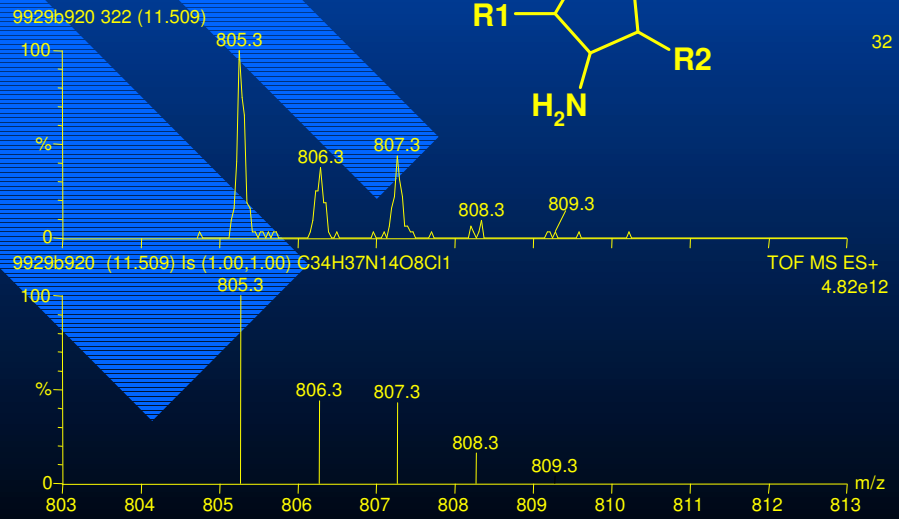


Doubly Charged Molecular Ion

- substituted adenine analog
- r.m.m. 804 daltons



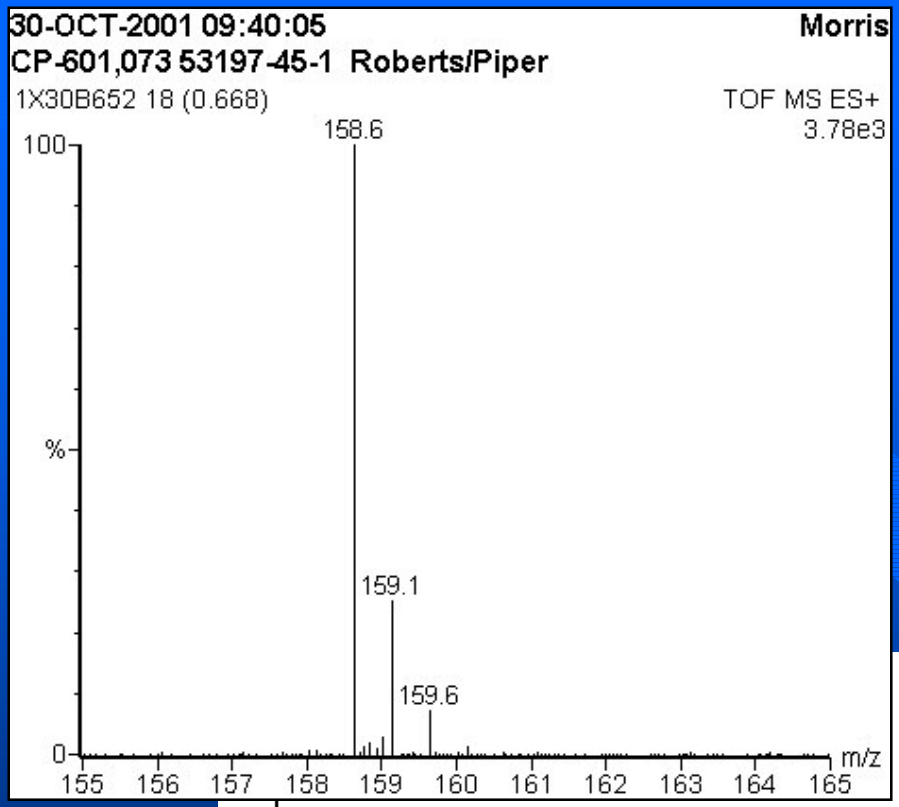


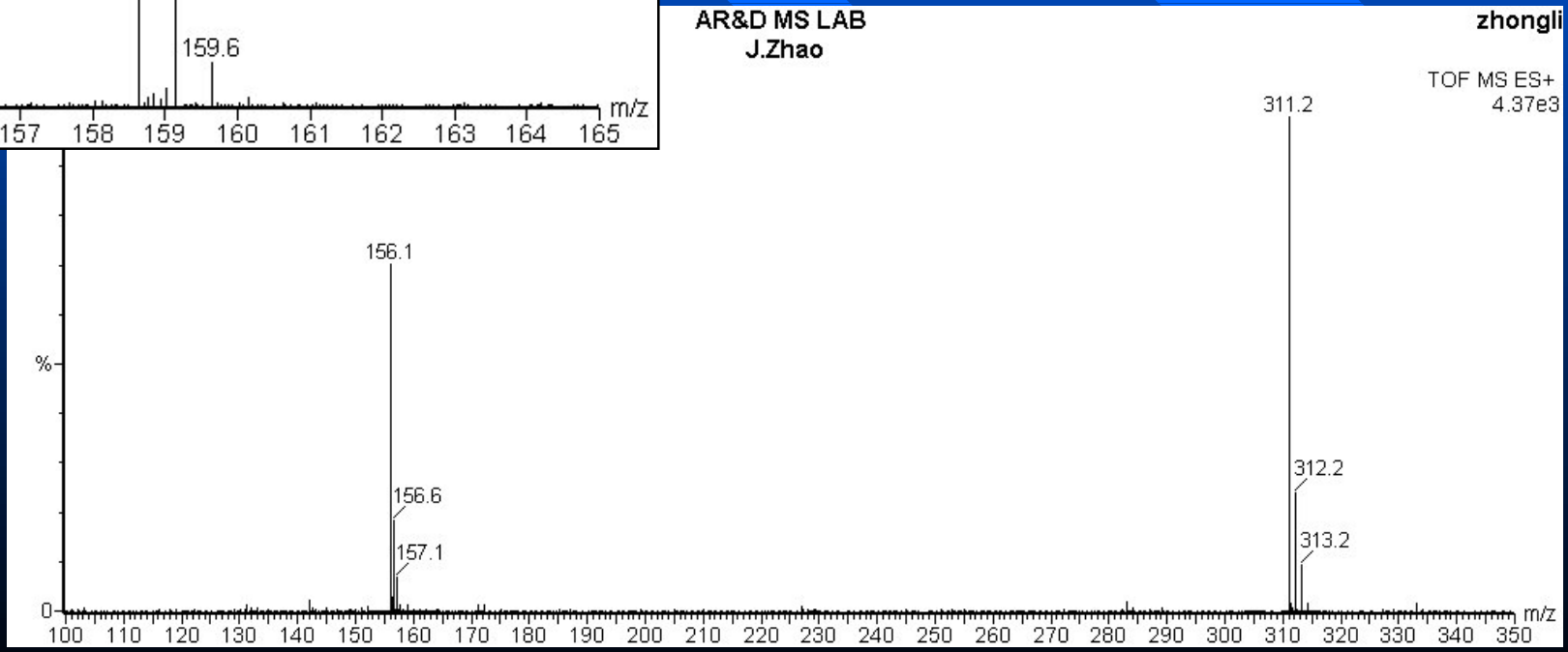


But how small can you go?

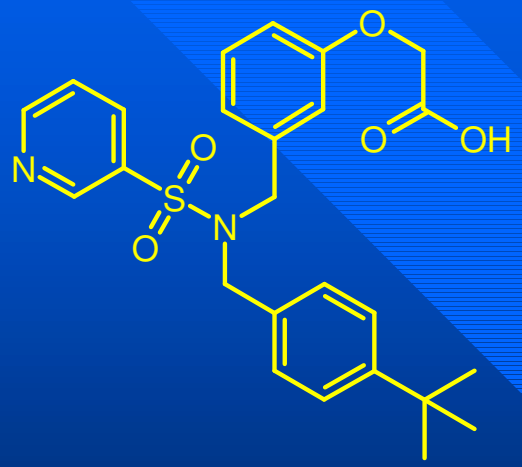
- Gaskell (1997) predicts most abundant charge state based on number of basic sites
  - 1885 Da peptide with 4 “basic” sites predicts +4 charge state most abundant
- peptides from digests -- as small as 950 Da
  - abundant $[M+2H]^{2+}$
  - dependent upon instrumentation
  - dependent upon operating conditions

How low can you go?





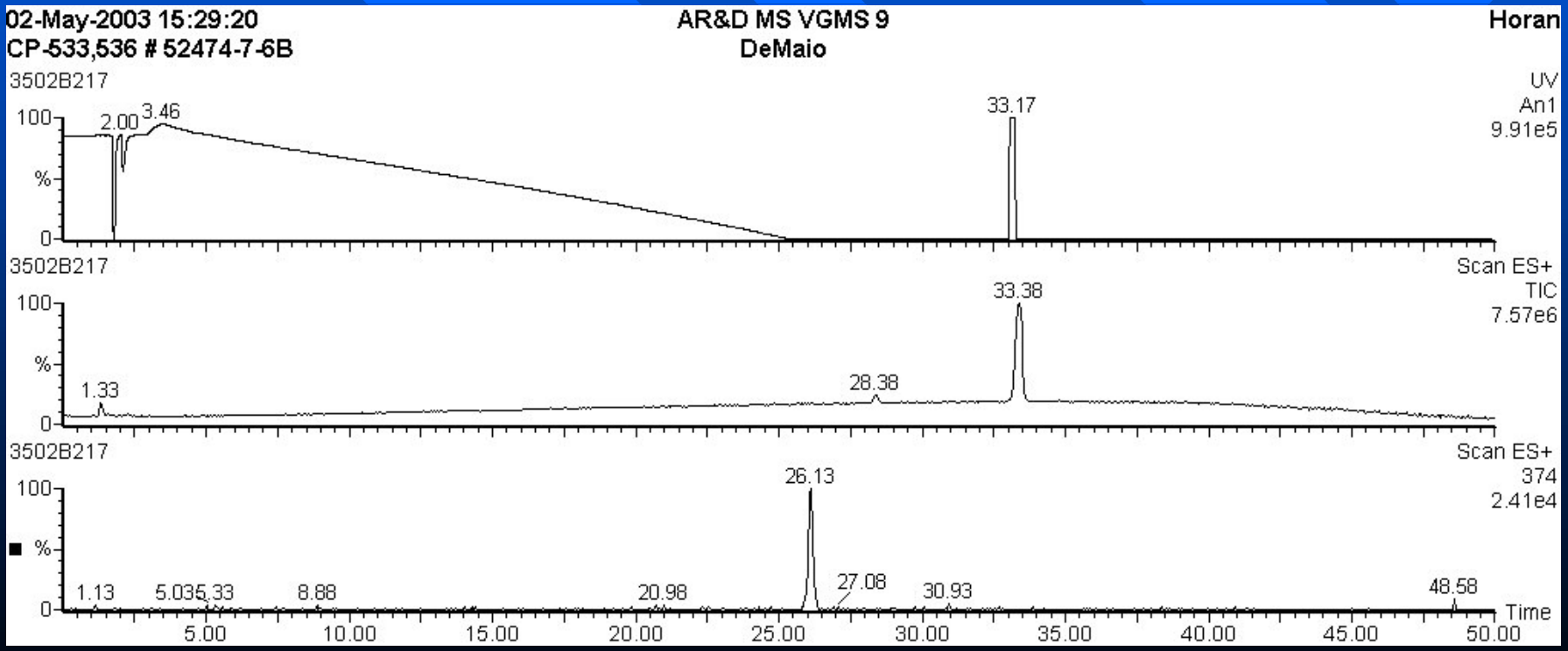
PGE2 agonist process-related impurity



- PGE2 agonist
  - $C_{25}H_{28}N_2O_5S$
  - r.m.m. 468

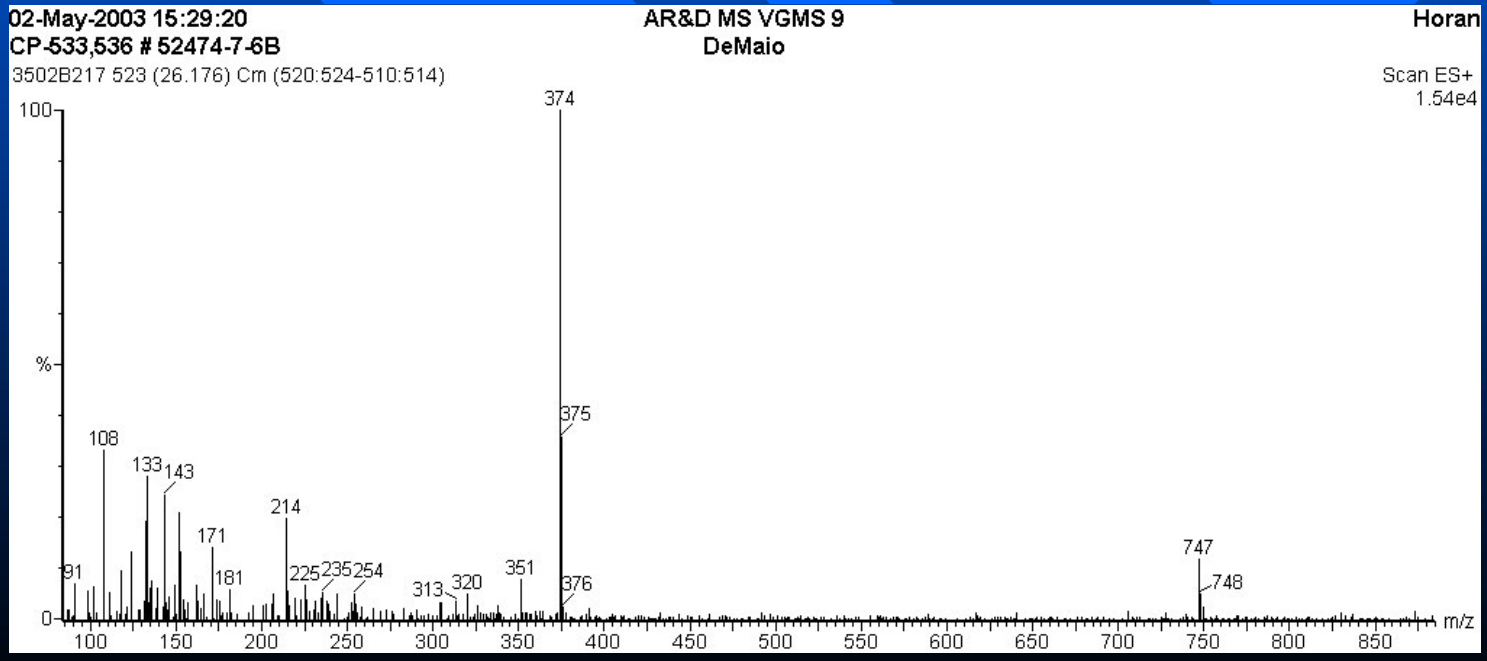
PGE2 agonist process-related impurity

- Found in an impurity screen for this drug substance
- Screen proposed m/z 374 as the $[M+H]^+$



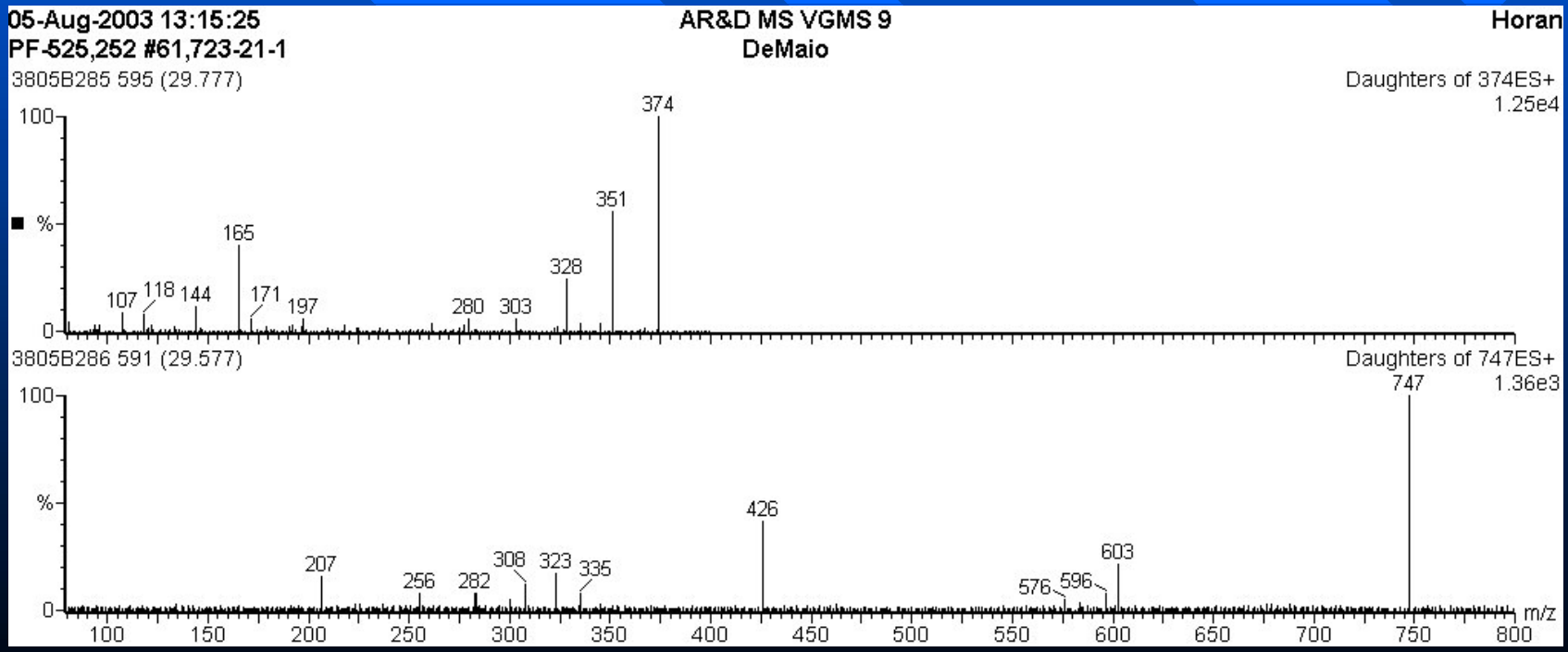
PGE2 agonist process-related impurity

- m/z 374 is indeed the most prominent feature, but is it the real molecular ion?
- molecular ion and proton-bound dimer?
- molecular ion and doubly charged mol ion?



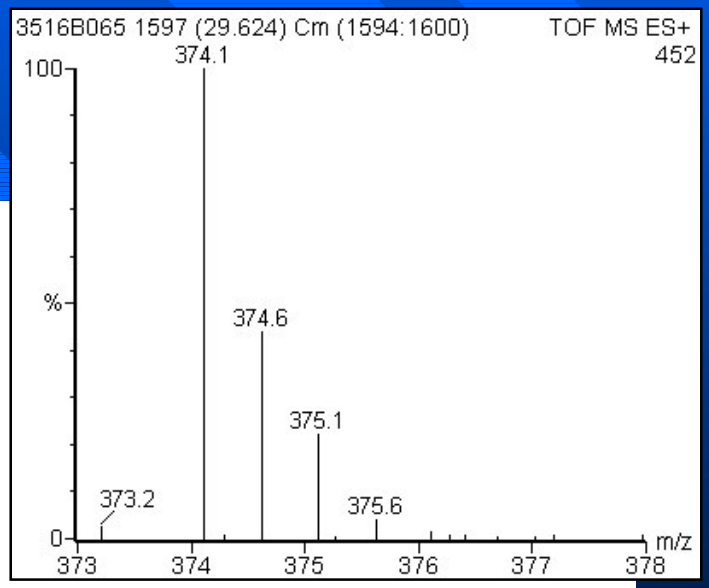
PGE2 agonist process-related impurity

- if m/z 747 is $[2M+H]^+$, ms-ms should generate m/z 374
- unusual 23 dalton differences in ms-ms of m/z 374

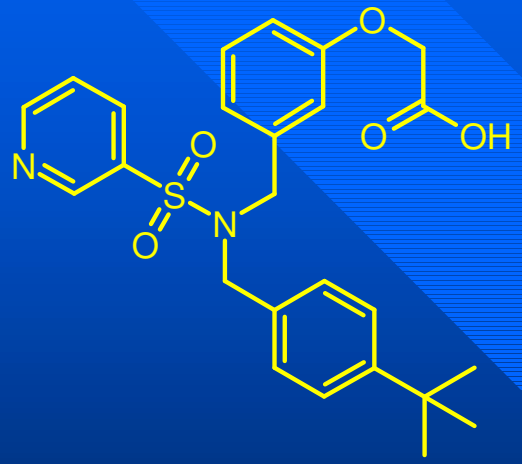


PGE2 agonist process-related impurity

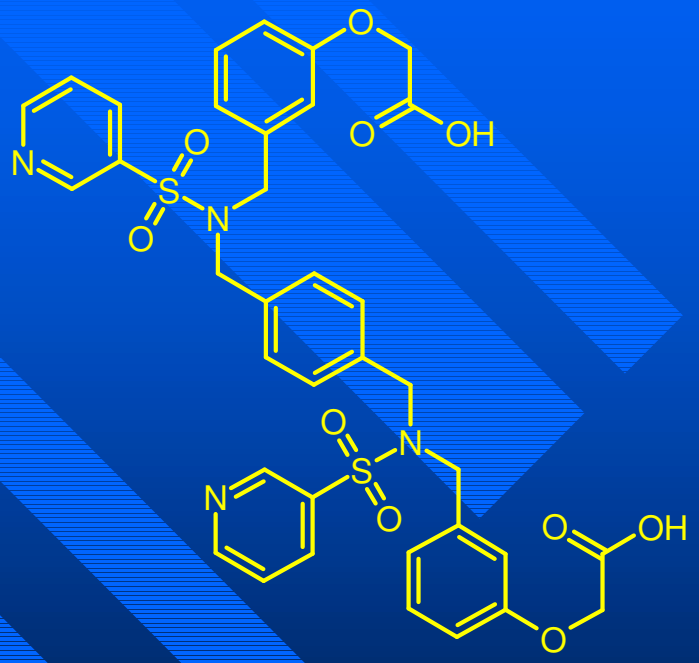
- m/z 374 is indeed the most prominent feature, but it's a doubly charged ion
- m/z 747 is the real $[M+H]^+$!



PGE2 agonist process-related impurity

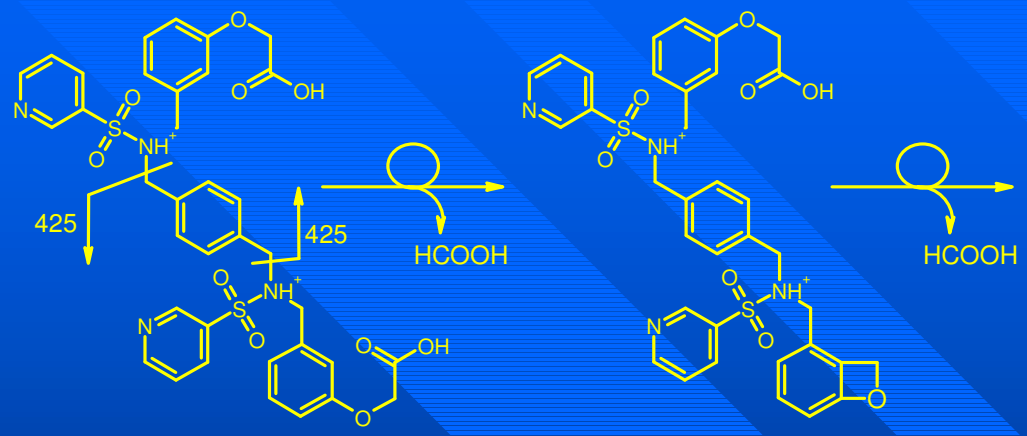


- PGE2 agonist
  - $C_{25}H_{28}N_2O_5S$
  - r.m.m. 468

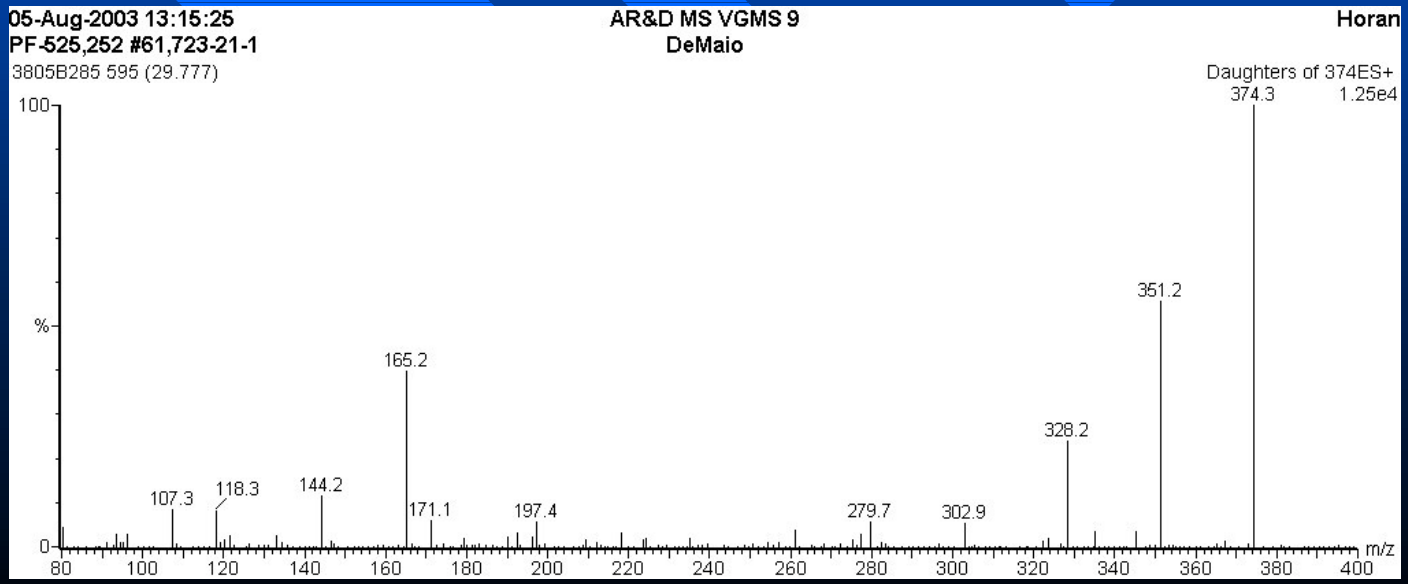


- impurity
  - $C_{36}H_{34}N_4O_{10}S_2$
  - r.m.m. 746

PGE2 agonist process-related impurity



$[M+2H]^{2+}$, m/z 374 m/z 351 m/z 328

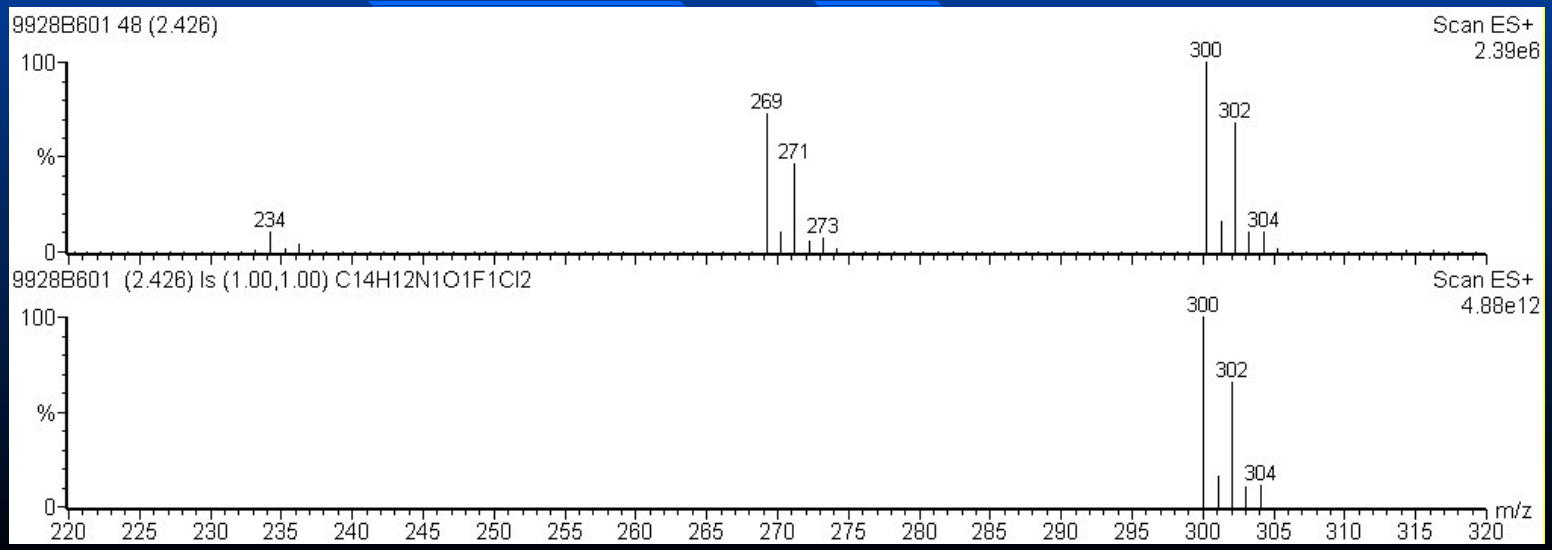


Polyisotopic Elements

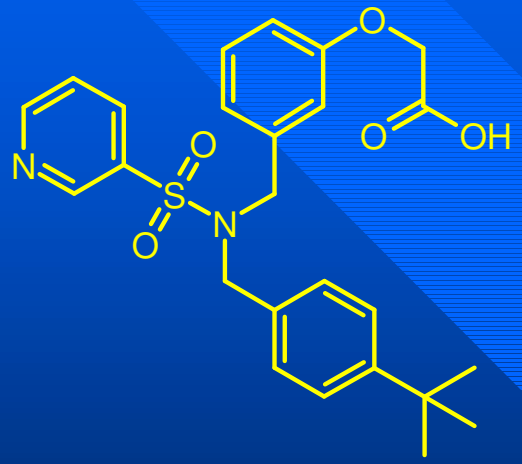
- Chemical atomic mass vs monoisotopic atomic mass
- Carbon -- 12.011 (periodic table)
  - ^{12}C - 12.0000 - 98.9%
  - ^{13}C - 13.0033 - 1.1%
- Chlorine -- 35.453 (periodic table)
  - ^{35}Cl - 34.9689 - 75.8%
  - ^{37}Cl - 36.9659 - 24.2%

Isotope patterns

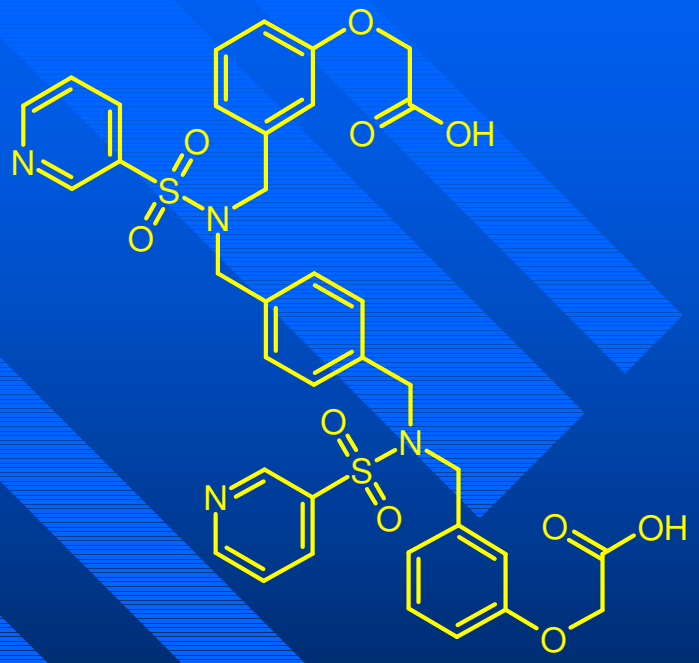
- for $C_{14}H_{12}NOFCl_2$, r.m.m. 299 Da.
- m/z 269 is neutral loss of methylamine
- m/z 234 is further loss of Cl_1
- isotope patterns match predictions



PGE2 agonist process-related impurity



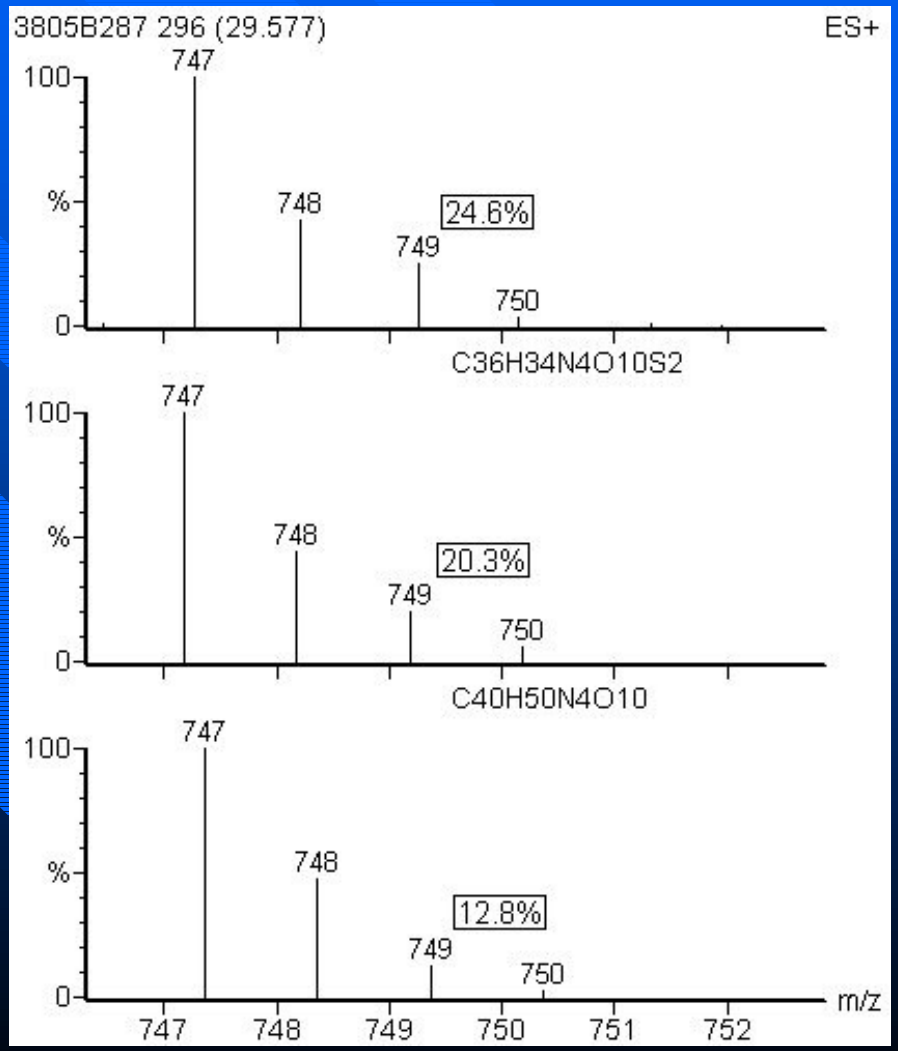
- PGE2 agonist
  - $C_{25}H_{28}N_2O_5S$
  - r.m.m. 468



- impurity
  - $C_{36}H_{34}N_4O_{10}S_2$
  - r.m.m. 746

PGE2 agonist process-related impurity

- Isotope pattern for the m/z 747 $[M+H]^+$. P+2 is 24.6% of P. ($^{34}S = 4.2\%$)
- prediction for elemental composition with two sulfur atoms
- prediction replacing sulfurs with carbon and hydrogen



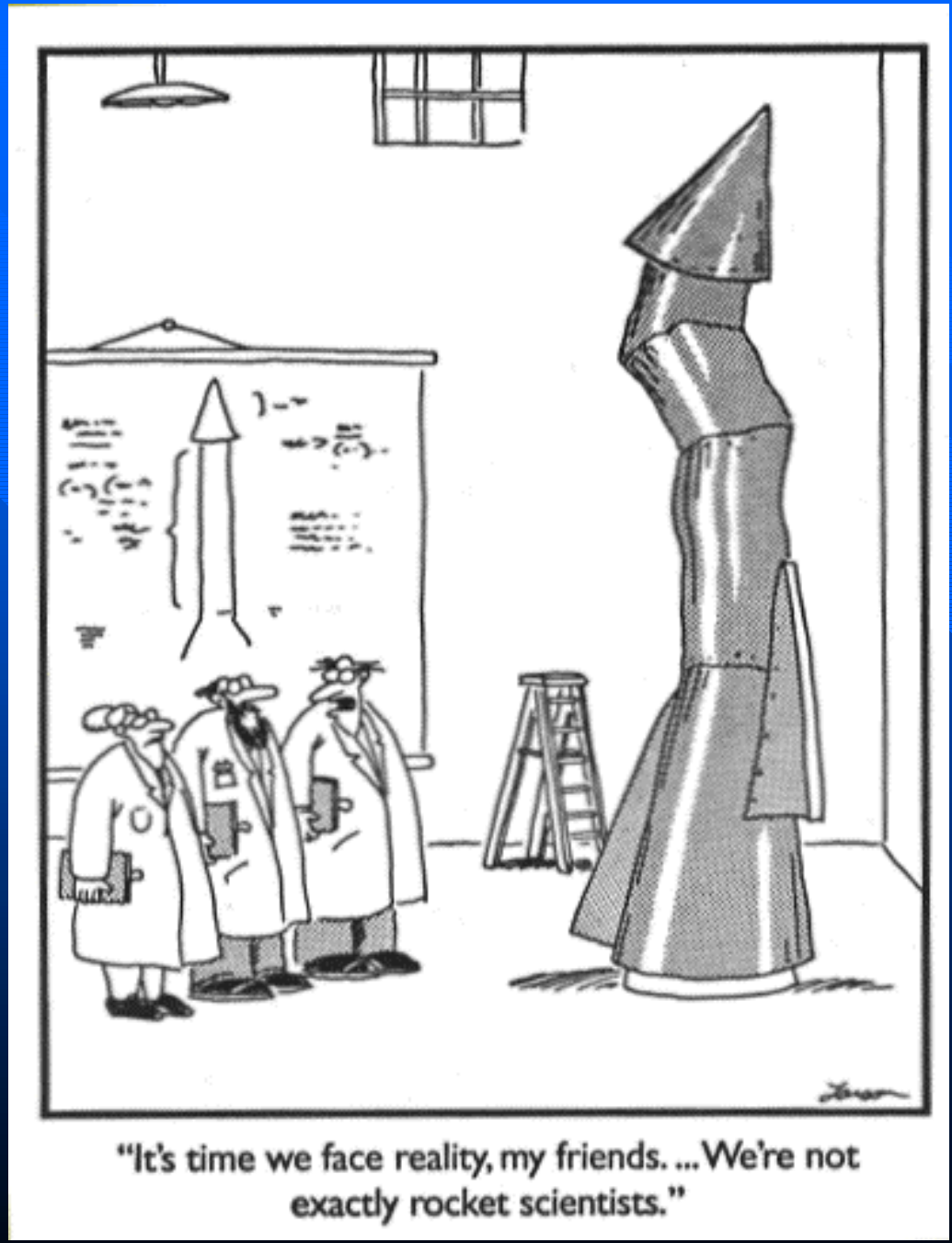
PGE2 agonist process-related impurity

Accurate mass measurements

m/z	assignment	Observed Acc Mass	Elemental composition	Expected Acc Mass	Agreement
747	$[M+H]^+$	747.1790	$C_{36}H_{35}N_4O_{10}S_2$	747.1795	-0.7 ppm
			$C_{42}H_{29}N_5O_7S$	747.1788	0.2 ppm.
			$C_{44}H_{31}N_2O_8S$	747.1801	-1.6 ppm
			$C_{39}H_{31}N_4O_{10}S$	747.1761	3.8 ppm
			$C_{39}H_{33}N_5O_7S$	747.1821	-4.3 ppm
351	$[M+2H-HCOOH]^{2+}$	351.0869	$C_{35}H_{34}N_4O_8S_2$	351.0909	11.4 ppm
328	$[M+2H-2(HCOOH)]^{2+}$	328.0868	$C_{34}H_{32}N_4O_6S_2$	328.0882	4.3 ppm

Concepts Clarified

- relative molecular mass
- chemical vs monoisotopic molecular mass
- what the mass spectrometer really measures
- isotope patterns
- accurate mass vs. high resolution
- accurate mass measurements
- contributions to a structure identification



"It's time we face reality, my friends. ... We're not exactly rocket scientists."

Words of Wisdom:

It is as important to understand what a technique CANNOT tell you as it is to understand what it can tell you.