Current Mass Spectrometry Methods for the Analysis of 7-Ketocholesterol and Related Sterols

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Abstract

In this article we summarise the current mass spectrometry methods used for analysis of 7-ketocholesterol and related sterols, focusing on the advantages of the different methods with emphasis on pre-analytical and analytical precautions to avoid artefactual *ex vivo* formation of these oxysterols.

Key Words: Oxysterol, GC-MS, LC-MS, Derivatisation, Autoxidation, Cholesterol

1. Introduction

7-Ketocholesterol (7-KC, also known as 7-oxocholesterol, 7-OC) has interested biochemists since the early 1940's. Studies by Bergström and Wintersteiner showed that 7-KC, along with 7α-hydroxycholesterol (7α-HC) and 7β-hydroxycholesterol (7β-HC) could be formed via *ex vivo* autoxidation of cholesterol (1-3). They identified 7-KC by optical rotation, UV absorption, reaction with Girard reagent and comparison to information on the synthetic compound. They stated "There is no reason why this type of autoxidation (of cholesterol) should not also occur *in vivo*" having, along with colleague Johana Ritzmann, isolated 7-KC from mare's serum, but did add "On the other hand, it is highly probable that at least a part of the isolated products were formed *in vitro* during the initial phases of the isolation procedure" (2). In later life Bergström's interests shifted to the new field of prostaglandins (4), culminating with his award of the 1982 Nobel Prize in Physiology or Medicine with Bengt Samuelsson and John Vane. Bergström's student, Jan Sjövall who worked with him on the isolation of the first prostaglandin in crystalline form (4, 5), continued Bergström line of cholesterol research, particularly in the bile acid field, and uncovered both C_{24} and C_{27} bile acids with a 7-oxo-5-ene function in the steroid ring structure, as in 7-KC (6, 7). While it is highly unlikely that 7-oxo-5-ene bile acids could be artefacts of *ex vivo* sample preparation, this is not the case for 7-KC itself, where cholesterol is often in a thousand-fold excess (8). The diligent researcher must always be aware that even 1% *ex vivo* autoxidation of cholesterol can produce appreciable quantities of 7-KC.

While many researchers in the sterol and oxysterol field, the authors included, have been suspicious of some of the results published related to endogenous quantities of 7-KC in biological samples (9, 10), its formation via endogenous autoxidation of cholesterol is now well established, particularly in the context of lysosomal storage disorders (11-16) and also enzymatically from 7-dehydrocholesterol (7-DHC) as in Smith-Lemli-Opitz syndrome (SLOS) (7, 17). However, it is the down-stream enzymatically formed metabolites of 7-KC that are perhaps more reliable markers of the formation of 7-KC as these are unlikely to be artefacts of sample work-up and handling, and may also be of more biological interest (6, 7, 18).

Mass spectrometry (MS) or tandem mass spectrometry (MS/MS or MS^2) combined with gas-chromatography (GC) or liquid chromatography (LC) have proved to be the techniques of choice for the analysis and quantification of 7-KC and related sterols. In the sections below these methods are discussed along with sample preparation procedures. We focus on human plasma and serum as these are the most studies biological samples.

2. Methods for Analysis of 7-KC

2.1. GC-MS

Many of the GC-MS methods in current use are based on the classic study of Dzeletovic et al (19). This method was developed for the analysis multiple oxysterols including 7-KC in human plasma. Key features were: (i) the presence of ethylenediaminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) in the plasma preparation process, EDTA as a chelator of metal ions and BHT as an antioxidant; (ii) the addition of isotope-labelled standards (including [$^2H_6$]7-KC) prior to saponification, and (iii) saponification under mild conditions i.e., a 1 to 10 ratio of plasma to ethanolic 0.35 M KOH, incubation at room temperature (22 ^oC) for 2 hr (Table 1). Dzeletovic et al found that 7-KC decomposed at temperatures above 22 ^oC, was lost at extended incubation times (50% after 14 hr) and also when the KOH concentration was doubled to 0.7 M (19). To minimise artefactual formation of 7-KC along with 7-hydroxycholesterols (7-HCs), hydrolysis was performed under an argon atmosphere, although this is seldom used today in modified versions of the protocol. After neutralisation and extraction into chloroform the sterol extract was dried down and re-dissolved in toluene prior to separation of oxysterols, including 7-KC, from cholesterol and similar hydrophobic sterols on a silica cartridge. 7-KC eluted in the oxysterol fraction in 0.5% propan-2-ol in hexane. This step prevents an artefactual formation of 7-KC and 7-HCs from cholesterol in subsequent steps. Prior to GC-MS analysis the dried oxysterol fraction was derivatised with pyridine:hexamethyldisilazane:trichloromethylsilane (3:2:1, v:v:v) at 60 ^oC for 30 min to give trimethylsilane (TMS) ethers (Figure 1A). GC-MS analysis was performed via selected ion monitoring (SIM), *m/z* 472, $[M]^{+\cdot}$, selected for 7-KC, and *m/z* 478, $[M]^{+\cdot}$, selected for [$^2H_6$]7-KC with quantification by the isotope-dilution. The method was also performed in the absence of base hydrolysis to allow the quantification of non-esterified 7-KC. The non-esterified or free component of 7-KC comprised 20 – 60% of the total 7-KC measured after base hydrolysis. In summary, Dzeletovic's method shows minimal artefactual formation of oxysterols, avoids the decomposition of 7-KC during base hydrolysis and provided good sensitivity for its day, i.e. limit of detection (LOD) for 7-KC of 2 ng/mL from 1 mL of plasma (see Table 1) (19).

In more recent years Schött and Lütjohann have updated the method, by increasing the quantity of BHT and EDTA, this allowed a higher base concentration (a 1 to 4 ratio of plasma to 1.5 N NaOH in 90% ethanol) for the 2 hr room temperature saponification (20). After neutralisation, extraction was into dichloromethane rather than chloroform but otherwise the method employed was essentially that of Dzeletovic et al (19), except derivatisation was with pyridine:hexamethyldisilazane:trichloromethylsilane (9:3:1, v:v:v) at 90 ^oC for 1.5 hr (20). Unsurprisingly, Schött and Lütjohann's method (20), performed using a more modern GC-MS instrument than that used by Dzeletovic et al (19), had better sensitivity (for 7-KC, LOD 0.1 ng/mL from 500 µL plasma, see Table 1). However, both Dzeletovic et al and Schött and Lütjohann's methods are to quote Dzeletovic et al "relatively laborious, limiting the number of samples that can be prepared simultaneously". With regard to 7-KC both methods are advantageous to the earlier GC-

MS method of Kudo et al where 7-KC partially decomposed under harsher solvolysis conditions (9).

2.2. LC-MS/MS

Many of the LC-MS/MS methods for oxysterol analysis are based on the method developed by McDonald et al (21). This method allowed the measurement of ten oxysterols, including 7-KC, in plasma and was increased to fourteen in later studies (22). The sample preparation protocol for plasma, typically 200 µL, was rather different from that of Dzeletovic et al (19), in that there was an initial lipid extraction into dichlorometane:methanol (1:1, v:v) containing internal standards, including [$^2H_7$]7-KC, and BHT, and after separation from pelleted protein, a saponification step via the addition of 10 N KOH to the extract in a ratio of 1 volume 10 N KOH to 20 volumes lipid extract in dichlorometane:methanol (21). Saponification was allowed to proceed at 25 ^{O}C for 1.5 hr. A second extraction step was then performed by adding 10 volumes of Dulbrecco's phosphate buffered saline. The organic layer was collected and the aqueous layer (10 volumes) re-extracted with 10 volumes of dichloromethane. The two organic layers were combined and dried under nitrogen. A sterol and oxysterol (including 7-KC) fraction was then separated from other lipids on an aminopropyl solid phase extraction (SPE) column. The lipid fraction was loaded onto the column and sterols and oxysterols eluted in 23:1 chloroform:methanol. This fraction was dried under nitrogen and re-dissolved in 90% methanol (400 µL for 200 µL of plasma).

LC-MS/MS was performed using a reversed phase column for separation coupled to an electrospray ionisation (ESI) tandem quadrupole mass spectrometer. A gradient of acetonitrile, propan-2-ol, water, containing 5 mM ammonium acetate was utilised to separate oxysterols. 7-KC ionised as the $[M+H]^+$ ion at *m/z* 401.3 and the multiple reaction monitoring (MRM) transition 401.3→383.3 (Figure 1B) used for identification and quantification along with the transition 408.3→390.3 for the [$^2H_7$]7-KC internal standard. The authors point out that of the 10 oxysterols studied, 7-KC gave the least good % relative standard error (RSE) with most oxysterols giving a RSE of less than 10% but 7-KC an RSE of 18% (21). Importantly, McDonald et al designed their protocol for multiple oxysterols, and it was not optimised for 7-KC in particular (21). Never-the-less a RSE of 18% is still quite respectable considering the multiple dry-down steps and that 7-KC was not separated from cholesterol except in the final LC-MS/MS analysis.

Others have developed less laborious methods for the analysis of 7-KC in the absence of a saponification step, involving only deproteinization in methanol and LC-ESI-MS/MS with MRM *m/z* 401→95. However, simple methods are not necessarily better than those which are more laborious (23).

2.3. LC-MS/MS with Derivatisation

Following the discovery of unusual bile acids (3β-hydroxy-7-oxochol-5-enoic acid) with a ring structure similar to that of 7-KC in urine of a patient with Niemann Pick type C (NPC) disease (6), an interest developed in 7-KC as a potential plasma diagnostic for NPC disease (11, 12, 24, 25).

2.3.1. Derivatisation with Dimethylglycine (DMG)

Jiang, Ory, Han and colleagues developed a derivatisation method to improve the ionisation of 7-KC and also cholestane-3β,5α,6β-triol (3β,5α,6β-triol) and exploited this in the study of NPC patient plasma. In the absence of a saponification step and without the addition of antioxidants, other than EDTA in the blood collection tube, they derivatised these oxysterols with dimethylglycine (DMG) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as a coupling reagent (Figure 1C) (11, 12). Specifically, oxysterols in 50 µL of plasma were extracted in 250 µL of methanol containing isotope labelled internal standards ([$^2H_7$]7-KC and [$^2H_7$] 3β,5α,6β-triol). After centrifugation the solution was dried down under nitrogen at 35 °C and redissolved in 20 µL of 0.5 M dimethylglycine, 2 M 4-(dimethylamino)pyridine in chloroform and 20 µL of EDC in chloroform. After 1 hr at 45 °C, 20 µL of methanol was added to quench the reaction and the solution dried down once more under nitrogen at 35 °C. The derivatised sample was redissolved in 200 µL of 80% methanol. LC-MS/MS was carried out using a reversed phase column and with a gradient of water and acetonitrile containing 0.015% trichloroacetic acid and 0.5 % acetic acid, ionisation was by atmospheric chemical ionisation (APCI) and MS/MS exploiting MRMs 486 → 383 (quantifier) 486 → 104 (qualifier) (12). Inter-run precision was better than 15% and the method gave a lower limit of quantitation (LOQ) of 2 ng/mL. Surprisingly, the authors claimed that "3β,5α,6β-triol and 7-KC markers were not increased in other neurodegenerative or lysosomal diseases" (12), which is in contrast to studies by others who found both analytes to be elevated in the lysosomal storage disorders acid sphingomyelin deficiency (ASMD, also known as NPB) and Wolman disease (13-15, 25).

2.3.2. Derivatisation with the Girard Reagent

Our preference for the analysis of 7-KC and related metabolites is derivatisation with the Girard P (GP) reagent (8, 13-16, 26). Typically for plasma analysis, 100 µL of plasma is extracted into 1.05 mL of absolute ethanol containing isotope-labelled standards, including [$^2H_7$]KC, and diluted to 70% ethanol with water. Oxysterols including 7-KC are separated from cholesterol on a reversed-phase SPE column. Cholesterol binds to the column while 7-KC and other oxysterols and bile acid precursors elutes in the flow through. This fraction is then dried down under vacuum and re-constituted in 150 µL of propan-2-ol. For the analysis of oxysterols *without a keto group*, we introduce such a group by *ex vivo* oxidation with cholesterol oxidase (0.264 units) in 1 mL of 50 mM KH_2PO_4, prior to addition of 2 mL of methanol, 150 µL of glacial acetic acid and 190 mg of [$^2H_5$]GP bromide salt. For the analysis of 7-KC and other sterols *with a native keto group*, the

procedure excludes cholesterol oxidase and 150 mg [$^2H_0$]GP chloride salt replaces [$^2H_5$]GP bromide. The derivatised molecules are finally separated from excess reagent by a second reversed phase SPE step and eluted in 2 mL of methanol. If total sterols are to be measured saponification of 100 µL of plasma is in 1.05 mL of ethanolic 0.35 M KOH for 2 hr at room temperature followed by neutralisation and reversed-phase SPE with the rest of the protocol as for free sterols (8).

GP-derivatised 7-KC separates from its isomer 7α-hydroxycholest-4-en-3-one on a C_{18} reversed-phase column with a methanol, acetonitrile, 0.1% formic acid gradient and fragments in MS/MS (534.4→455.4, see Figure 1D) to give the [M-pyridine]$^+$ ([M-Py]$^+$) ion suitable for MRM on tandem quadrupole instruments (Figure 2).

If tribrid instruments e.g. Orbitrap IDX, are used the fragment-ion at 455.4 ([M-Py]$^+$) can be fragmented further by MS/MS/MS (or MS^3). 7-KC and its metabolites give characteristic [M-Py-43]$^+$, [M-Py-59]$^+$ and [M-Py-90]$^+$ fragment ions (Figure 3B) (13). This fragmentation has been exploited in the discovery of 27-hydroxy-7-ketocholesterol (also called 26-hydroxy-7-oxocholesterol), 3β-hydroxy-7-ketocholest-5-enoic acid (also called 3β-hydroxy-7-oxocholest-5-en-26-oic acid) and 3β-hydroxy-7-ketochol-5-enoic acid (also called 3β-hydroxy-7-oxochol-5-enoic acid) in plasma of patients suffering from the lysosomal storage disorders NPC, ASMD and Wolman disease (13-16).

We have similarly used the GP derivatisation protocol to measure 7-KC in CSF (26) and tissue samples including brain (27).

3. Discussion

7-KC can be formed via *ex vivo* oxidation of cholesterol. This is problematic as cholesterol is often 1,000 times more abundant than 7-KC, so measured values of 7-KC should always be treated with caution. However, the endogenous formation of 7-KC has now been unequivocally established, (i) as an enzymatic product of CYP7A1 oxidation of 7-DHC in the disorder Smith-Lemli-Opitz syndrome where 7-DHC is elevated and (ii) non-enzymatically in the lysosomal disorders NPC, ASMD and Wolman disease where the lysosomal transport proteins NPC1 or NPC2 are deficient NPC type 1 and type 2 disease, respectively, the lysosomal enzyme acid sphingomyelinase is deficient in ASMD, and the enzyme lysosomal acid lipase is inactive in Wolman disease. In addition the discovery of 7-KC metabolites in an unusual bile acid biosynthesis pathway further provides proof of the endogenous nature of 7-KC (14).

It is worth considering the optimal precautions to avoid *ex vivo* formation of 7-KC. Our preference is to collect blood in EDTA lavender top tubes to minimise the Fenton reaction, separate oxysterols from cholesterol early in the sample preparation protocol and where possible dry samples under vacuum. In our experience dried samples containing cholesterol held in an air atmosphere provides the major source of artefactual 7-KC. Multiple freeze thaw cycles are also a danger point.

4. Conclusion

For a long time the importance of 7-KC in biochemistry was dismissed by many in the oxysterol field. In hindsight this was clearly a mistaken view point. It has now been proved unequivocally that 7-KC can be formed endogenously. However, its measurement can prove challenging as *ex vivo* autoxidation of cholesterol is always a possibility, potentially distorting the quantitative measurement of 7-KC.

Human Samples

Plasma was from a patient diagnosed with lysosomal storage disorders or a laboratory control sample. All participants or their parents provided written informed consent in accordance with the Declaration of Helsinki and the study was conducted with institutional review board approval.

Acknowledgements

We are grateful for funding from UKRI (grant numbers MR/Y008057/1, BB/S019588/1) and the European Union through European Structural Funds (ESF), as part of the Welsh Government funded Academic Expertise for Business project. For the purpose of Open Access, the authors have applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission. We thank Dr Christopher Titman and Shimadzu UK Limited for providing access to instrumentation and generating tandem mass spectrometry data.

Competing Interest Statement

WJG and YW are listed as inventors on the patent “Kit and method for quantitative detection of steroids” US9851368B2. WJG and YW are shareholders in CholesteniX Ltd.

Figure Captions

Figure 1. Derivatisation and mass spectrometry analysis of 7-KC. (A) Conversion of 7-KC to its TMS ether by the method of Dzeletovic et al in blue (19) or Schött and Lütjohann in green (20). (B) MRM transition utilised by McDonald et al (21). (C) Derivatisation of 7-KC to its DMG ester and MRMs exploited by Jiang, Ory and Han (11). (D) Derivatisation of 7-KC to its GP hydrazone and fragmentation by MS/MS and MS^3 (13).

Figure 2. LC-ESI-MS/MS MRM chromatogram of GP-derivatised 7-KC with separation from 7α-hydroxycholest-4-en-3-one (7α-HCO) from a control plasma sample. Separation was achieved on a Hypersil Gold C_{18} column, exploiting a 37 min methanol, acetonitrile and 0.1% formic acid gradient. MS/MS was recorded on a Shimadzu LCMS-8060RX triple quadrupole instrument. The MRM transition 534.4 → 455.4 is applicable to both 7-KC and 7α-HCO. 7α-HCO gives two peaks due of cis trans isomerisation at the C-3 hydrazone group.

Figure 3. LC-ESI-MS(MS^3) analysis of 7-KC from a ASMD patient plasma sample. (A) Separation of 7-KC from 7α-HCO on a Hypersil Gold C_{18} column exploiting a 17 min methanol, acetonitrile and 0.1% formic acid gradient. Upper panel, reconstructed-ion chromatogram of 534.4054 ± 5 ppm corresponding the $[M]^+$ ions of 7-KC and 7α-HCO. Data acquired in the Orbitrap of an Orbitrap IDX instrument. Lower panel, MRM-like chromatogram for the 534.4→455.4→ transition. Data acquired in the ion-trap of the Orbitrap IDX. (B) MS^3 (534.4→455.4→) spectrum of 7-KC. Key fragment ions are highlighted in red. (C) MS^3 (534.4→455.4→) spectrum of 7α-HCO. Fragment ion nomenclature is described in (8).

Table 1. Summary of mass spectrometry methods typically used for analysis of 7-KC.

Method	Technology	Saponification	Extraction (before or after solvolysis)	Derivatisation	Sensitivity	Concentration in Control Plasma (ng/mL, mean ± SD or range)
Dzeletovic et al (19)	GC-MS (SIM)	1:10, plasma:0.35 M KOH in EtOH, 22 °C, 2 hr	1. Organic (after) 2. SPE (after)	TMS ether, 60 °C, 30 min	LOD 2 ng/mL from 1 mL plasma	22 ± 14 (total)[a] 8 ± 4 (free)[b]
Schött & Lutjohann (20)	GC-MS (SIM)	1:4, plasma:1.5 M NaOH in 90% EtOH, rt, 2 hr	1. Organic (after) 2. SPE (after)	TMS ether, 90 °C, 90 min	LOD 0.1 ng/mL, LOQ 0.3 ng/mL from 0.5 mL plasma	61 – 138 (total)[c]
McDonald et al (21)	LC-ESI-MS/MS (MRM)	1:20, plasma extract:10 M KOH, 25 °C, 90 min	1. Organic (before) 2. Organic (after) 3. SPE (after)	N/A	LOD < 1 ng/mL from 200 µL plasma	24 (total)[d] 84 ± 4.5 (total)[e]
Lin et al (23)	LC-ESI-MS/MS	N/A	1. Organic	N/A	LOQ 1 ng/mL from 25 µL plasma	5 (mean, free)[f] <1 – 23 (range, free)[f]
Jiang et al (11, 12)	LC-APCI-MS/MS	N/A	1. Organic	DMG, 45 °C, 1 hr	LOQ 2 ng/mL from 50 µL plasma	29 (mean, free)[g] 11 – 44 (range, free)[g]
Yutuc et al (8) Ali Asgari et al (26)	LC-ESI-MS^n	1:10, plasma:0.35 M KOH in EtOH, rt, 2 hr	1. Organic 2. SPE (after) 3. SPE (after)	GP, rt, 12 hr	LOQ 3.7 ng/mL from 100 µL plasma	30 (total)[d] 6.5 (free)[d] 4.0 ± 2.5 (free)[h]

Abbreviations: rt, room temperature; N/A, not applicable.

[a] n = 31

[b] n = 6

[c] n = 5

[d] NIST SRM 1950 (28)

[e] mean ± RSE, n = 200

[f] n = 314

[g] n = 89

[h] n = 83

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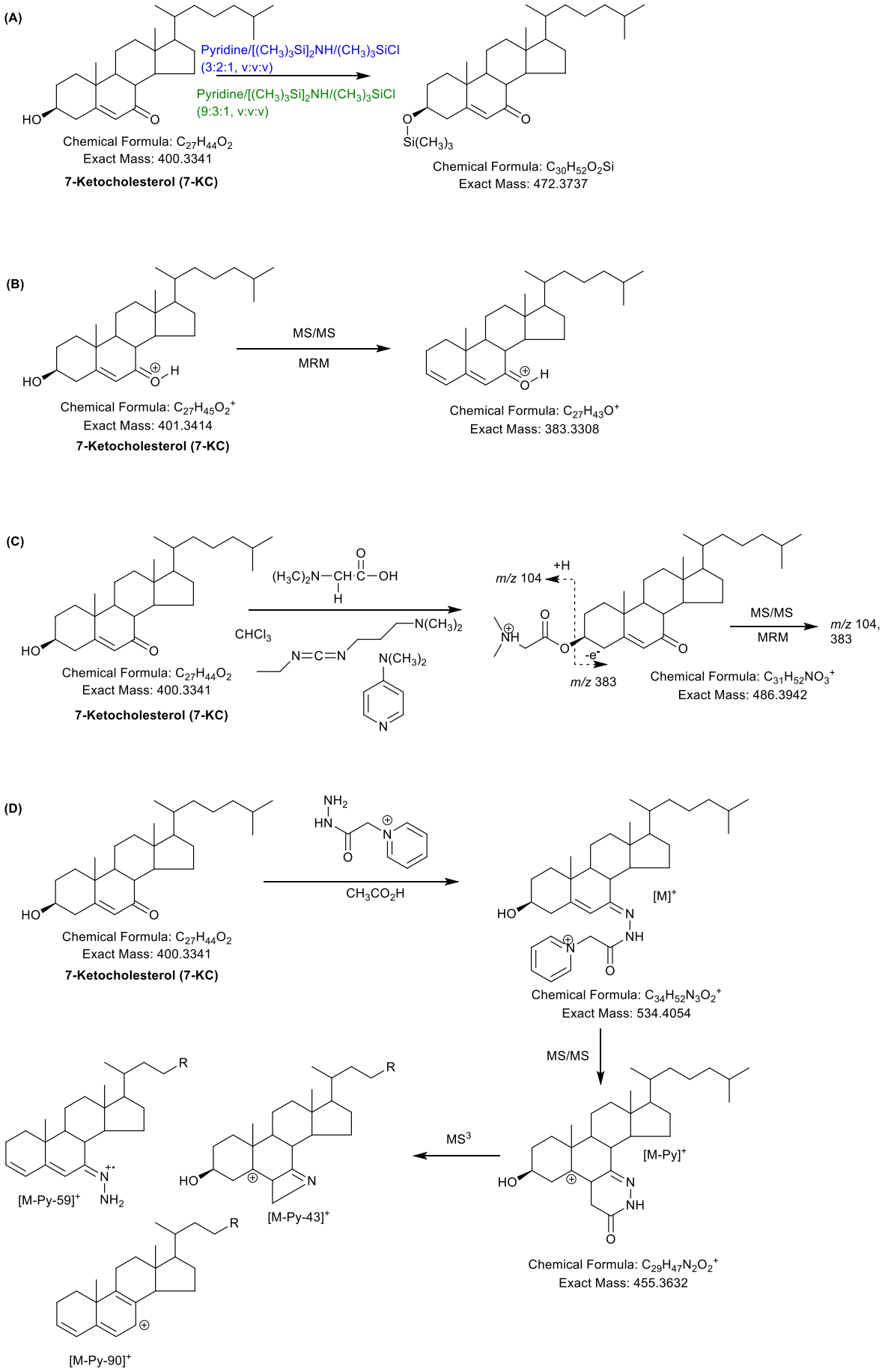
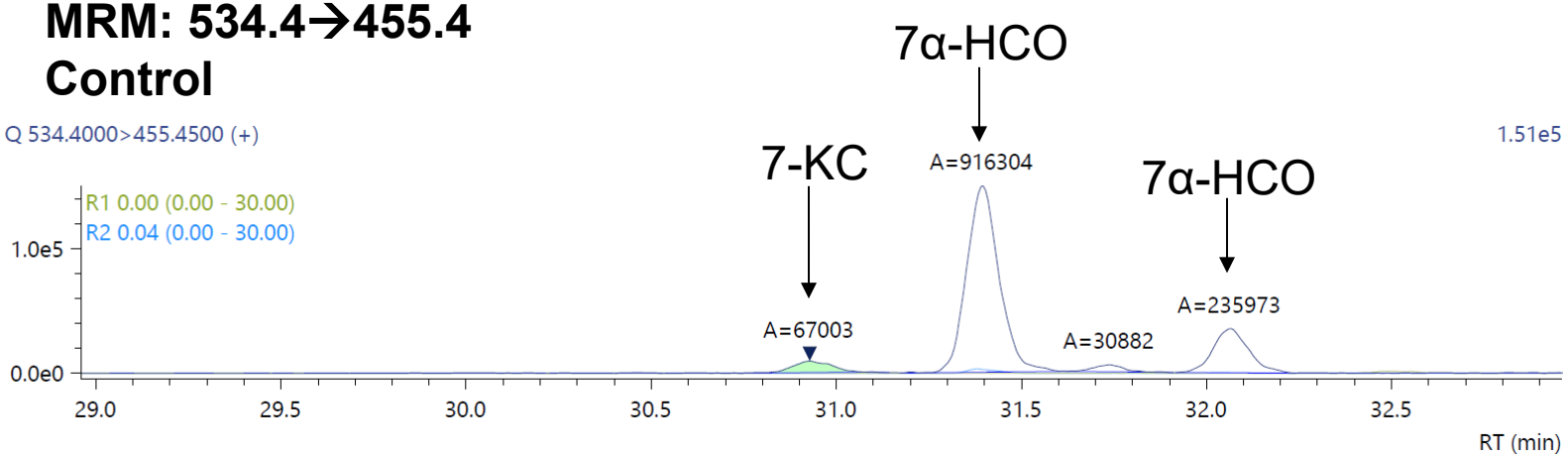
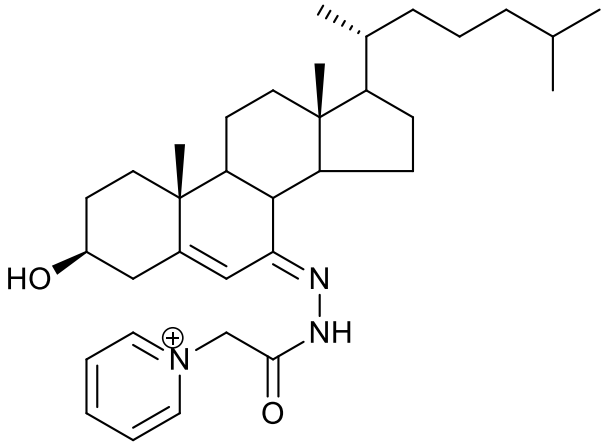
1


(A)
Pyridine/[(CH3)3Si]2NH/(CH3)3SiCl
(3:2:1, v:v:v)
Pyridine/[(CH3)3Si]2NH/(CH3)3SiCl
(9:3:1, v:v:v)
HO
O
O
Si(CH3)3
Chemical Formula: C27H44O2
Exact Mass: 400.3341
7-Ketocholesterol (7-KC)
Chemical Formula: C30H52O2Si
Exact Mass: 472.3737
(B)
MS/MS
MRM
HO
O
H
Chemical Formula: C27H45O2+
Exact Mass: 401.3414
7-Ketocholesterol (7-KC)
Chemical Formula: C27H43O+
Exact Mass: 383.3308
(C)
(H3C)2N—CH·C—OH
H
O
CHCl3
N(CH3)2
N=C=N
N(CH3)2
N
HO
O
Chemical Formula: C27H44O2
Exact Mass: 400.3341
7-Ketocholesterol (7-KC)
m/z 104
+H
NH
O
O
-e-
m/z 383
MS/MS
MRM
m/z 104, 383
Chemical Formula: C31H52NO3+
Exact Mass: 486.3942
(D)
NH2
HN
N
O
CH3CO2H
HO
O
Chemical Formula: C27H44O2
Exact Mass: 400.3341
7-Ketocholesterol (7-KC)
[M]+
HO
N
NH
N
O
Chemical Formula: C34H52N3O2+
Exact Mass: 534.4054
MS/MS
[M-Py]+
HO
N
NH
O
Chemical Formula: C29H47N2O2+
Exact Mass: 455.3632
MS3
R
[M-Py-59]+
N+·
NH2
R
HO
N
[M-Py-43]+
R
[M-Py-90]+

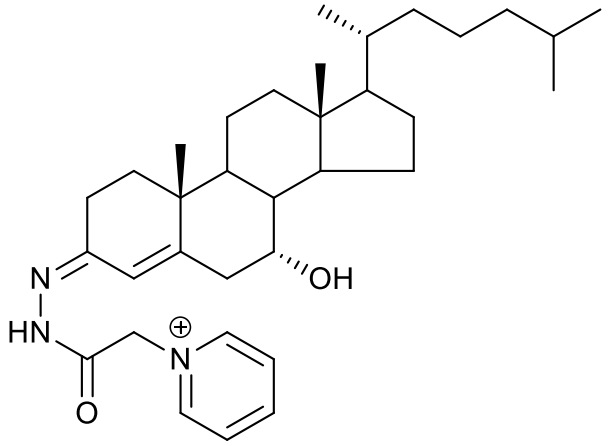
2

MRM: 534.4→455.4
Control

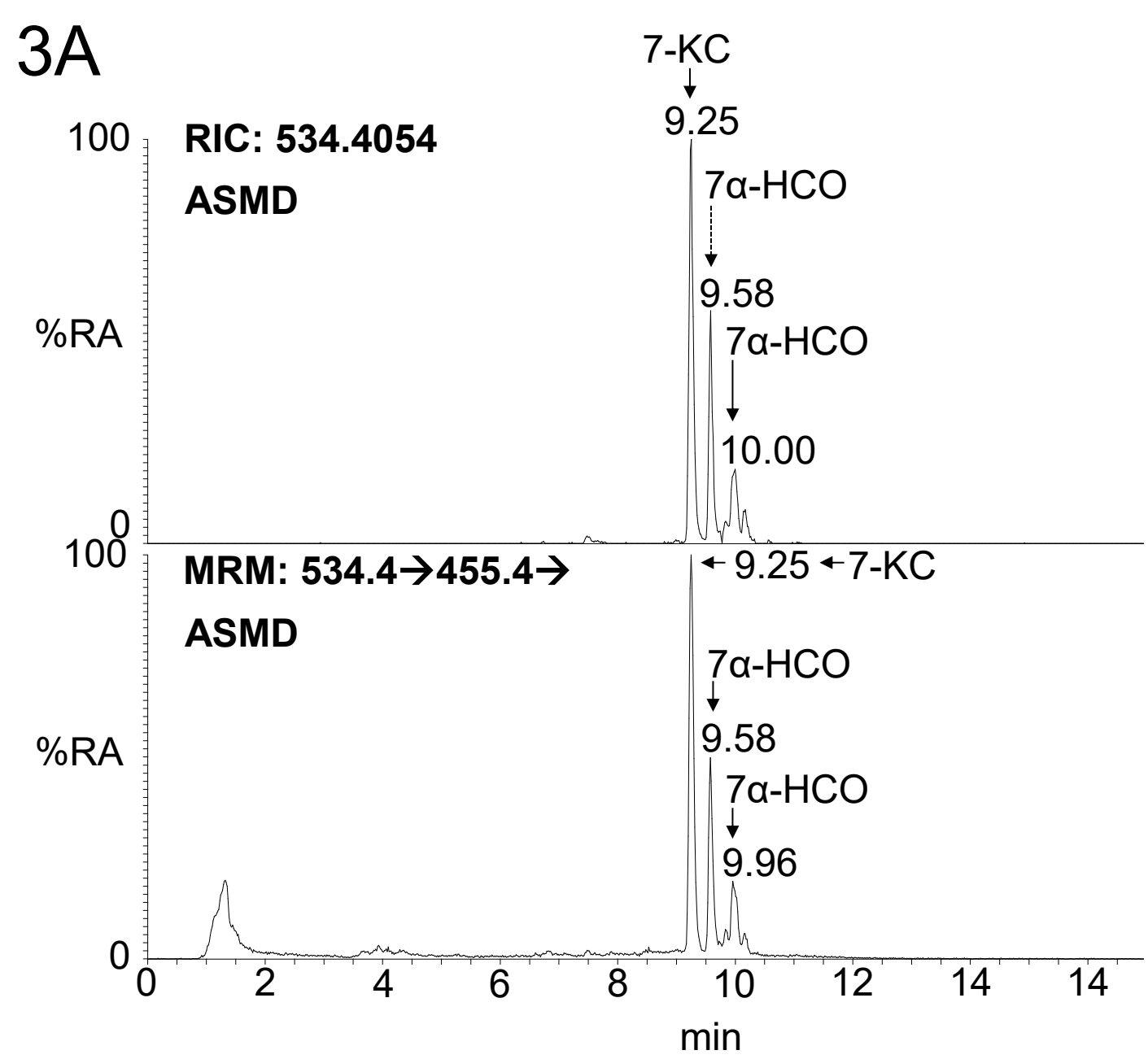


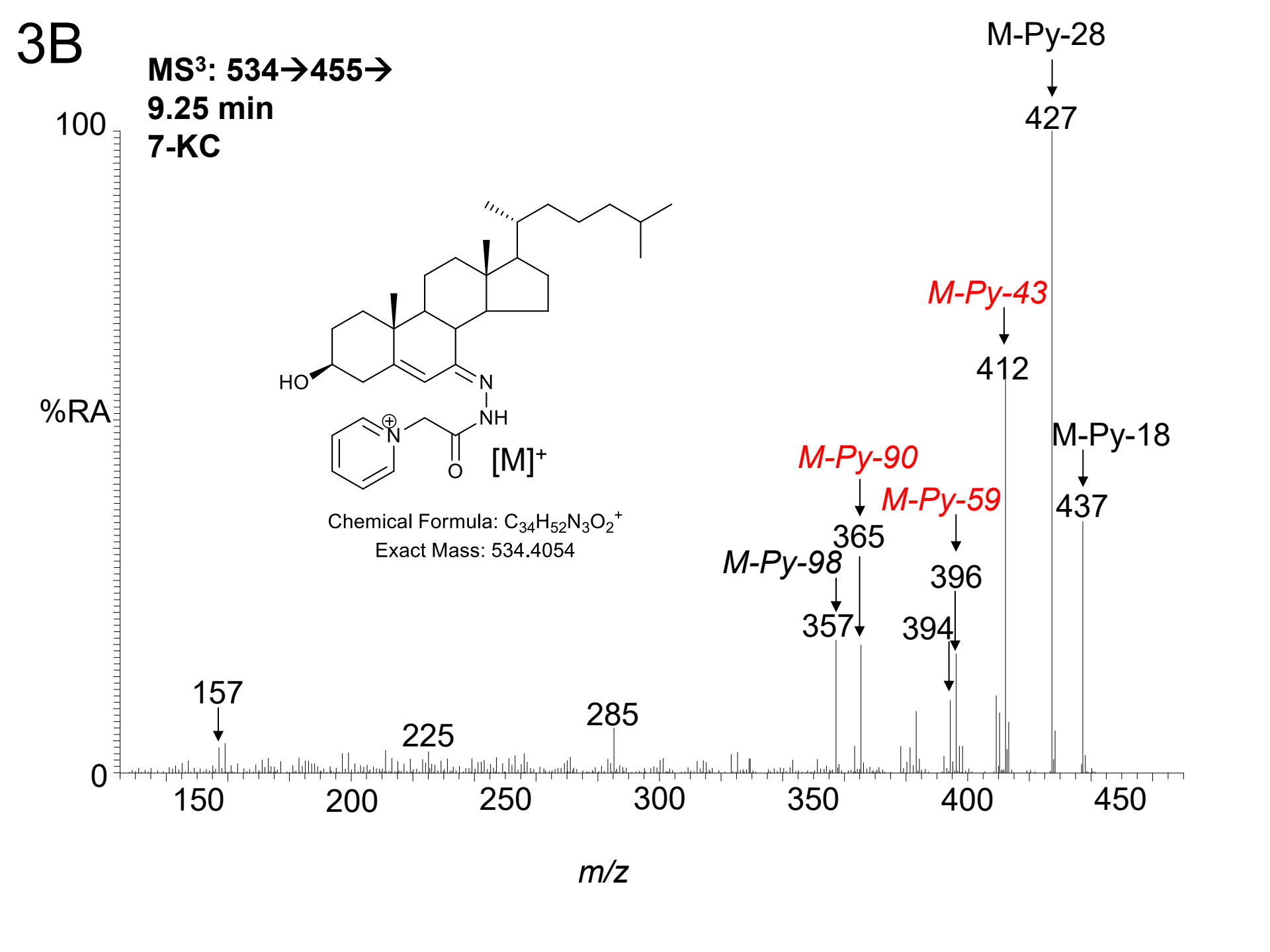


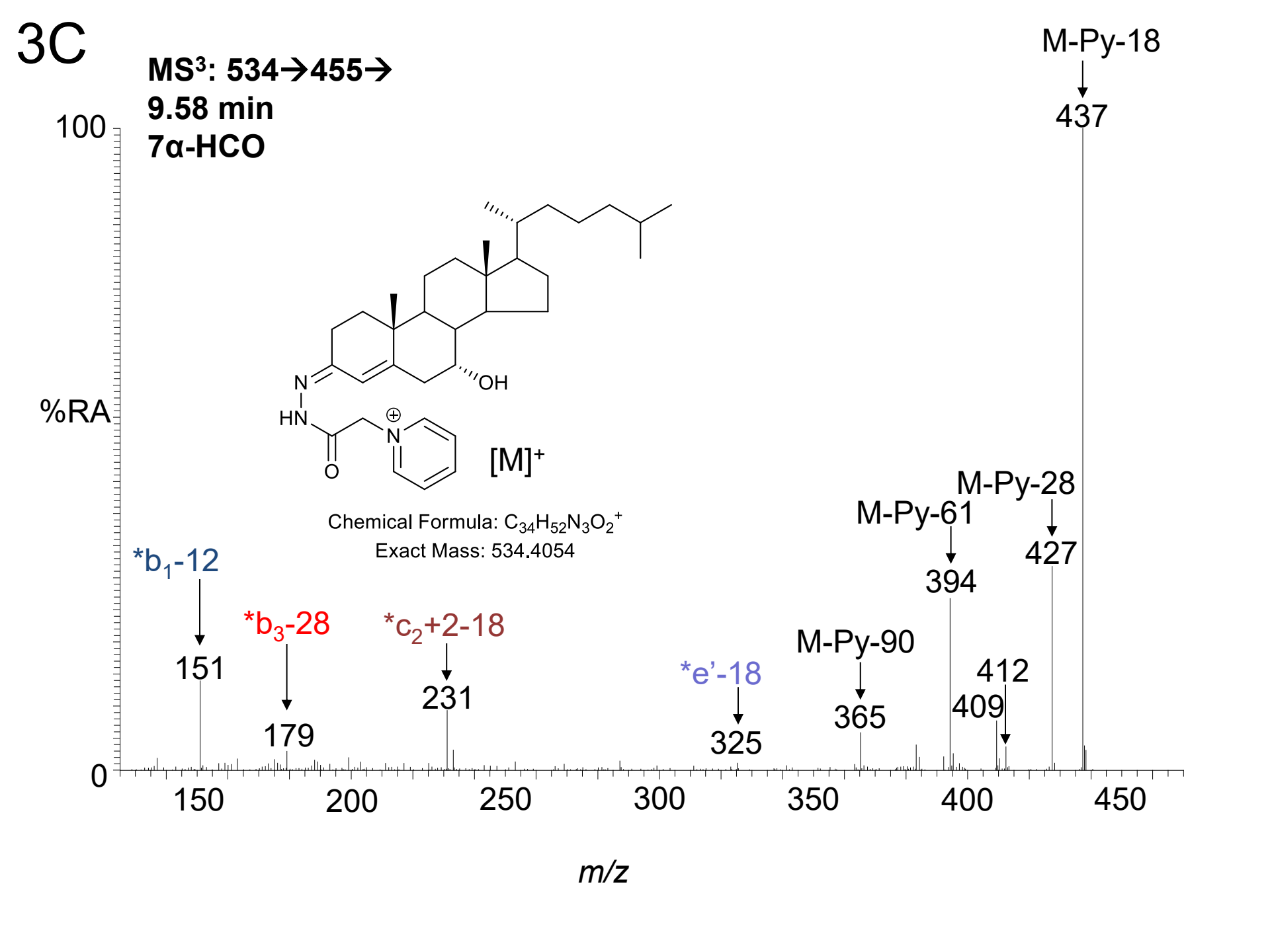
Chemical Formula: $C_{34}H_{52}N_3O_2^+$
Exact Mass: 534.4054



Chemical Formula: $C_{34}H_{52}N_3O_2^+$
Exact Mass: 534.4054


3A
7-KC
9.25
RIC: 534.4054
ASMD
100
%RA
7α-HCO
9.58
7α-HCO
10.00
0
MRM: 534.4→455.4→
9.25 7-KC
ASMD
100
7α-HCO
9.58
%RA
7α-HCO
9.96
0
0 2 4 6 8 10 12 14 14
min


3B
MS³: 534→455→
9.25 min
7-KC
100
%RA
0
HO
N
NH
N
O
[M]+
Chemical Formula: C34H52N3O2+
Exact Mass: 534.4054
M-Py-28
427
M-Py-43
412
M-Py-18
437
M-Py-90
365
M-Py-59
396
M-Py-98
357
394
157
225
285
150
200
250
300
350
400
450
m/z


3C
MS³: 534→455→
9.58 min
7α-HCO
100
%RA
0
N
HN
O
OH
N
[M]⁺
Chemical Formula: C34H52N3O2+
Exact Mass: 534.4054
M-Py-18
437
M-Py-28
427
M-Py-61
394
M-Py-90
365
412
409
*b1-12
151
*b3-28
179
*c2+2-18
231
*e'-18
325
150
200
250
300
350
400
450
m/z