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Review

New methods for analysis of oxysterols and related compounds by LC–MS

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ABSTRACT

Oxysterols are oxygenated forms of cholesterol or its precursors. They are formed enzymatically and *via* reactive oxygen species. Oxysterols are intermediates in bile acid and steroid hormone biosynthetic pathways and are also bioactive molecules in their own right, being ligands to nuclear receptors and also regulators of the processing of steroid regulatory element-binding proteins (SREBPs) to their active forms as transcription factors regulating cholesterol and fatty acid biosynthesis. Oxysterols are implicated in the pathogenesis of multiple disease states ranging from atherosclerosis and cancer to multiple sclerosis and other neurodegenerative diseases including Alzheimer's and Parkinson's disease. Analysis of oxysterols is challenging on account of their low abundance in biological systems in comparison to cholesterol, and due to the propensity of cholesterol to undergo oxidation in air to generate oxysterols with the same structures as those present endogenously. In this article we review the mass spectrometry-based methods for oxysterol analysis paying particular attention to analysis by liquid chromatography–mass spectrometry (LC–MS).

Contents

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1. Introduction

Cholesterol is the archetypical sterol, being an essential component of cell membranes throughout the animal kingdom [1]. The first step of cholesterol metabolism is oxidation to an oxysterol [2]. Similarly, precursors of cholesterol can be oxidised to oxysterols [3]. Consequently, oxysterols are a large family of molecules where cholesterol or its precursors have additional oxygen functionalities at one or more positions in the ring system or on the side-chain. Oxysterol analysis dates back to the 1940s and even at that time the potential of cholesterol to undergo nonenzymatic oxidation *ex vivo* was noted [4]. This can be problematic as cholesterol can be oxidised in air to give identical products to some of those formed enzymatically. Throughout the years, from the 1940s onwards, there have been many reports where oxysterols formed by nonenzymatic autoxidation *ex vivo* have been confused by those formed enzymatically *in vivo* [2]. The situation is further complicated by the fact that oxysterols can be formed endogenously by reactive oxygen species (ROS) [5,6] with the same structures as those formed *ex vivo* or enzymatically *e.g.* 7-oxocholesterol (7-OC), 7β-hydroxycholesterol (7β-HC) [7,8]. Oxysterols are key members of the bile acid and steroid hormone biosynthesis pathways [9]. Mutations in enzymes responsible for oxysterol formation can lead to disease in humans. One example is cytochrome P450 (CYP) 27A1, a sterol (25R) 26-hydroxylase, whose deficiency is the cause of cerebrotendinous xanthomatosis (CTX), a rare disease which can present in early infancy with cholestatic liver disease [10], in early childhood with chronic diarrhoea and cataracts, in later childhood with tendon xanthomata, learning difficulties or psychiatric illness, and in adult life with spastic paraparesis, a fall in IQ or frank dementia, ataxia and/or dysarthria [11]. CTX is usually diagnosed only in the adolescent and adult. Deficiency in another enzyme, CYP7B1, an oxysterol 7α-hydroxylase, can lead to liver disease in infants and spastic paraplegia in adults (hereditary spastic paraplegia type 5, SPG5) [12]. Deficiency in CYP7A1, cholesterol 7α-hydroxylase, can lead to hyperlipidemia and gallstones [13].

The brain contains a high proportion of the body's cholesterol [1], the majority of which is present in myelin sheaths of oligodendrocytes but much of cholesterol metabolism is in neurons where CYP46A1 oxidises it to 24S-hydroxycholesterol (24S-HC) [14]. Unlike cholesterol, 24S-HC can pass the blood brain barrier (BBB) and be exported from brain [15]. This makes 24S-HC a target for analysis in plasma in the hope of early diagnosis of neurodegenerative disease *e.g.* Alzheimer's disease (AD), Parkinson's disease (PD) [16,17].

Oxysterol synthesis is an important part of the immune response to pathogens. Macrophages, a type of white blood cell which play a critical role in immunity can make high levels of 25-hydroxycholesterol (25-HC), through up-regulation of cholesterol 25-hydroxylase (CH25H), when stimulated by bacteria [18,19] or viruses [20], and its metabolite 7α,25-dihydroxycholesterol (7α,25-diHC) can activate a G protein-coupled receptor (GPCR) called Epstein–Barr virus-induced gene 2 (EBI2, GPR183) and oxysterol gradients guide migration of EBI2 expressing immune cells [21,22] many of which have been implicated in shaping the adaptive and innate immune response. The exact role of 25-HC itself in immunity is yet to be uncovered, although Reboldi et al. showed that 25-HC is a mediator of negative feedback towards interleukin 1 (IL-1) family cytokine production and inflammasome activity, through binding to INSIG (insulin induced gene) and antagonising the sterol response element-binding protein-2 (SREBP-2) driven mevalonate pathway, thereby, reducing *Il1b* transcription and repressing IL-1-activating inflammasomes [23].

Most recently, oxysterols have been implicated in neurogenesis [24] and their relatives, cholestenoic acids, in the regulation of survival of motor neurons [25]. Their involvement in these processes is through interaction with the liver X receptors (LXRs), known receptors of oxysterols [26,27].

2. Metabolism of cholesterol and its precursors to oxysterols

2.1. Enzymatic metabolism of cholesterol

The first step in steroid hormone biosynthesis is oxidation of cholesterol to 22R-hydroxycholesterol (22R-HC) and on to 20R,22R-dihydroxycholesterol (20R,22R-diHC) and ultimately to pregnenolone by the enzyme CYP11A1 [28]. Interestingly, 22R-HC and 20R,22R-diHC have been reported to be present in newborn and adult mouse brain supporting the concept to steroidogenesis in brain [29,30] (Fig. 1A, Supplementary Table S1). Both these oxysterols activate LXRs [28]. The first step of the neutral pathway of bile acid biosynthesis is oxidation of cholesterol to 7α-hydroxycholesterol (7α-HC) by hepatic CYP7A1, 7α-HC is then oxidised to 7α-hydroxycholest-4-en-3-one (7α-HCO) by hydroxysteroid dehydrogenase (HSD) 3B7 or alternatively 7α-HC is further hydroxylated to 7α,(25R)26-dihydroxycholesterol (7α,(25R)26-diHC) by CYP27A1 (Fig. 1B). Note, introduction of a hydroxy group at the terminal carbons, C-26 or C-27, of the cholesterol side-chain introduces R or S stereochemistry at C-25. To avoid confusion we define the stereochemistry when known, and use the systematic numbering of carbon atoms according to IUPAC rules [31]. 7α-HCO can be similarly hydroxylated by CYP27A1 and 7α,(25R)26-diHC can be oxidised by HSD3B7, both to give 7α,(25R)26-dihydroxycholest-4-en-3-one (7α,(25R)26-diHCO). Alternatively, hydroxylation can be at C-12α through CYP8B1 to generate 7α,12α-dihydroxycholest-4-en-3-one (7α,12α-diHCO) on the pathway to cholic acid, and oxidation of both 7α,12α-diHCO and 7α,(25R)26-diHCO by CYP27A1 can occur to generate the (25R)26 acids, 7α,12α-dihydroxy-3-oxocholest-4-en-(25R)26-oic (7α,12α-diH,3O-CA) and 7α-hydroxy-3-oxocholest-4-en-(25R)26-oic (7αH,3O-CA) acids, respectively. Each of these metabolites can be observed in human and in mouse plasma (Table 1, Table S1) [32–36]. Prior to formation of (25R)26 acids the 3-oxo-4-ene group can be reduced by two successive aldoketo reductase (AKR) enzymes, 1D1 and 1C4, to a 3α-hydroxy-5β-hydrogen configuration, but metabolites with these structures are not routinely observed in plasma. Of the enzymatically formed oxysterols, 7α-HC can also be formed by autoxidation of cholesterol *ex vivo*, so there is always a concern over concentration reported in the literature for this oxysterol [2]. The first step of the acidic pathway of bile acid biosynthesis is the conversion of cholesterol to (25R)26-hydroxycholesterol ((25R)26-HC) by CYP27A1 (Fig. 1B). (25R)26-HC can be oxidised further to the (25R)26 acid, 3β-hydroxycholest-5-en-(25R)26-oic acid (3β-HCA), by CYP27A1 or to 7α,(25R)26-diHC by CYP7B1. Both of these

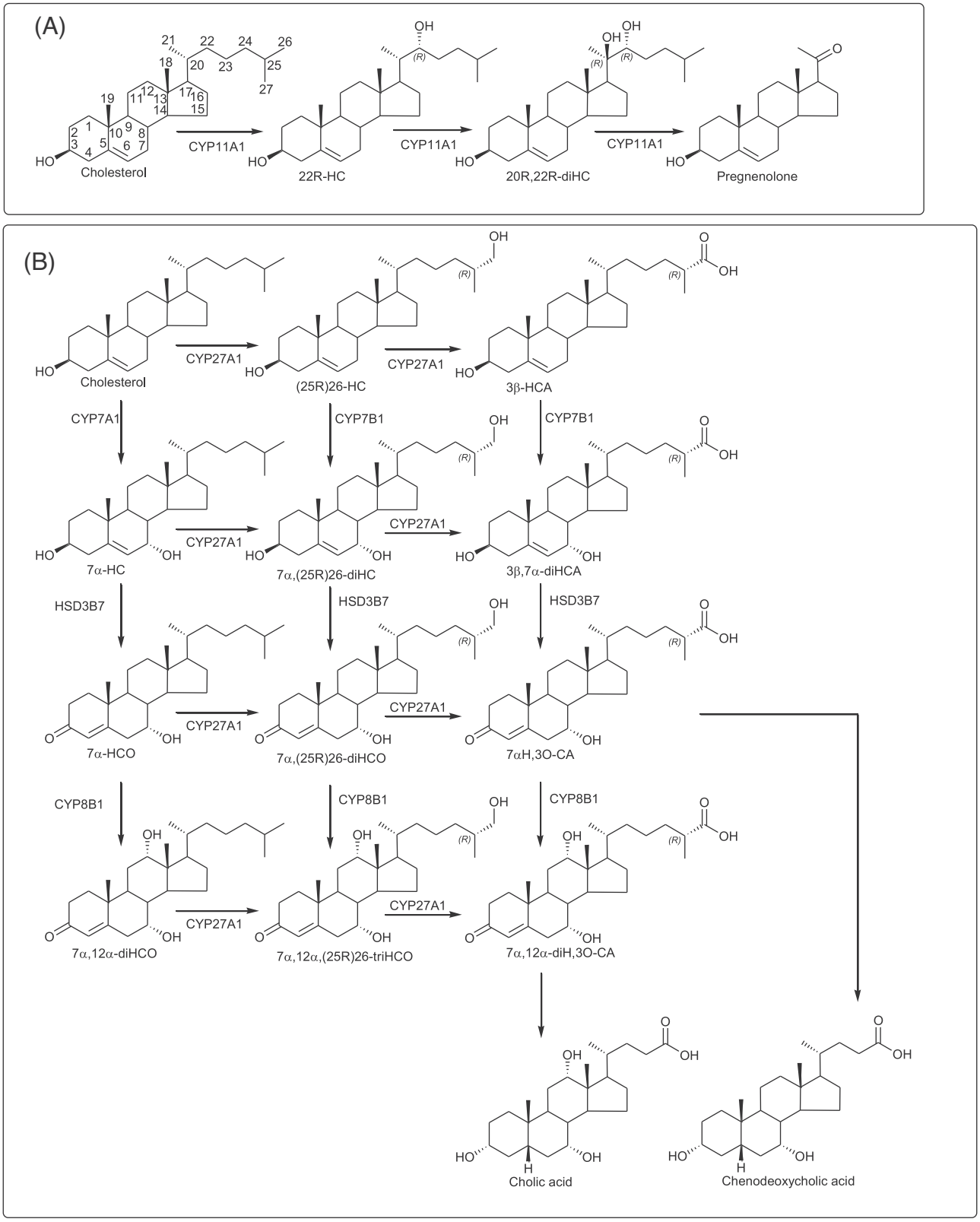

Fig. 1. (A) Early steps of steroid hormone biosynthesis. (B) Conversion of cholesterol to bile acids.

metabolites can be converted to 3β,7α-dihydroxycholest-5-en-(25R)26-oic acid (3β,7α-diHCA) or alternatively 7α,(25R)26-diHC can be oxidised to 7α,(25R)26-diHCO by HSD3B7. 7α,(25R)26-diHCO is then further oxidised to 7αH,3O-CA by CYP27A1 while is 3β,7α-diHCA is oxidised by HSD3B7 also to 7αH,3O-CA. While the neutral pathway of bile acid biosynthesis is hepatic, the acidic pathway can proceed extrahepatically [37]. (25R)26-HC is largely biosynthesised in lung [38]. Both 7α,(25R)26-diHCO and 7αH,3O-CA are exported from brain while (25R)26-HC is imported to brain from the circulation indicating that the bile acid biosynthesis

Table 1
Sterols including oxysterols, cholenoic and cholestenoic acids in human plasma/serum and CSF. All concentrations in ng/mL.

Sterol systematic name (common name)	Plasma or serum					
	Free sterols LC–MS with EADSA[a,b,c,d]	NIST SRM 1950 *Free* sterols LC–MS with EADSA[h]	NIST SRM 1950 *Free* or **Total** sterols LC–MS[i,j]	Cooper Inst[k], Dallas Heart[l], **Total** sterols LC–MS	*Free* or **Total** sterols LC–MS[m,n,o,p,q,r,s,t,u,v]	*Free* or **T** sterols GC–MS[c,w]
3β-Hydroxycholest-5-en-24-one (24-Oxocholesterol)	*0.59 ± 0.85*[a,e] *0.24 ± 0.06*[d,e]	*0.14 ± 0.03*[h,e]	**3.7**[i]	**6.0 ± 2.2**[k] **5(0.4–116)**[l]	–	–
3β-Hydroxycholest-5-en-24S,25-epoxide (24S,25-Epoxycholesterol)	*0.59 ± 0.85*[a,f] *0.24 ± 0.06*[d,f]	0.14 ± 0.03[h,f]	**1.8**[i]	**1.8 ± 4.4**[k] **1(0.1–56)**[l]	**2 ± 2**[m]	–
7α-Hydroxy-26-*nor*-cholest-4-ene-3,24-dione	*<0.2*[a,g] *0.37 ± 0.05*[b,g] *0.2 ± 0.2*[d,g]	0.04 ± 0.02[g,h]	–	–	–	–
Cholest-5-ene-3β,4β-diol (4β-Hydroxycholesterol)	–	–	*5.46 ± 0.19*[j] **22.1 ± 0.8**[j] **31.4**[i]	**53.1 ± 2.4**[k] **36(9–500)**[l]	**77 ± 40**[m] **22.5 ± 9.5**[n] **48.5 ± 2.0**[o] **51.9 ± 2.4**[s]	**29 ± 10**[w] **12.37 ± 7** **14.15 ± 0**
Cholest-5-ene-3β,24S-diol (24S-Hydroxycholesterol)	*6.86 ± 0.31*[a] *12.88 ± 0.45*[b] *7.00 ± 3.15*[c] *7.11 ± 0.40*[d]	*5.46 ± 0.05*[h]	*2.55 ± 0.12*[j] **19.7±0.8**[j] **44.6**[i]	**56.1 ± 2.1**[k] **57(10–314)**[l]	*12.3 ± 4.79*[r] *8.64*[t] **51 ± 12**[m] **64 ± 14**[p] **60.4 ± 1.6**[o] **64.4 ± 1.8**[s]	*36 ± 2.82* **64 ± 24**[x] **33.18 ± 1** **55.46 ± 2**
Cholest-5-ene-3β,25-diol (25-Hydroxycholesterol)	*4.06 ± 0.22*[a] *1.18 ± 0.07*[b] *1.25 ± 0.82*[c] *3.96 ± 0.27*[d]	0.70 ± 0.02[h]	*0.36 ± 0.02*[j] **8.1 ± 1.2**[j] **5.7**[i]	**11.8 ± 2.4**[k] **8(1–56)**[l]	**31 ± 11**[m] **14.0 ± 1.1**[o] **14.9 ± 1.1**[s]	*0.82 ± 0.* **2 ± 3**[x] **5.83 ± 2.** **6.96 ± 0.**
Cholest-5-ene-3β,(25R)26-diol ((25R)26Hydroxycholesterol)	*19.12 ± 0.70*[a] *23.95 ± 0.60*[b] *11.63 ± 7.37*[c] *18.99 ± 0.85*[d]	*10.33 ± 0.60*[h]	*5.14 ± 0.29*[j] **216±16**[j] **131.0**[i]	**151.4 ± 2.0**[k] **150(25–990)**[l]	*17.7 ± 8.5*[r] **117 ± 35**[m] **120 ± 30**[p] **129.9 ± 4.1**[o] **139.0 ± 4.7**[s]	*31 (13–4* *13.86 ± 1* **154 ± 43** **43.67 ± 3** **203.58 ±**

Table 1 (*Continued*)

Sterol systematic name (common name)	Plasma or serum					
	Free sterols LC–MS with EADSA[a,b,c,d]	NIST SRM 1950 *Free* sterols LC–MS with EADSA[h]	NIST SRM 1950 *Free* or **Total** sterols LC–MS[i,j]	Cooper Inst[k], Dallas Heart[l], **Total** sterols LC–MS	*Free* or **Total** sterols LC–MS[m,n,o,p,q,r,s,t,u,v]	*Free* or **T** sterols GC–MS[c,x]
Cholest-5-ene-3β,7β-diol (7β-Hydroxycholesterol)	*0.00 ± 0.32*[a] *1.09 ± 0.13*[b] *0.33 ± 0.61*[c] *1.02 ± 0.58*[d]	*0.48 ± 0.28*[h]	–	–	–	*0.45 ± 0.* **3 ± 5**[x] **13.05 ± 1** **34.68 ± 9**
3β-Hydroxycholest-5-en-7-one (7-Oxocholesterol)	*3.77 ± 1.29*[a] *3.14 ± 0.14*[b] *3.58 ± 4.00*[c] *4.98 ± 2.25*[d]	*0.59 ± 0.33*[h]	*5.51 ± 3.16*[j] **49.1 ± 33.4**[j] **24.0**[i]	**84 ± 4.5**[k] **39(8–375)**[l]	*29(11.4–44.4)*[q]	*13.64 ± 3* **22 ± 14**[x] **10.74 ± 6** **69.54 ± 2**
7α-Hydroxycholest-4-en-3-one	*2.29 ± 0.25*[a] *8.61 ± 0.47*[b] *0.88 ± 2.09*[c] *2.43 ± 0.37*[d]	*10.01 ± 1.65*[h]	–	–	*26.1 ± 2.9*[o] *6.4 ± 5.3*[u] *38 ± 18*[v] **27.5 ± 3.0**[s]	*24.4 ± 5.4* *10 (3–14*
Cholest-5-ene-3β,7α-diol (7α-Hydroxycholesterol)	*0.82 ± 0.39*[a] *3.64 ± 0.29*[b] *0.33 ± 0.41*[c] *1.30 ± 0.39*[d]	*9.52 ± 2.64*[h]	*4.68 ± 0.63*[j] **135 ± 27**[j] **91.8**[i]	**158.7 ± 3.2**[k] **90(12–2762)**[l]	**145 ± 82**[n] **136.5 ± 12.0**[s]	*14 (7–15* *7.31 ± 2.1* **43 ± 48**[x] **8.99 ± 9.** **65.84 ± 8**
Cholest-4-ene-3β,6-diol or Cholest-5-ene-3β,6-diol (6-Hydroxycholesterol)	*1.68 ± 1.07*[a] *0.28 ± 0.48*[c] *1.96 ± 0.50*[d]	–	–	–	–	–
7α,25-Dihydroxycholest-4-en-3-one	*1.24 ± 0.20*[a] *0.96 ± 0.05*[b] *1.46 ± 1.29*[c] *1.10 ± 0.32*[d]	*1.10 ± 0.04*[h]	–	–	–	–
Cholest-5-ene-3β,7α,25-triol (7α,25-Dihydroxycholesterol)	*<0.20*[a] *0.23 ± 0.01*[b] *0.35 ± 0.13*[c]	0.12 ± 0.04[h]	–	–	–	–
7α,(25R)26-Dihydroxycholest-4-en-3-one	*5.55 ± 0.41*[a] *2.69 ± 0.11*[b] *2.22 ± 1.04*[c] *5.10 ± 0.56*[d]	*3.63 ± 0.16*[h]	–	–	–	*3 (2–10)*

Cholest-5-ene-3β,7α,(25R)26-triol (7α,(25R)26-Dihydroxycholesterol)	*1.65 ± 0.39*[a] *0.46 ± 0.02*[b] *0.60 ± 0.34*[c] *0.92 ± 0.49*[d]	*0.26 ± 0.09*[h]	**9.6**[i]	**10 ± 2.4**[k] **10(2–90)**[l]	–	*<1*[y]
7α,12α-Dihydroxycholest-4-en-3-one	–	–	–	–	*0.4 ± 0.27*[u]	–
Cholest-5-ene-3β,7α,12α-triol (7α,12α-Dihydroxycholesterol)	–	–	–	–	–	–
3β-Hydroxychol-5-en-24-oic acid	*1.78 ± 0.25*[a] *2.46 ± 0.09*[b] *1.03 ± 0.99*[c] *0.83 ± 0.14*[d]	*2.48 ± 0.32*[h]	–	–	–	–
7α-Hydroxy-3-oxochol-4-en-24-oic acid	*1.91 ± 0.26*[a] *2.43 ± 0.13*[b] *1.77 ± 1.24*[c] *1.17 ± 0.23*[d]	*2.03 ± 0.27*[h]	–	–	–	–
3β,7α-Dihydroxychol-5-en-24-oic acid	*4.13 ± 0.77*[a] *1.83 ± 0.10*[b] *1.00 ± 2.11*[c] *1.52 ± 0.34*[d]	*1.04 ± 0.10*[h]	–	–	–	–
3β-Hydroxycholest-5-en-(25R)26-oic acid	*82.60 ± 3.50*[a] *83.83 ± 2.98*[b] *50.26 ± 25.17*[c] *81.12 ± 4.31*[d]	*62.48 ± 2.39*[h]	–	–	–	*67.2 ± 27* *75.5 ± 5.* *57(39–64*
3β,7β-Dihydroxycholest-5-en-26-oic acid	*5.36 ± 0.81*[a] *2.79 ± 0.09*[b] *2.72 ± 1.27*[c] *1.67 ± 0.32*[d]	*2.74 ± 0.18*[h]	–	–	–	–
3β,22,25-Trihydroxycholest-5-en-24-one	*9.63 ± 1.46*[a,g] *4.07 ± 0.22*[b,g] *5.37 ± 0.64*[d]	*8.02 ± 0.72*[h,g]	–	–	–	–
7α-Hydroxy-3-oxocholest-4-en-(25R)26-oic acid	*63.89 ± 5.44*[a] *50.73 ± 2.07*[b] *50.68 ± 19.90*[c] *65.27 ± 6.22*[d]	*58.27 ± 5.48*[h]	–	–	–	*81.7 ± 27* *27.4 ± 3.* *85(67–9*
3β,7α-Dihydroxycholest-5-en-(25R)26-oic acid	*47.42 ± 6.61*[a] *19.30 ± 0.73*[b] *18.57 ± 10.29*[c]	*19.22 ± 1.92*[h]				*38.9 ± 25* *29.5 ± 4.* *30(25–3*

Table 1 (*Continued*)

Sterol systematic name (common name)	Plasma or serum					
	Free sterols LC–MS with EADSA[a,b,c,d]	NIST SRM 1950 *Free* sterols LC–MS with EADSA[h]	NIST SRM 1950 *Free* or **Total** sterols LC–MS[i,j]	Cooper Inst[k], Dallas Heart[l], **Total** sterols LC–MS	*Free* or **Total** sterols LC–MS[m,n,o,p,q,r,s,t,u,v]	*Free* or **T** sterols GC–MS[c,w]
	39.40 ± 3.95[d]		–	–	–	

Values for free sterols are given in *italics*, total sterols (free + esterified with fatty acids) in **bold**. – Signifies not measured. SE = standard error, SD = standard deviation.

[a] From Griffiths et al. [93],*n* = 84, mean ± SE.
[b] From Crick et al. [32], pool of 8 adults, mean ± SE, *n* = 5 measurements.
[c] From Iuliano et al. [34], *n* = 18, coronary heart disease patients, mean ± SD.
[d] From Theofilopoulos et al. [25], *n* = 50, mean ± SE.
[e] In EADSA analysis 24S,25-epoxycholesterol isomerises to 24-oxocholesterol.
[f] Total 24S,25-epoxide given including products of EADSA side-reactions.
[g] Authentic standard not available. Presumptive identification based on retention time, exact mass and MS^n spectra.
[h] From Griffiths et al. [60], *n* = 3 independent sample preparations, mean ± SD.
[i] From McDonald et al. [35].
[j] From Quehenberger et al. [69], *n* = 3, mean ± SE.
[k] From McDonald et al. [35], *n* = 200, mean ± RSE.
[l] From Stiles et al. [36], *n* = 3230, median (range).
[m] From Honda et al. [73], *n* = 19, mean ± SD.
[n] From van de Merbel [108], *n* = 129, mean ± SD.
[o] From Ikegami et al. [109], *n* = 36, mean ± SE.
[p] From Burkard et al. [71], *n* = 22, mean ± SD.
[q] From Jiang et al. [77], *n* = 89, mean(range).
[r] From Bandaru et al. [70], *n* = 12, mean ± SD.
[s] From Ikegami et al. [74], *n* = 113, mean ± SE.
[t] From Sidhu et al. [75], pooled plasma sample.
[u] From DeBarber et al. [79], *n* = 20, mean ± SD.
[v] From DeBarber et al. [80], *n* = 9, mean ± SD.
[w] From Bodin et al. [110], *n* = 125, mean ± SD.
[x] From Dzeletovic et al. [40], *n* = 31, mean ± SD, free oxysterol levels re-calculated from% of unesterified oxysterols (*n* = 6).
[y] From Axelson and Sjövall [111], *n* = 20, median (upper quartile–lower quartile) for acids, *n* = 6 for hydroxysterols average (range).
[z] From Meaney et al. [39], *n* = 9, mean ± SE, blood taken from brachial artery, values not significantly different from the jugular vein except of 7α-hydroxy-3-oxocholest-4-enoic ac
blood. 7α-Hydroxycholest-4-en-3-one measured by LC–UV.
[A] From Axelson et al. [37], *n* = 11, mean ± SD.
[B] From Schött et al. [65], representative plasma sample, mean ± SD, *n* = 6 measurements.
[C] From Ogundare et al. [95], *n* = 9, mean ± SD.
[D] From Theofilopoulos et al. [25], pool of 15CSF samples, mean ± SE, *n* = 3 measurements.
[E] From Theofilopoulos et al. [25], *n* = 18, mean ± SE.
[F] From Sidhu et al. [75], pooled CSF sample.
[G] From Leoni et al. [63], *n* = 24, median (25–75% confidence interval).
[H] From Saeed et al. [68], *n* = 8, mean ± SD.

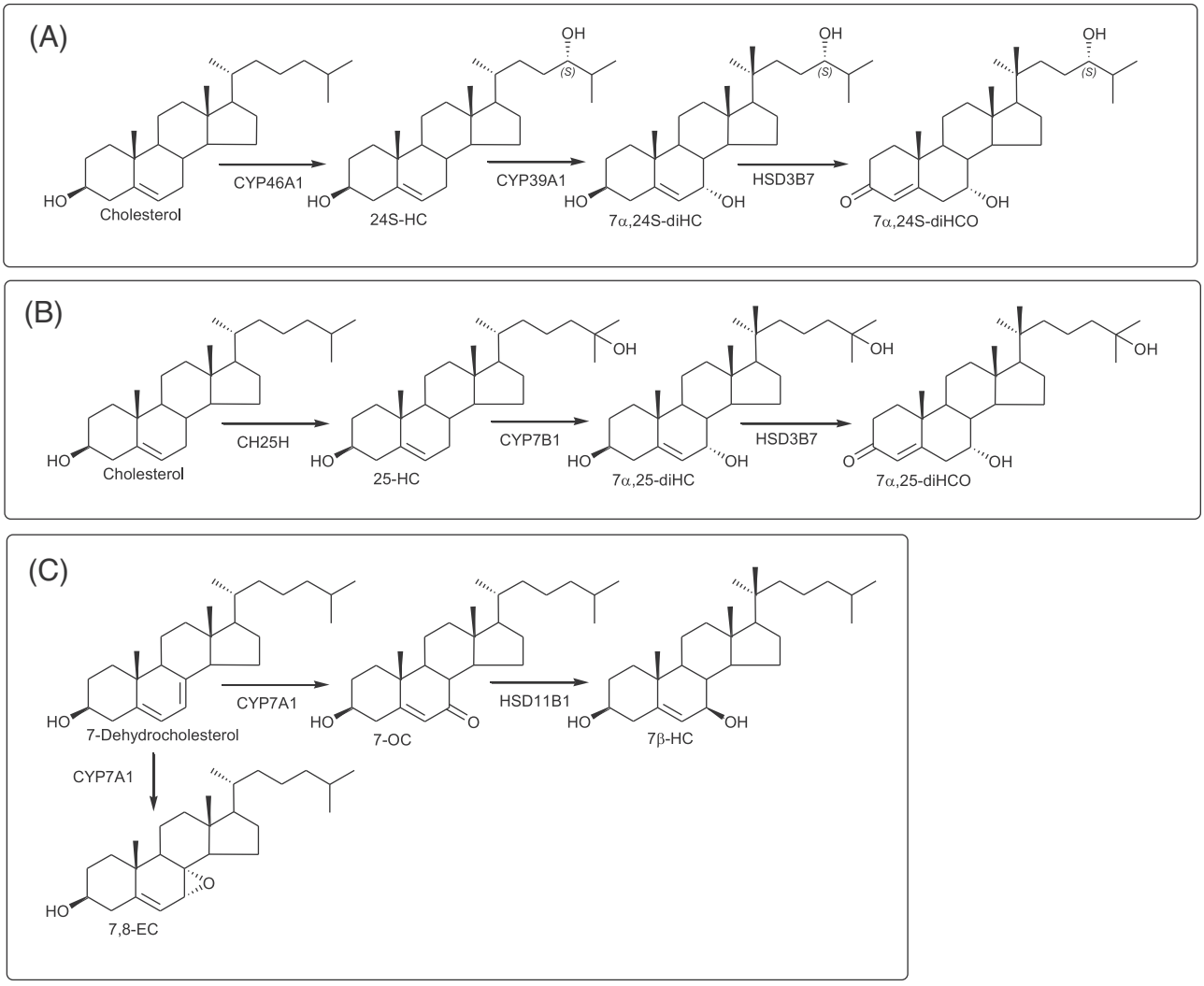

Fig. 2. Biosynthesis and metabolism of (A) 24S-HC, (B) 25-HC and (C) 7-OC and 7,8-EC.

pathway is active in brain [34,39]. All of the above metabolites of the acidic pathway of bile acid biosynthesis are observed in human plasma [32,34,36]. In plasma oxysterols are found predominately esterified to fatty acids, the ratio of esterified to free oxysterols is of the order of about 10:1 [40].

The major oxysterol exported from brain is 24S-HC, this is metabolised in liver to 7α,24S-dihydroxycholesterol (7α,24S-diHC) by CYP39A1 and further to 7α,24S-dihydroxycholest-4-en-3-one (7α,24S-diHCO) (Fig. 2A) [9]. Low levels of 7α,24S-diHC or 7α,24S-diHCO have been observed in mouse cerebrospinal fluid (CSF) (Table S1) [33], and we have recently identified 7α,24S-diHCO in mouse and porcine brain [Griffiths, unpublished data]. *Cyp39a1* is reported to be expressed in the somatosensory cortex of adult mouse brain [41]. The Allen Brain Atlas [41], http://www.brain-map.org/, is an excellent resource for gene expression data in brain. An alternative route of metabolism of 24S-HC is through conjugation with sulphuric and/or glucuronic acids and excretion in urine [42,43]. 25-HC is usually present at low levels in biological samples (Table 1, Table S1). As is the case with 7α-HC, it can be formed enzymatically and by autoxidation, and again there is always doubt whether the 25-HC observed is formed *in vivo* or *ex vivo* during sample handling or storage [2]. High levels of 25-HC are formed by macrophages following bacterial or viral challenge, in which case plasma levels are elevated [18–20]. 25-HC is metabolised by CYP7B1 to 7α,25-diHC and on to 7α,25-dihydroxycholest-4-en-3-one (7α,25-diHCO) by HSD3B7 (Fig. 2B).

Recently it was reported by Nelson et al. [44] and by Wu et al. [45] that (25R)26-HC (commonly referred to by the non-systematic name 27-hydroxycholesterol) is associated with ER-positive breast cancer. Earlier studies had established (25R)26-HC as a selective estrogen receptor modulator [46] and Nelson et al. showed that (25R)26-HC increased ER-dependent growth and LXR-dependent metastasis in a mouse model of breast cancer [44]. High grade tumours, tumour cells and tumour associated macrophages were found to express high levels of CYP27A1 [44]. Wu et al. [45] found that the (25R)26-HC content of normal breast tissue was increased in ER+ breast cancer patients compared to cancer-free controls, and in tumours (25R)26-HC was further elevated. Both Nelson et al. and Wu et al. found that increased (25R)26-HC concentrations correlated with reduced expression of CYP7B1, the enzyme responsible for metabolism of (25R)26-HC to 7α,(25R)26-diHC [44,45]. It is now timely for detailed studies of the oxysterolome in relation to cancer in general and breast cancer in particular.

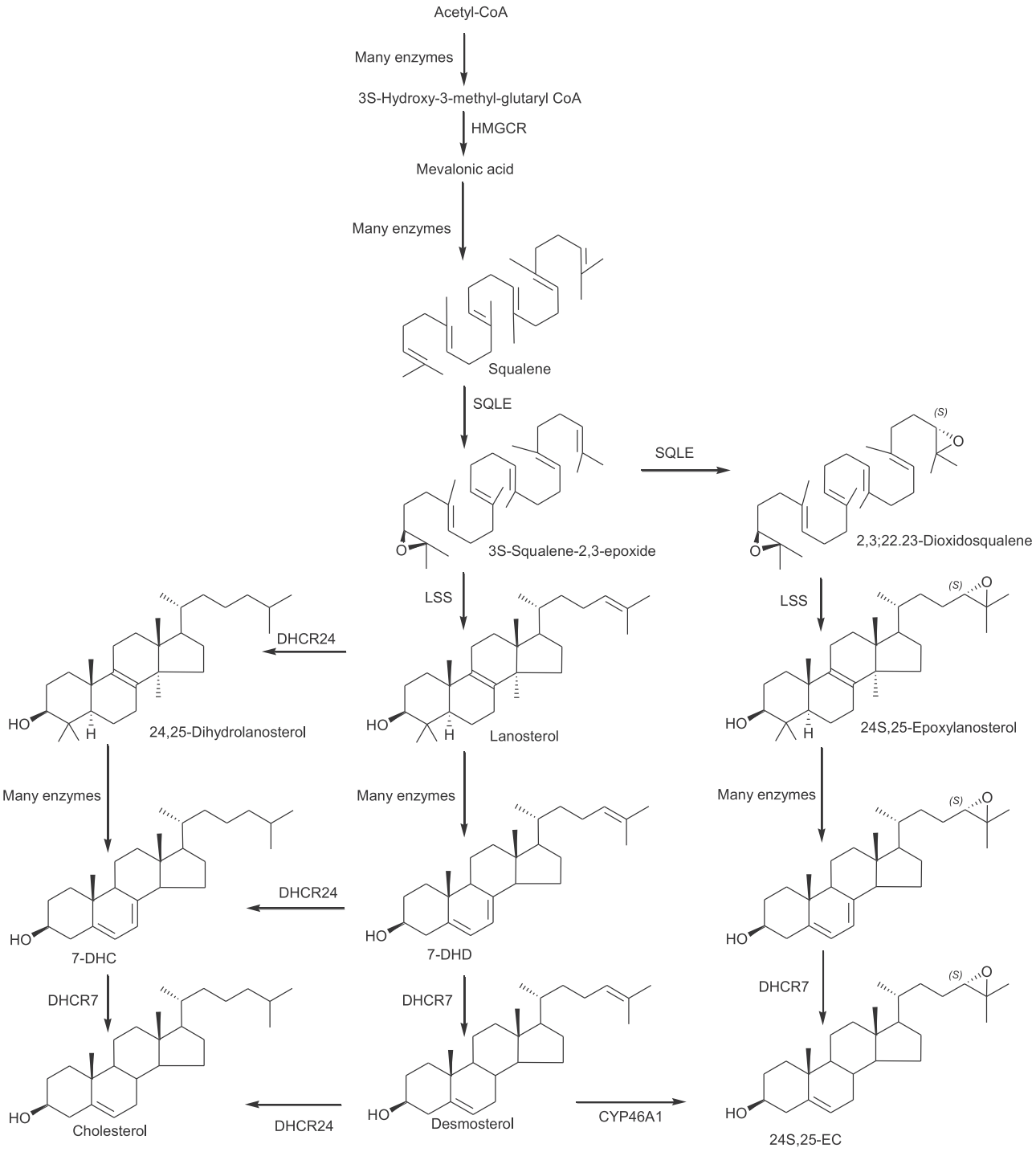

Fig. 3. Simplified version of the cholesterol biosynthesis pathway.

2.2. Enzymatic metabolism of cholesterol precursors

7-OC is a notorious autoxidation product [2], however, it has recently been shown that it can be formed from the cholesterol precursor 7-dehydrocholesterol (7-DHC) by the enzyme CYP7A1 [8]. 7-OC can be metabolised by HSD11B1 to 7β-hydroxycholesterol (7β-HC) [47]. 7β-HC can also be formed by autoxidation and is another oxysterol that can be formed endogenously and *ex vivo* (Fig. 2C).

24S,25-Epoxycholesterol (24S,25-EC) is an unusual oxysterol in that it is formed *via* a shunt of the cholesterol biosynthesis pathway, also known as the mevalonate pathway (Fig. 3) [48]. In the cholesterol synthesis pathway, prior to cyclisation to lanosterol, squalene is oxidised to 2,3S-oxidosqualene (squalene-2,3S-epoxide) by squalene epoxidase (SQLE). SQLE can introduce a second oxygen atom with the formation of 2,3S;22S,23-dioxidosqualene. 2,3S;22S,23-Dioxidosqualene is then cyclised by lanosterol synthase (LSS) to give 24S,25-epoxylanosterol. Lanosterol

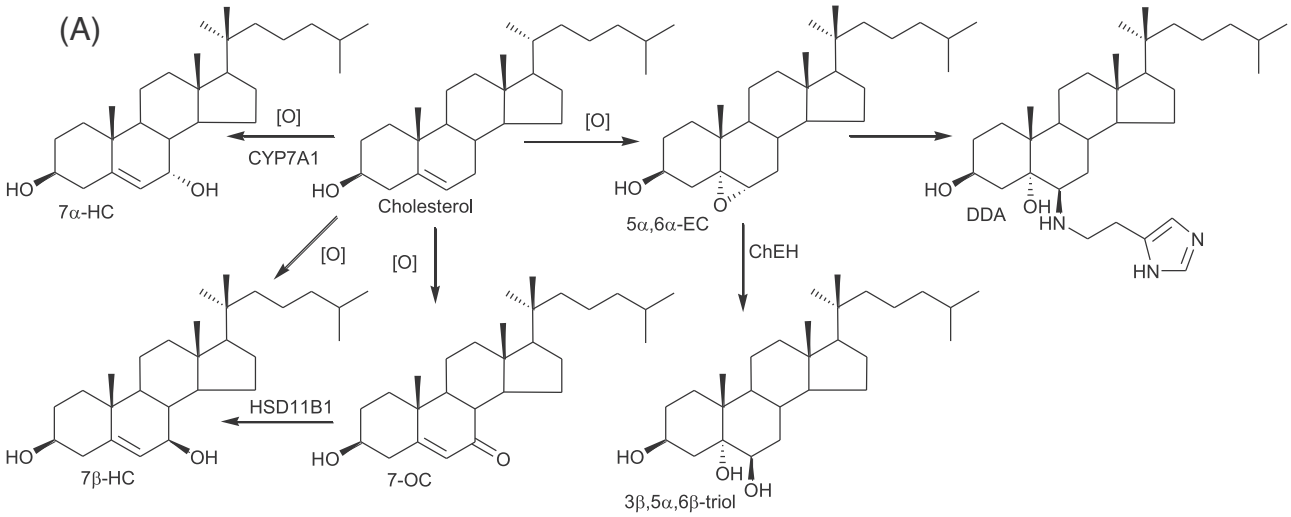

Fig. 4. Oxysterols formed by reactive oxygen species or autoxidation *ex vivo* .

itself is metabolised through multiple enzymes to cholesterol, while 24S,25-epoxylanosterol follows a parallel pathway, but with the exclusion of the enzyme 24-dehydrocholesterol reductase (DHCR24), to give 24S,25-EC as the ultimate product [49]. Recently, it has been reported that 24S,25-EC can also be formed from desmosterol by CYP46A1 [50]. 24S,25-EC is a ligand to the LXRα and LXRβ nuclear receptors [24,26,27]. It is a prominent oxysterol in developing and newborn brain, where levels of 24S-HC are low, (Table S1) [24,51,52] and has been ascribed neurogenerative properties in the ventral midbrain [24]. Evidence from the *Cyp7b1* knockout mouse where 24S,25-EC levels are elevated in brain indicates that 24S,25-EC is metabolised through 7α-hydroxylation by CYP7B1 [52]. Interestingly, levels of 24S,25-EC are reduced in the *Cyp46a1* knockout mouse both in brain and plasma [30], [Griffiths unpublished data], while elevated in the *CYP46A1* over expressing mouse [Griffiths unpublished data]. These observations may be explained by 24S,25-EC being formed from desmosterol by CYP46A1 or alternatively through differential regulation of the mevalonate pathway by negative-feedback by cholesterol whose synthesis along with that of desmosterol is reduced in brain of the *Cyp46a1* knockout mouse in response to its reduced metabolism [30,53]. 24S,25-EC is technically difficult to analyse as it is susceptible to acid catalysed hydration and methanolysis.

Besides being a substrate for CYP46A1, desmosterol is also a substrate for CYP7A1 and CYP27A1 with the formation of 7α-hydroxydesmosterol (7α-HD) and 26-hydroxydesmosterol (26-HD), respectively (Table S1) [29,54].

2.3. *Metabolism* via *reactive oxygen species*

7-OC, 7α-HC, 7β-HC, 5α,6α-epoxycholesterol (5α,6α-EC) and 5β,6β-EC have been proposed to be formed *via* ROS *in vivo* while cholestane-3β,5α,6β-triol (3β,5α,6β-triol) is formed from 5,6-EC by the enzyme cholesterol epoxide hydrolase (ChEH). As the *in vivo* ROS-formed sterols can also be formed from cholesterol *ex vivo* analysis is challenging [55]. Both 7-OC and 3β,5α,6β-triol have been linked to the lipid storage disease Niemann-Pick disease type C1 (NPC1) (Fig. 4). An alternative mechanism for metabolism of 5α,6α-EC is aminolysis with histamine to give dendrogenin A (DDA, Fig. 4). Low levels of endogenous DDA are found in brain and also in other tissues and plasma [56]. DDA is not detected in cancer cell lines and is present at lower concentrations in breast tumours than normal tissue suggesting that DDA may be deregulated during carcinogenesis [56,57].

3. Classical analytical methods

3.1. *Avoiding* ex vivo *autoxidation*

Whether the ultimate analytical method is gas chromatography–mass spectrometry (GC–MS) or liquid chromatography (LC)–MS precautions need to be taken to minimise *ex vivo* autoxidation. For example, for plasma or serum analysis collection of blood into EDTA containing vacutainers is preferred, EDTA chelates Fe^{2+} and thereby prevents Fenton chemistry and the generation of free radicals. Following centrifugation to prepare *e.g.* plasma/serum the antioxidant butylated hydroxytoluene (BHT) is often added. An alternative is to add the peroxide reducing agent triphenylphosphine (TPP) in addition to BHT [58]. To minimise oxidation from atmospheric oxygen sample preparation steps can be performed under Ar. The precautions taken in the authors laboratory to minimise *ex vivo* autoxidation are to separate sterols from oxysterols in a first step of sample preparation, thereby minimising cholesterol and 7-DHC *ex vivo* autoxidation artefacts at source, and to perform all drying steps under vacuum. It may also be beneficial to evaluate sample preparation protocols by adding exogenous [$^2H_7$]cholesterol and [$^2H_7$]7-DHC to the sample matrix and following the formation of heavy-isotope labelled oxysterols during sample preparation [58,59].

3.2. *Gas chromatography–mass spectrometry (GC–MS)*

The classical method for oxysterol analysis is by GC–MS. The limitation of this method is that it is not applicable to oxysterol conjugates, requiring hydrolysis of fatty acid esters, solvolysis of sulphates, and hydrolysis of glucuronic acid acetals. Never-theless, GC–MS provides the “gold standard” method for oxysterol analysis [60]. The “gold standard” method originates from studies in the laboratory of Björkhem and Diczfalusy and is described in the classic publication by Dzeletovic et al. [40]. This methodology has been adopted by many groups worldwide [61–65]. In brief, the method for plasma analysis is as follows. Blood is collected in EDTA containing vacutainers, and following centrifugation to prepare plasma, antioxidant BHT is added as are deuterated internal standards. Alkaline hydrolysis is performed under argon using 0.35 M ethanolic KOH for 2 h at room temperature. The solution is neutralised with phosphoric acid and sterols extracted into chloroform. Solvent is evaporated and the residue dissolved in toluene. Oxysterols are then separated from cholesterol on a silica

cartridge, eluted and dried down and derivatised to trimethylsilyl (TMS) ethers with pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1, v/v/v) at 60 °C for 30 min (Fig. 5A). The product is dried down and re-dissolved in hexane for injection onto GC–MS. Analysis is usually by selected ion monitoring (SIM) on a single quadrupole MS. This method with minor modifications is also applicable to analysis of CSF [63], brain tissue [66] and cells [67]. Sensitivity is of the order of 1 ng/mL from 0.5 mL of CSF [63] and <0.5 ng/mL from 0.5 mL of serum [65].

Björkhem and colleagues have modified their GC–MS method so that it is also applicable to cholestenoic acids, specifically 7αH,3O-CA in CSF [68]. Cholestenoic acids are extracted from CSF into ether following acidification with 0.1 M HCl. After washing of the ether phase with water until neutral the ether is evaporated and the cholestenoic acids methylated with TMS-diazomethane in hexane in a solution of methanol and toluene (Fig. 5B). After 5 min the solvent and excess reagent is removed by evaporation. The methylated acids are converted to TMS ethers as above and analysed by GC–MS. The method is sufficiently sensitive to detect ng/mL concentrations of 7αH,3O-CA from 1 mL of CSF [68]. An important consideration for sterols with a 3-oxo-4-en-7-ol group is the lability of the hydroxy group both in acid and base solution. This precludes the use of an alkaline hydrolysis step and requires careful use of any acids. To correct for any loses by dehydration Saeed et al. [68] included an isotope labelled version of 7αH,3O-CA during sample preparation.

4. Newer analytical methods

4.1. Liquid chromatography–mass spectrometry (LC–MS) without derivatisation

McDonald, Russell and co-workers have developed a high throughput LC–MS method for oxysterol analysis [35,69] and most impressively implemented the method in the analysis of 3230 serum samples from a clinically well-defined cohort, the Dallas Heart Study (Table 1) [36]. Their method is as follows. In brief, sterols and oxysterols are extracted into dichloromethane/methanol (1/1, v/v) containing BHT and deuterated internal standards. Following centrifugation the supernatant is hydrolysed with KOH in dichloromethane/methanol (1/1, v/v) for 1.5 h at 35 °C. PBS is added to give a two phase system and the organic phase retained. Solvent is evaporated and the residue re-dissolved in hexane and sterols and oxysterols separated from other lipids on an aminopropyl cartridge. The sterol and oxysterol fraction is reconstituted in 90% methanol for injection on a reversed phase LC column. McDonald and Russell use a Poroshell 120 SB-C_{18} HPLC column (150 × 2.1 mm, 2.7 μM particle size) and an acetonitrile/propan-2-ol/5% ammonium acetate gradient. Detection is by electrospray ionisation (ESI) tandem mass spectrometry (MS/MS) exploiting multiple reaction monitoring (MRM) [35]. The MRM transitions utilised are $[M+NH_4]^+ \rightarrow [MH - nH_2O]^+$ or $[M+H]^+ \rightarrow [MH\text{-}nH_2O]^+$. The sensitivity of the method allows the detection of 1 ng/mL oxysterol from 200 μL of plasma. McDonald and Russell using this method analysed a pooled plasma sample from NIST representative of the population of the USA (NIST Standard Reference Material (SRM) 1950) [35,69] and reported the concentrations of 10 oxysterols (Table 1). Using this method they went on to analyse 3230 samples from the Dallas Heart Study. The method is applicable to >60 sterols and vitamins D metabolites, and they routinely quantified 29 sterols including 15 oxysterols in serum [36]. There are numerous other LC–MS protocols for oxysterol analysis [70–72], but none are as extensive as that developed by McDonald and Russell or have been exploited on such a high numbers of samples [36].

4.2. 2 LC–MS with derivatisation

Although the LC–MS method described above can be used on a high throughput format, 50 samples could be extracted in a single day [35], it relies on chromatographic separation of isomeric oxysterols and the availability of authentic standards. This is a consequence of $[M+NH_4]^+$ or $[M+H]^+$ ions of isomeric oxysterols giving a similar MS/MS spectrum. This is evident in the excellent library of MS/MS spectra found on the Lipid Maps website http://www.lipidmaps.org/data/standards/. In addition, oxysterols are neither basic or acidic, hence do not readily form $[M+H]^+$, $[M+NH_4]^+$ or $[M-H]^-$ ions and do not give strong signals in ESI, atmospheric pressure chemical ionisation (APCI) or other desorption ionisation methods. In an effort to improve signal, derivatisation methods have been designed which in some cases also provide added structural information.

4.2.1. Derivatisation to picolinyl esters

Honda et al. [73] have developed a sensitive LC–MS/MS method exploiting derivatisation of oxysterols to picolinyl esters which is applicable to 5 μL of serum (Fig. 5C). Alkaline hydrolysis (1 N KOH at 37 °C, 1 h) is carried out after the addition of BHT and isotope labelled standards, then oxysterols are extracted into *n*-hexane. To the dried extract, freshly prepared derivatisation mixture of 2-methyl-6-nitrobenzoic anhydride (1 mg), 4-dimethylaminopyridine (3 mg), picolinic acid (8 mg), pyridine (150 μL) and triethylamine (20 μL) is added. After heating for 60 min at 80 °C, the derivatised oxysterols are extracted into *n*-hexane and separated from insoluble material by centrifugation. The supernatant is dried down and reconstituted into acetonitrile for LC-ESI–MS/MS analysis using a Hypersil Gold (150 × 2.1 mm, 3 μm particle size) reversed phase column and an acetonitrile/methanol/0.1% acetic acid gradient. Hydroxycholesterols tend to give dipicolinates and $[M+Na]^+$ ions in ESI. When MS/MS is performed the dominant fragment ions are $[M+Na\text{-}123]^+$ and *m*/*z* 146 which correspond to the loss of picolinic acid from the $[M+Na]^+$ ion, and to sodiated picolinic acid, respectively. These fragmentations can be exploited for MRM analysis. Honda et al. [73] were able to detect oxysterols at a level of 2 ng/mL from only 5 μL of serum. Using this protocol Honda et al. measured the concentration of seven oxysterols in human serum. Ikegami et al. [74] have subsequently exploited their method in the investigation of a large cohort of patients with hepatitis C virus infection ($n = 55$) and healthy controls ($n = 113$). They determined the serum concentration of 6 oxysterols and found that the levels of 7α-HC, 4β-hydroxycholesterol (4β-HC) and 25-HC were elevated in patient samples. The concentrations of these oxysterols fell after treatment with anti-viral therapy. In contrast the concentrations of 24S-HC, 26-HC and 7α-HCO did not vary between patient and control samples.

4.2.2. Derivatisation to nicotinyl esters

Nicotinic acid (pyridine-3-carboxylic acid) has a very similar structure to picolinic acid (pyridine-2-carboxylic acid) and has been used as a derivatisation reagent by Sidhu et al. to measure free 24S-HC in plasma and CSF (Fig. 5D) [75]. They found that hydroxycholesterols give double derivatives which ionise as $[M+H]^+$ and $[M+2H]^{2+}$ ions in ESI. They exploited the $307.2^{2+} \rightarrow 124.0^+$ MRM transition corresponding to $[M+2H]^{2+}$ fragmenting to protonated nicotinic acid. Sidhu et al. suggest that free 24S-HC in CSF is adsorbed by polypropylene surfaces based on an experiment in which they transferred a CSF sample to five consecutive polypropylene tubes and achieved a recovery of only 45%. To avoid this they suggest collecting CSF in tubes preloaded with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). Their method for sample preparation is as follows. Plasma (50 μL) with added internal standards is diluted with 50 mM ammonium acetate, 1%

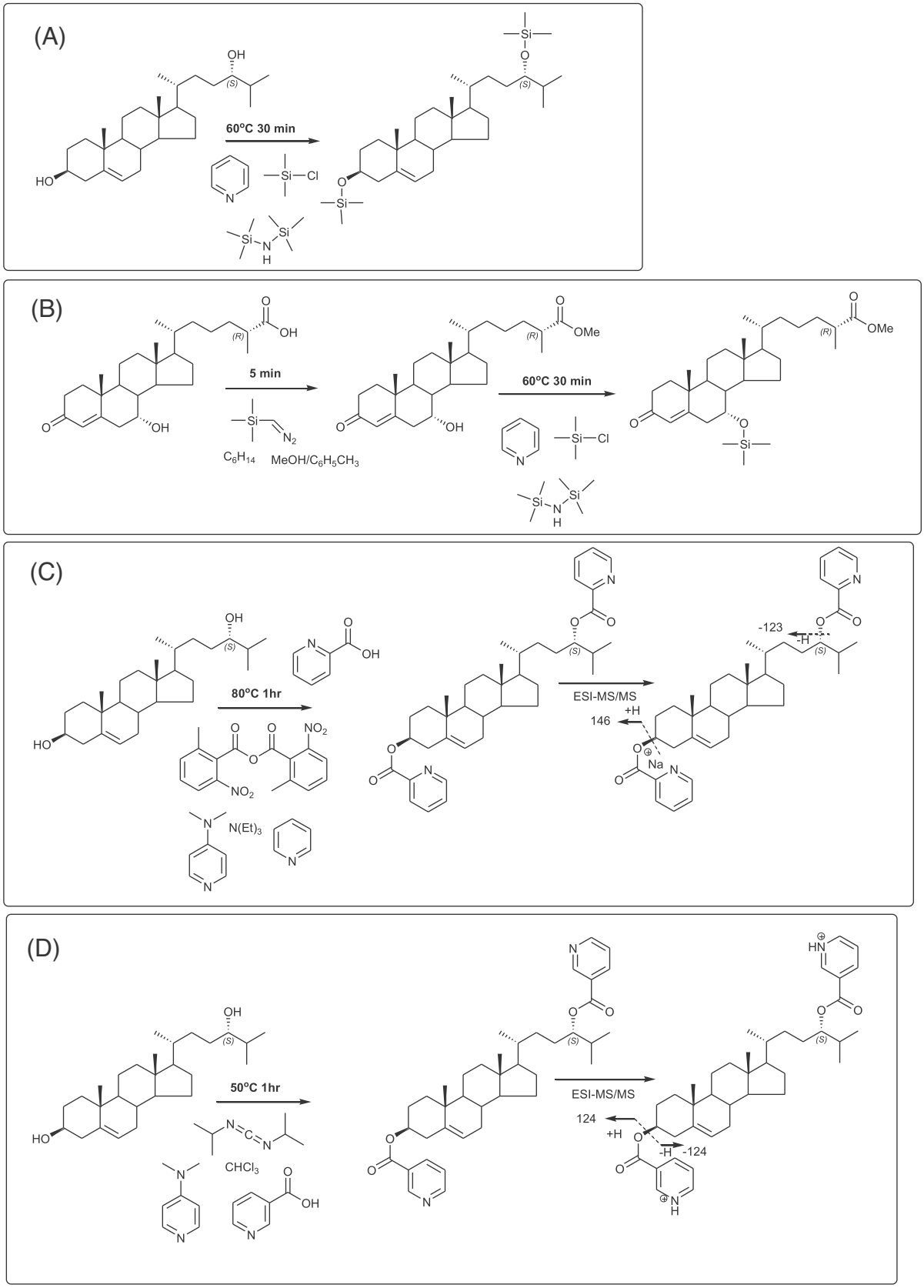

Fig. 5. Derivatisation of oxysterols or cholestenoic acids to (A) TMS ethers [40], (B) TMS ether methyl esters [68], (C) *bis* picolinyl esters [73], (D) *bis* nicotinyl esters [75], (E) *N,N*-dimethylglycyl esters [76], (F) *O*- (3-trimethylammonium-propyl) oximes [79], (G) Girard hydrazones. Fragment ions formed by MS/MS are indicated. See Fig. S1 for fragmentation mechanisms suggested by Murphy [107] for GP-derivatives.

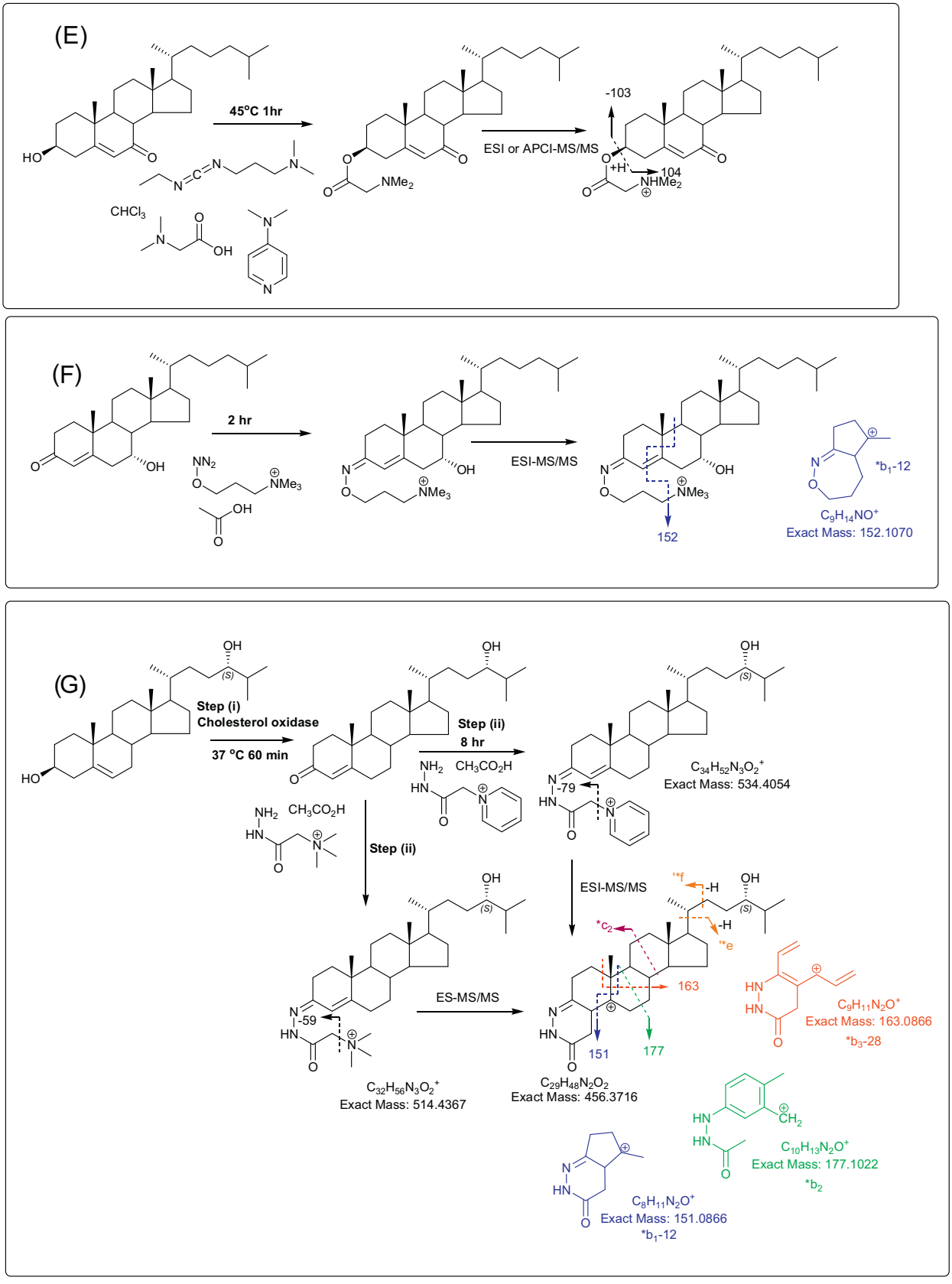

Fig. 5. (Continued)

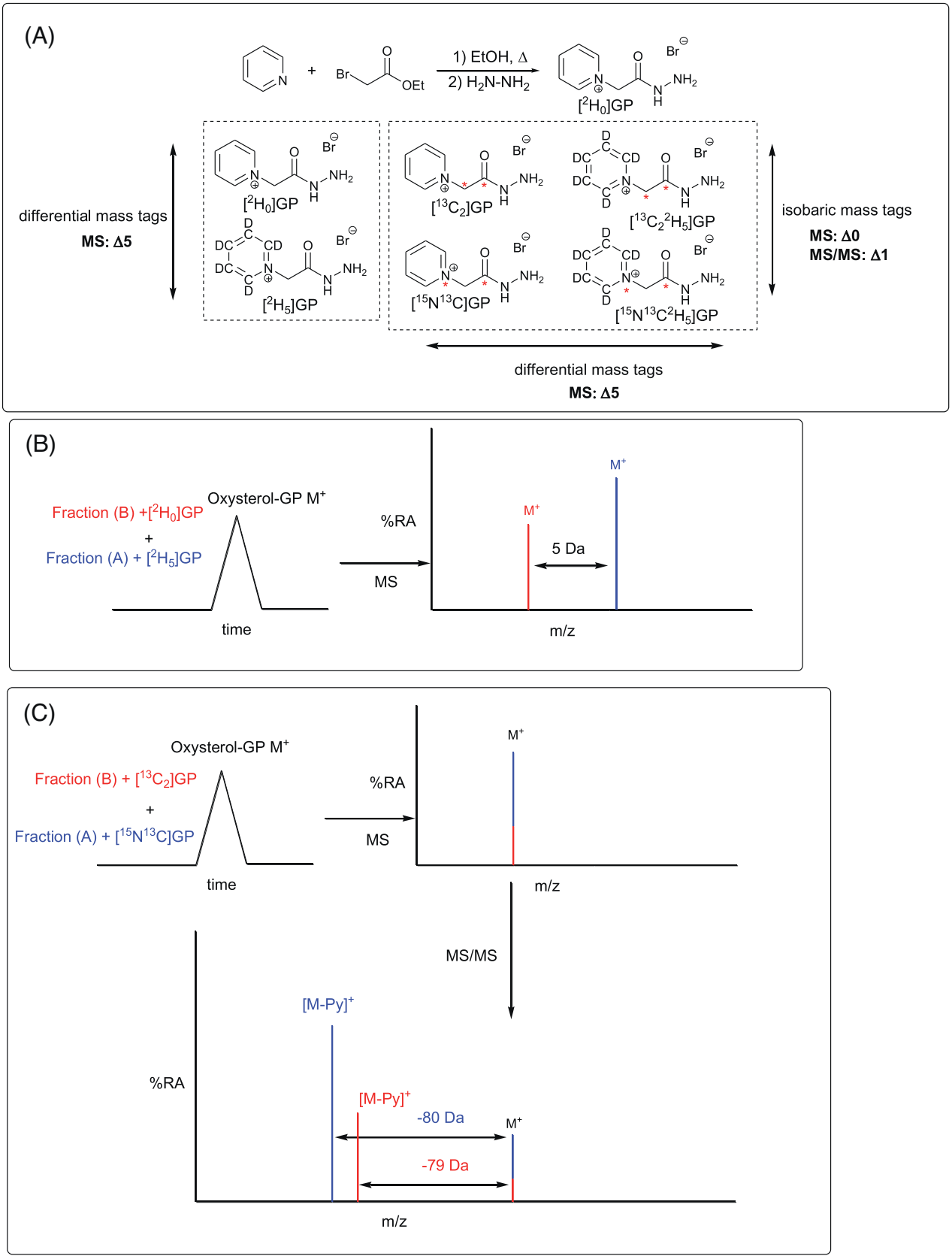

Fig. 6. (A) Synthesis of the GP reagent and different isotope labelled versions of the GP reagent (* = ^{15}N or ^{13}C) [32]. Schematic illustrating application of (B) differential mass tags and (C) isobaric mass tags. Modified from Crick et al. [32].

formic acid buffer and oxysterols extracted into methyl-*tert*-butylether (MTBE). Likewise for CSF, internal standards are added to CSF (200 μL) and oxysterols extracted into MTBE. The MTBE is evaporated and a derivatisation mixture consisting of 63 mg *N,N*-diisopropylcarbodiimide, 62 mg nicotinic acid, and 61 mg of 4-(dimethylamino) pyridine in chloroform added. After heating at 50 °C for 1 h, chloroform is evaporated and the residue re-dissolved in methanol for LC-ESI–MS/MS analysis. Sidhu et al. used a trap column (Phenomenex C_{18}, 4 × 3 mm) prior to an analytical column (Eclipse XDB-C_{18}, 3 × 100 mm, 3.5 μm particle size), both reversed phase, and a water/acetonitrile/methanol/0.1% formic acid gradient [75]. From 50 μL of plasma and 200 μL of CSF they were able to detect and quantify 24S-HC at 1 ng/mL and 0.025 ng/mL concentrations, respectively. They indicate that they have analysed over 1700 samples using this method, but in their 2015 publication just gave data for three patient samples, one pooled plasma sample and one pooled CSF sample [75]. The pooled plasma sample had a 24S-HC concentration of 8.64 ng/mL while the pooled CSF concentration was 0.244 ng/mL. These values are in moderate agreement with earlier data for free 24S-HC determined by others, see Table 1. Very interestingly, when CSF was analysed from patients suffering from NPC1 disease, a lysosomal storage disorder, who were administered HP-β-CD intracerebroventricularly as part of a clinical trial in NPC1 patients the CSF level of 24S-HC increased 20–50 fold [75].

4.2.3. Derivatisation to N,N-*dimethylglycine esters*

Jiang and Han have also utilised derivatisation to improve the LC–MS/MS analysis of oxysterols (Fig. 5E) [76,77]. They exploited derivatisation to *N,N*-dimethylglycine esters and APCI. In brief, oxysterols are extracted from plasma (50 μL) into methanol containing internal standards and after centrifugation to remove proteins the extract is dried down. The derivatisation mixture (20 μL) of 0.5 M dimethylglycine, 2 M 4-dimethylaminopyridine in chloroform and 1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in chloroform is added to the extract and heated for 1 h at 45 °C, then quenched with methanol. The resulting mixture is dried down, re-dissolved in 40% methanol and analysed by LC–APCI–MS/MS using a reversed phase column (Betasil C_{18}, 100 × 2.1 mm, 5 μm particle size) with an acetonitrile/0.5% acetic acid/0.015% trichloroacetic acid gradient. $[M+H]^+$ ions are formed in APCI which fragment in MS/MS to $[M+H\text{-}103]^+$ and to *m/z* 104 corresponding to the loss of dimethylglycine and to protonated dimethylglycine, respectively. Jiang et al. [77] have used this method for the analysis of plasma from patients suffering from NPC1 disease. They used MRM analysis to quantify 7-OC and 3β,5α,6β-triol from control ($n = 89$), heterozygotes ($n = 45$) and homozygous NPC1 patients ($n = 109$). For 7-OC they found remarkably large differences in the three groups rising from 29 ng/mL in controls to 43.8 ng/mL in heterozygotes to 229 ng/mL in NPC1 patients. 3β,5α,6β-Triol was similarly increased in NPC1 patients. Although 7-OC can be formed by *ex vivo* autoxidation of cholesterol and no attempt was made to remove cholesterol during sample workup, the difference in

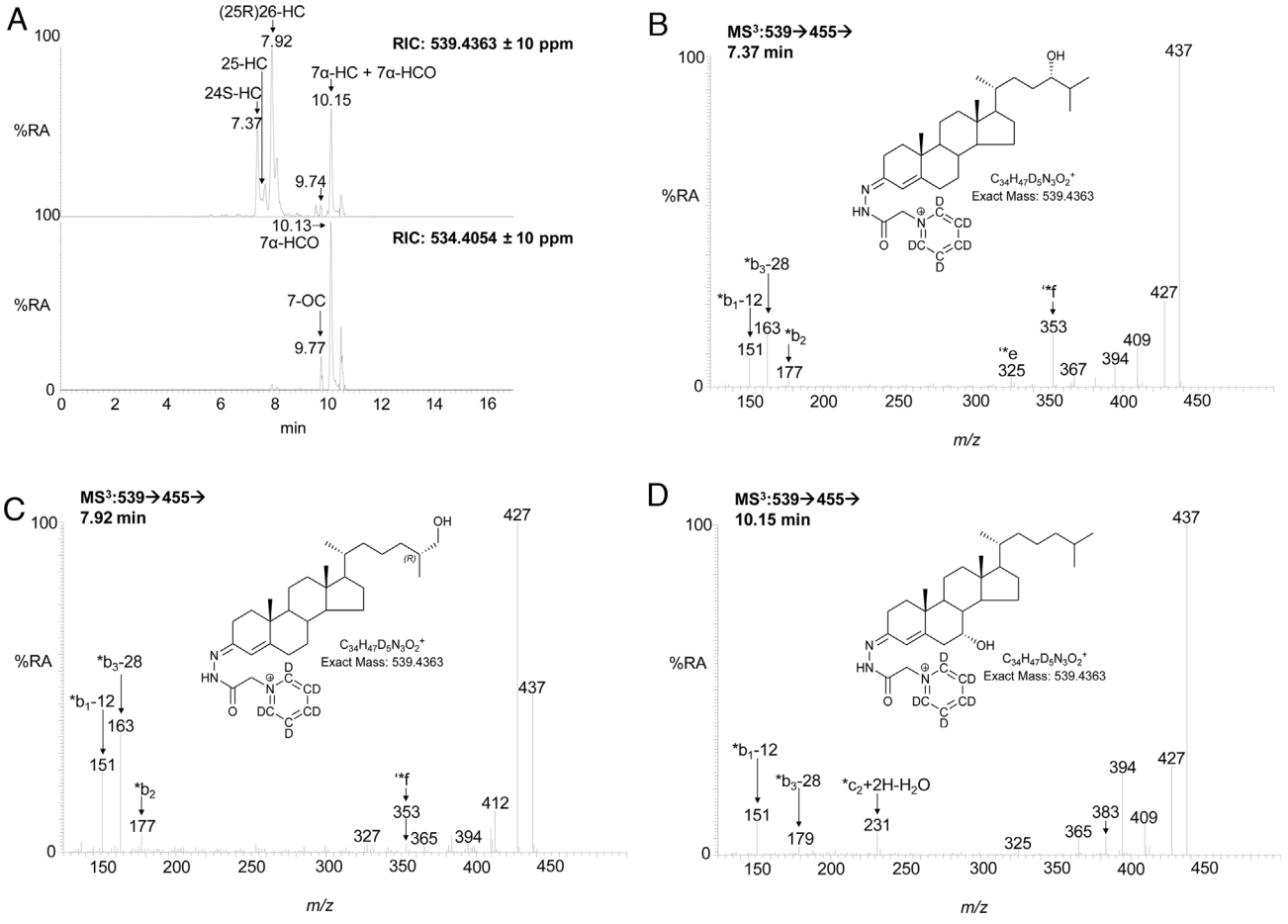

Fig. 7. LC–MS(MS^3) analysis of typical human plasma sample using EADSA technology [32]. (A) Upper panel, fraction A, with cholesterol oxidase. Lower panel, fraction B, without cholesterol oxidase. MS^3 fragmentation, $[M]^+ \rightarrow [M\text{-}Py]^+ \rightarrow$, of peaks eluting at (B) 7.37 min, 24S-HC; (C) 7.92 min, (25R) 26-HC; and (D) 10.15 min, 7α-HC plus 7α-HCO. Data generated on an Orbitrap Elite. Py corresponds to pyridine. Fragmentation mechanisms suggested by Murphy [107] are shown in Supplementary Fig. S1.

concentration for both target analytes in NPC1 and controls is striking.

4.2.4. Derivatisation to oximes

A number of oxysterols posses a carbonyl group, *e.g.* 7α-HCO, 7α,12α-diHCO as does the acid 7αH,3O-CA. Carbonyl groups will react with hydroxylamine-based reagents to give oximes. This reaction has been exploited for decades in GC–MS and LC–MS studies of oxosteroids [78]. Recently, DeBarber et al. have utilised the *O*-(3-trimethylammonium-propyl) hydroxylamine reagent, also called quaternary amonoxy (QAO) reagent, to derivatise oxysterols possessing an oxo group [79,80] (Fig. 5F). They exploited this method for the diagnosis of patients with CTX. CTX is a disease resulting from sterol (25R)26-hydroxylase (CYP27A1) deficiency. Derivatisation is simple; QAO reagent (210 μg) is added in methanol containing 5% acetic acid to 4 μL of plasma or to a dried blood spot, after 2 h at room temperature the mixture is analysed by LC-ESI–MS/MS exploiting the MRM transition $[M]^+ \rightarrow 152^+$. The likely structure of the ion at *m/z* 152 is shown in Fig. 5F, based on fragmentation studies of other carbonyl reactive reagents [81]. DeBarber et al. use a reversed phase column (Luna C_8, 50 × 2.1 mm, 5 μm particle size) and a water/acetonitrile/0.1% formic acid gradient in a 6 min run. Sensitivity was 1 pg on-column, and for 4 μL of plasma the lower limit of quantification (LLOQ) was 20 ng/mL. Using this methodology DeBarber et al. found 7α,12α-diHCO to be diagnostic of CTX being elevated by a factor of over 3000 in adults and by a factor of 10 in newborns.

4.2.5. Derivatisation to Girard hydrazones

Perhaps the most popular derivatisation method for LC–MS analysis of oxysterols is derivatisation to Girard hydrazones [82]. This method has been extensively used by Roberg–Larsen and Wilson in Norway [83–85], Shevchenko and colleagues in Germany [86], DeBarber and co-workers in Canada [87,88], and Soroosh et al. in the USA [89] and our own group in the UK [29,30,32,33,51,52,90–96].

The Girard reagents have been used for decades in steroid analysis [82]. They react with a carbonyl group of a steroid to give a Girard hydrazone (Fig. 5G, step ii). Girard hydrazones are positively charged on account of the presence of a quaternary amine group, and give enhance steroid solubility in water, a property widely exploited to aid steroid extraction from organic solvents [97]. The charged nature of the Girard hydrazones makes them very suitable for high sensitivity ESI–MS analysis [51,90,91,94,95,98]. Many oxysterols have a 3β-hydroxy-5-ene group which can be treated with cholesterol oxidase to give a 3-oxo-4-ene (Fig. 5G, step (i) [99], suitable for subsequent Girard derivatisation. Once the 3-oxo-4-ene group is in place derivatisation with the Girard reagents is

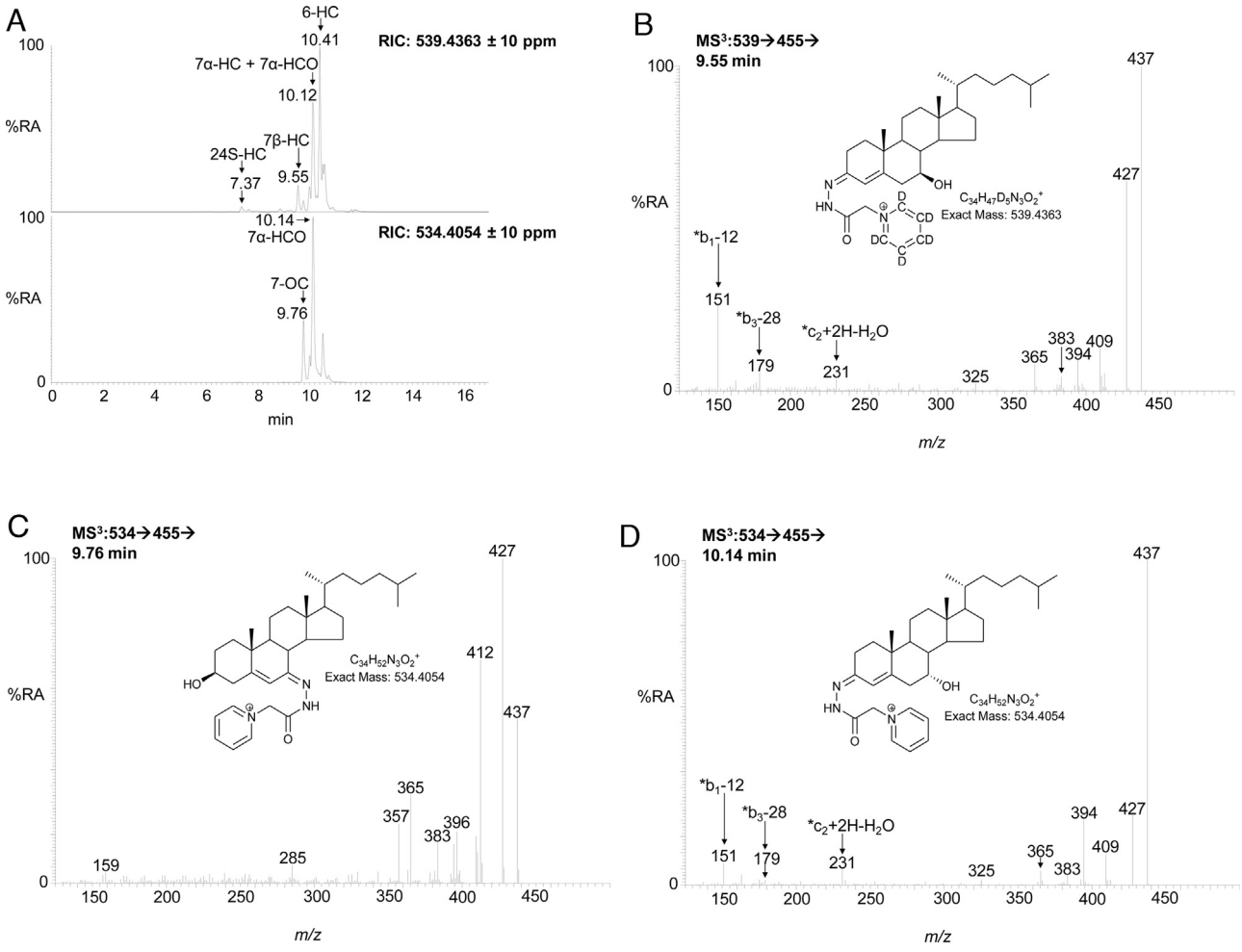

Fig. 8. LC–MS(MS^n) analysis using EADSA technology of a typical CTX patient plasma sample [32]. (A) Upper panel, fraction A, with cholesterol oxidase. Lower panel, fraction B, without cholesterol oxidase. MS^3 fragmentation, $[M]^+ \rightarrow [M\text{-}Py]^+ \rightarrow$, of peaks eluting at (B) 9.55 min, 7β-HC; (C) 9.76 min, 7-OC; and (D) 10.14 min, 7α-HCO. Data generated on an Orbitrap Elite. For comparison to normal plasma, levels of 24S-HC are similar in Figs. 7 and 8. The MS^3 spectrum of the peak eluting at 10.41 min, 6-HC, is shown in Fig. S2.

rapid. Oxysterols with a 3β-hydroxy-5α-hydrogen are also oxidised to 3-oxo compounds but the 3-oxo products react with Girard reagents at a slower rate than the corresponding 3-oxo-4-ene compounds [97]. There are two types of Girard reagent generally employed; Girard T (GT) (2-hydrazinyl-2-oxoethyl)-trimethylazanium chloride salt and Girard P (GP) 1-(hydrazinocarbonylmethyl) pyridinium chloride or bromide salt. Our preference is for the GP reagent, although most other groups use the GT reagent. We have exploited enzymatic oxidation and GP derivatisation, in a methodology we call enzyme-assisted derivatisation for sterol analysis (EADSA) in the study of rodent brain, rodent and human CSF and plasma/serum and also cells and medium (Table 1, Table S1) [20,24,25,29,32,33,93–95].

Our preference is to extract oxysterols from tissue or fluids into ethanol to which isotope labelled standards are added, centrifuge, and dilute the supernatant to 70% ethanol with water. The solution is passed through a C_{18} solid phase extraction (SPE) column; oxysterols and more polar sterols/steroids pass through the column while cholesterol is retained. Oxysterols are then dried and re-dissolved in propan-2-ol then treated with cholesterol oxidase in KH_2PO_4 buffer. After 60 min at 37 °C the reaction is quenched with methanol and GP reagent added with acetic acid catalyst. After overnight incubation at room temperature oxysterols are separated from un-reacted reagent by a second SPE step, this time using an Oasis HLB column. Oxysterols are eluted in methanol.

Our current method of LC–MS analysis exploits reversed phase LC on a Hypersil Gold C_{18} column (50 × 2.1 mm, 1.9 μm particle size) with a methanol/acetonitrile/0.1% formic acid gradient and detection with a ion trap-Orbitrap hybrid high resolution mass spectrometer. Analyte identification is achieved by using exact mass, measured in the high resolution Orbitrap, and by multiple stage fragmentation (MS^n) in the ion trap. A significant advantage of GP derivatisation is that the derivatised oxysterols give distinct fragmentation patterns (Fig. 5G) allowing isomer differentiation. A further advantage is that the method is applicable to down-stream metabolites of oxysterols including cholestenoic and cholenoic acids. The website http://sterolanalysis.org.uk a useful resource displaying MS^3 spectra of GP derivatised sterols. The sensitivity of the method allows oxysterols to be detected at the ng/mL level from 100 μL of plasma and the 30 pg/mL level from 250 μL of CSF. It is also applicable to vitamins D analysis [100].

Roberg-Larsen et al. [83,84] have adopted a similar approach but using the GT reagent and exploiting a trap column, to remove excess derivatisation reagent, prior to a miniaturised analytical column (Ace C_{18}, 150 × 1 mm or Ace C_{18} 150 × 0.1 mm). They have used both micro-LC and nano-LC to improve sensitivity. Using their nano-LC-ESI–MS/MS method they were able to achieve fg detection limits and profile oxysterols in extracts of only 10,000 cells [84]. Ecdysteroids are polyhydroxylated sterols found in insects, and Shevchenko and co-workers have exploited GT derivatisation for the pg detection by LC-ESI–MS/MS of these molecules [86].

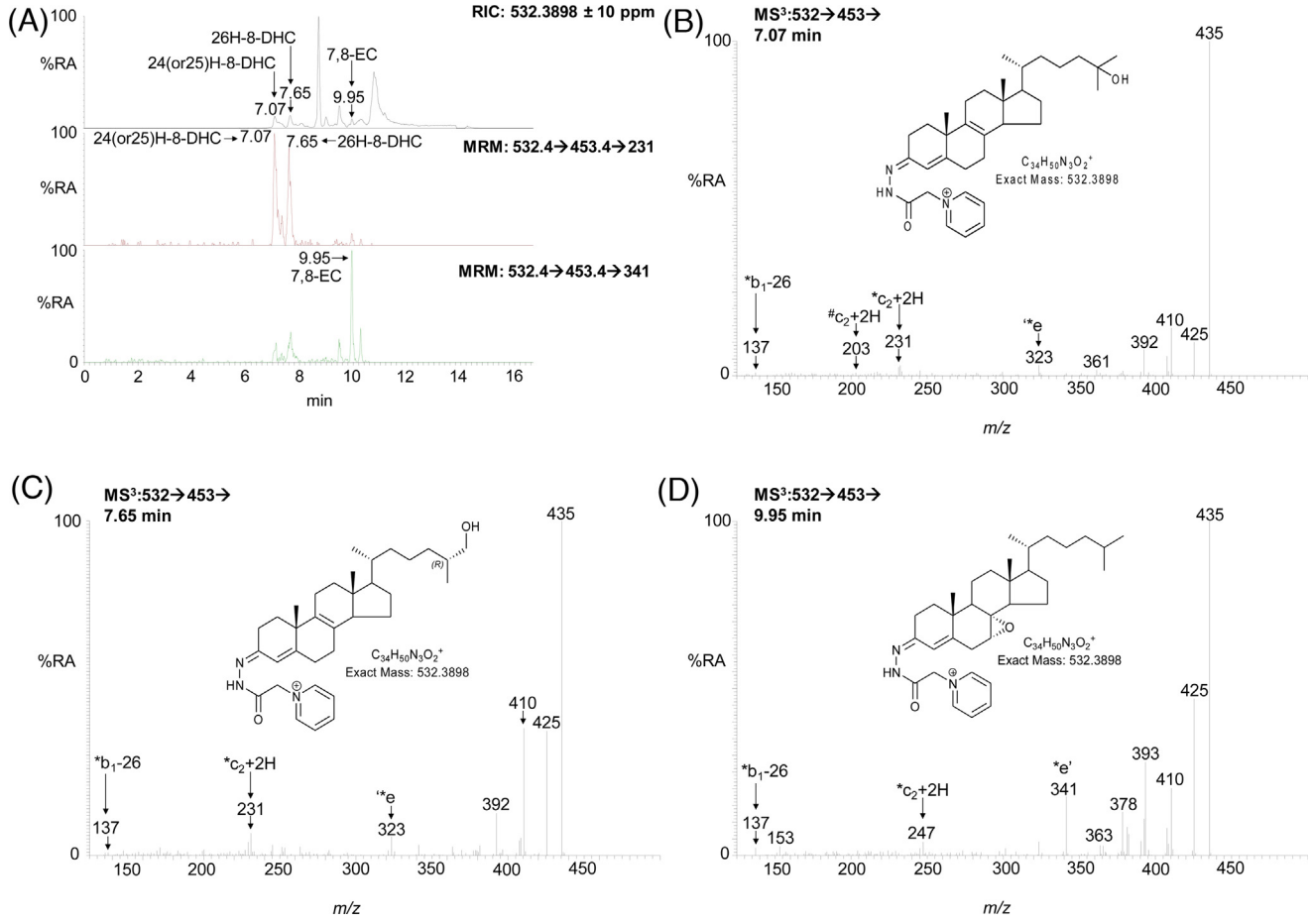

Fig. 9. LC–MS(MS^3) analysis using EADSA technology of a typical SLOS patient plasma sample [32,101]. (A) Upper panel, fraction A, with cholesterol oxidase. Central panel, reconstructed ion chromatograms (RIC) targeted on hydroxy-8-dehydrocholesterol (24H-8-DHC, 25H-8-DHC, 26H-8-DHC). Lower panel, RIC targeted on 7,8-EC. MS^3 fragmentation, $[M]^+ \rightarrow [M\text{-}Py]^+ \rightarrow$, of peaks eluting at (B) 7.07 min, 24H-8-DHC or 25H-8-DHC; (C) 7.65 min, 26H-8-DHC, (D) 9.95 min, 7,8-EC. Data generated on an Orbitrap Elite. Proposed structures of fragment ions are shown in Fig. S3.

4.3. New developments: Isotope coded derivatisation reagents

A disadvantage of the EADSA strategy as outlined above is that some oxysterols and cholestenoic acids naturally posses a 3-oxo-4-ene function *e.g.* 7α-HCO, 7αH,3O-CA; this makes them indistinguishable from equivalent molecules oxidised with cholesterol oxidase to contain this function *i.e.* 7α-HC, 3β,7α-diHCA. To solve this conundrum we divide the oxysterol fraction eluting from the first SPE column into (A) and (B) fractions. The (A) fraction is treated with cholesterol oxidase, while the (B) fraction is not. The (B) fraction will give information about molecules with a *natural* oxo group, the (A) fraction will give information about molecules *oxidised* to contain a 3-oxo group *and* those *naturally* containing a 3-oxo group. Deconvolution of (B) from (A) reveals the quantity of molecules *oxidised* to contain a 3-oxo group, *i.e.* those with a *natural* 3β-hydroxy group. This procedure requires the recording of two independent LC–MS runs with the obvious knock-on effects on sample throughput. To streamline analysis we have introduced isotope coded derivatisation reagents or quantitative charge-tags [32].

The GP reagent is straight forward to synthesise as are isotope labelled versions (Fig. 6A) [82]. For example, by reaction of [$^2H_5$] pyridine with ethyl bromoacetate and then hydrazine, [$^2H_5$]GP is synthesised [32]. In our EADSA methodology we now use [$^2H_5$]GP to derivatise (A) fractions and [$^2H_0$]GP to derivatise (B) fractions and then combine the two fractions immediately before LC–MS. Analytes in the (A) fraction are 5 Da heavier than their equivalents in the (B) fraction so easily distinguishable in a single LC–MS run (Fig. 6B). We now use the concept of differential mass tags in all our analysis (Fig. 7). The GP skeleton is also applicable to developing isobaric tags where two tags have the same nominal mass but fragment in MS/MS to give ions of different *m/z* . These fragment ions can then be used for relative quantification of (A) and (B) fractions (Fig. 6A and C). By generating a quadruplex it is possible to analyse two samples as both (A) and (B) fractions in a single LC–MS/MS run [32].

5. Applications of LC–MS for oxysterol analysis

5.1. Sterols in plasma from CTX patients

DeBarber et al. have used GP derivatisation without enzymatic oxidation to identify and quantify sterols with a 3-oxo group in plasma of CTX patients [87,88]. They scaled down the derivatisation method for 10 μL of plasma, omitting cholesterol removal and enzymatic oxidation and removing excess reagent *via* an HPLC trap column prior to the analytical column [88]. They developed both LC-ESI–MS/MS with MRM and LC-ESI–MS with MS^3 methods for

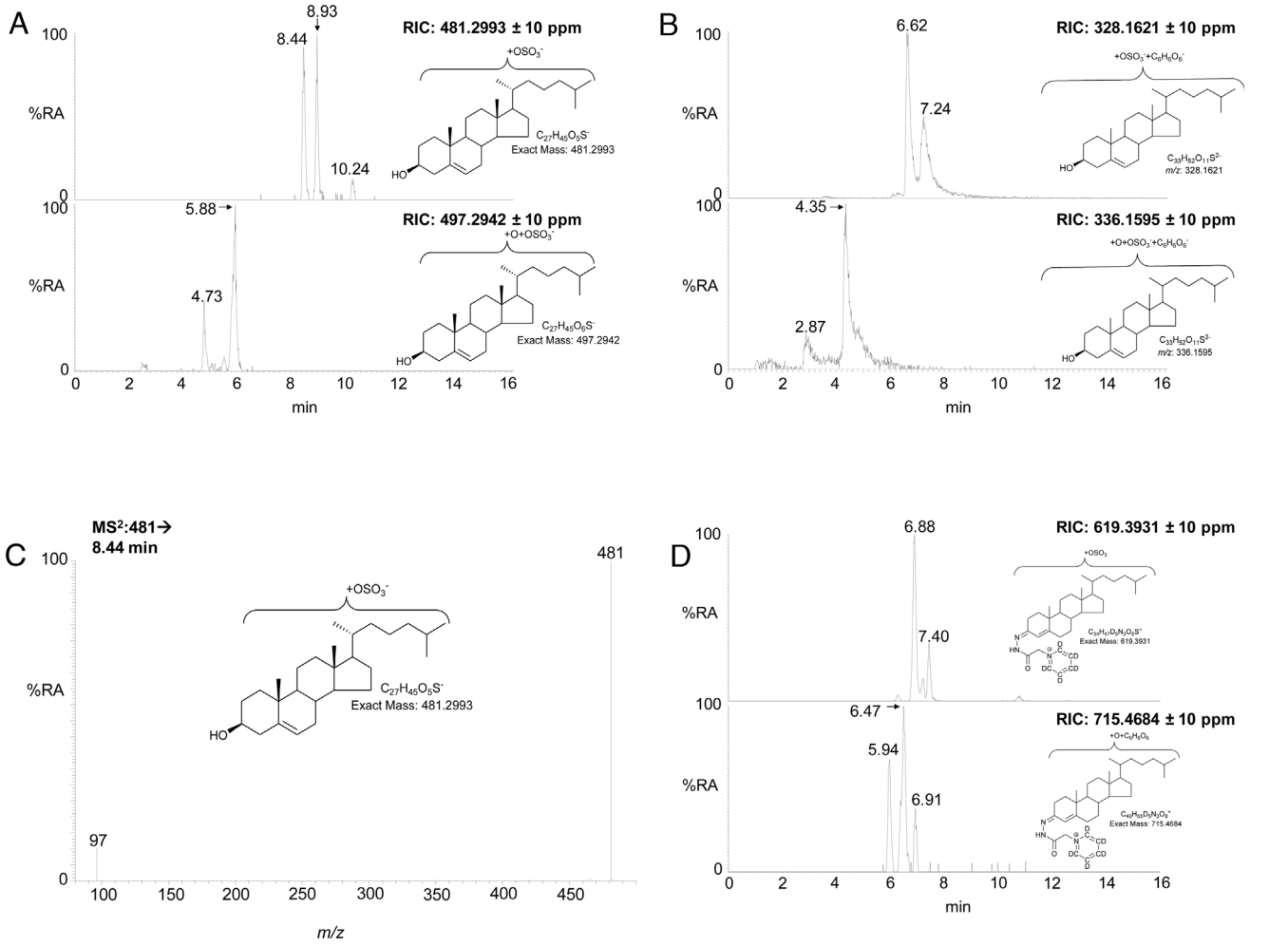

Fig. 10. LC–MS(MS^n) analysis of plasma from an infant suffering from oxysterol 7α-hydroxylase deficiency [32]. (A) RICs showing peaks corresponding to, upper panel, hydroxycholesterol sulphates and, lower panel, dihydroxycholesterol sulphates. (B) RICs showing peaks corresponding to, upper panel, monohydroxycholesterol sulphate glucuronides, and dihydroxycholesterol sulphate glucuronides, lower panel. (C) MS/MS spectrum of the peak eluting at 8.44 min. Data in (A)–(C) generated by LC-negative-ion-ESI on an Orbitrap Velos. (D) LC–MS analysis using EADSA technology of the infant plasma sample. Upper panel, RIC of hydroxycholesterol sulphates; lower panel hydroxycholesterol glucuronides. Data generated by positive-ion ESI on the Orbitrap XL. MS^2 spectra of peaks eluting at 6.88 and 5.94 min are shown in Fig. S4.

analysis of 7α-HCO, cholest-4-en-3-one and 5α-cholestan-3-one, and LC-ESI–MS^3 also for cholest-4,6-dien-3-one, 7α,12α-diHCO and 7α,12α-dihydroxy-5β-cholestan-3-one. Each of these metabolites is greatly elevated in CTX plasma compared to healthy individuals. We have found similar results when we have analysed CTX plasma, but by including the cholesterol oxidase step we could also show the almost complete absence of (25R)26-HC, 3β-HCA and 3β,7α-diHCA from CTX plasma (Fig. 8) [32,93].

5.2. Unusual oxysterols in plasma

Some patients with CTX and all patients with Smith–Lemli–Opitz syndrome (SLOS) have high levels of 7-DHC in plasma and tissues [101]. SLOS results from 7-dehydrocholesterol reductase (DHCR7) deficiency (see Fig. 3). Recently a new mechanism for the formation of 7-OC was described where 7-DHC is oxidised by CYP7A1 to 7-OC with cholesterol-7,8-epoxide (7,8-EC) as a side product (Fig. 2C) [8]. Using EADSA to profile plasma of SLOS patients we are able to identify 7,8-EC and confirm its absence in plasma from control individuals (Fig. 9) [101]. Further profiling of plasma from patients with SLOS allowed the identification of another unusual oxysterol, 26-hydroxy-8-dehydrocholesterol (26H-8-DHC) first reported in plasma by Wassif et al. [102], presumably formed from 8-DHC in a CYP27A1 catalysed reaction (Fig. 9) [Griffiths unpublished data]. Honda et al. [103] also identified 26H-8-DHC in liver mitochondrial incubations from a rat model of SLOS.

5.3. Oxysterol sulphates and glucuronides in plasma

At the beginning of this section we wrote that a limitation of GC–MS was its inability to detect oxysterols conjugated with glucuronic or sulphuric acids. In contrast, these oxysterols are readily detected *without* derivatisation by LC-ESI–MS/MS when operated in the negative ion mode [104]. This is illustrated by analysis of plasma from an infant suffering from oxysterol 7α-hydroxylase (CYP7B1) deficiency (Fig. 10) [12]. EADSA introduces the GP group at position 3, so conjugates at this location are not observed with the EADSA method. However, if sulphation or glucuronidation is elsewhere the conjugated oxysterol can be observed (Fig. 10). These modes of conjugation may be important in different disease states.

5.4. Oxysterols in CSF

The levels of oxysterols in CSF are low, however, the cholestenoic acids 3β-HCA, 3β,7α-diHCA and 7αH,3O-CA are far higher (Table 1) [25,68,95]. Using our EADSA methodology we not only identify these three acids in CSF, but also find peaks corresponding to down-stream acids (Fig. 11). In our earlier

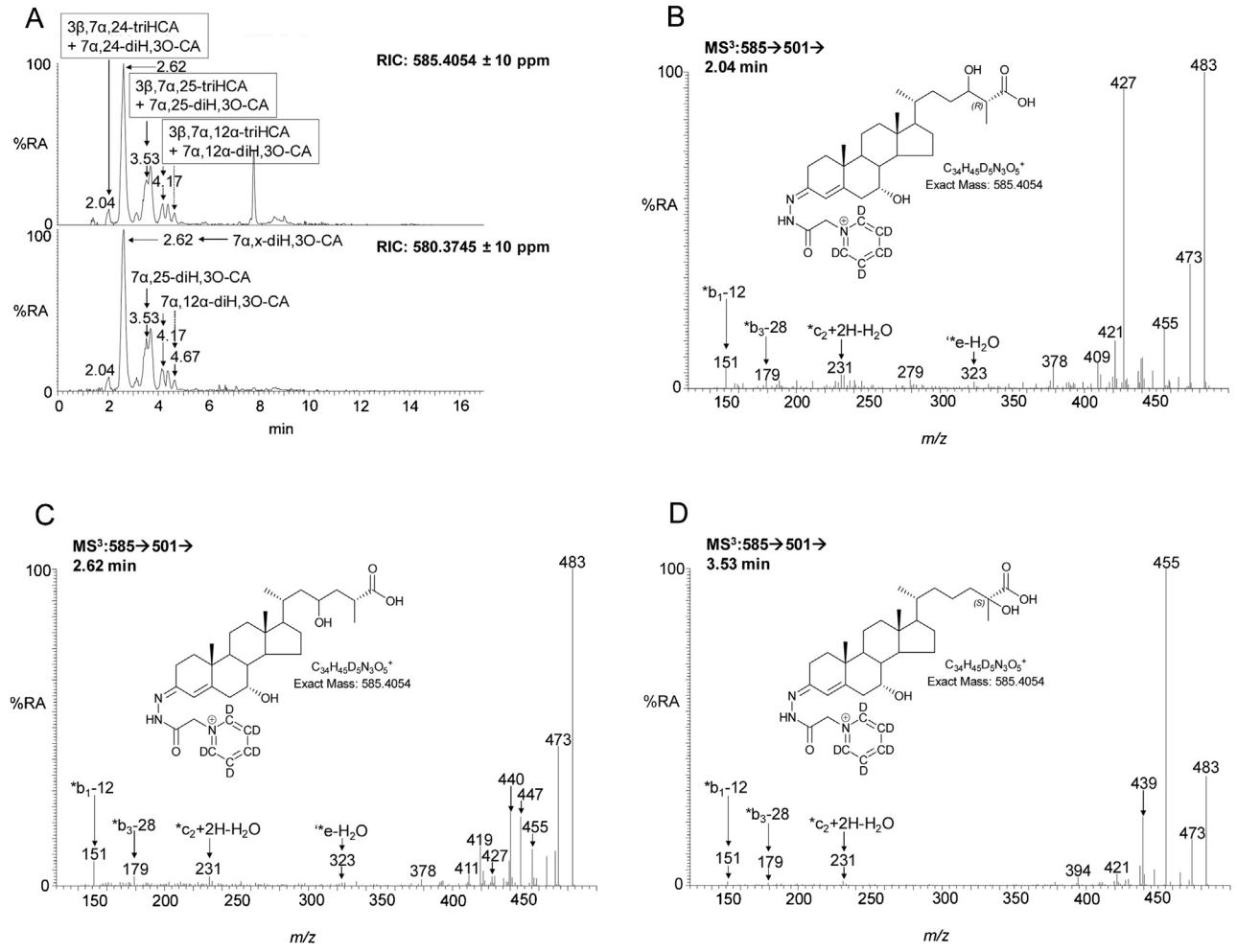

Fig. 11. LC–MS(MS^3) analysis of typical human CSF using EADSA technology [95]. (A) Upper panel, fraction A, with cholesterol oxidase. Lower panel, fraction B, without cholesterol oxidase. MS^3 fragmentation, $[M]^+ \rightarrow [M\text{-}Py]^+ \rightarrow$, of peaks eluting at (B) 2.04 min, 7α,24-diH,3O-CA; (C) 2.62 min, 7α,x-diH,3O-CA; and at (D) 3.53 min, 7α,25-diH,3O-CA. The MS^3 spectrum of the peak eluting at 4.17 min is shown in Fig. S5. Data generated on an Orbitrap Elite.

publications we tentatively identified the peaks eluting at 2.62 min and 3.53 min in the chromatograms shown in Fig. 11A to correspond to 7α,24S-dihydroxy-3-oxocholest-4-en-26-oic (7α,24-diH,3O-CA) and 7α,25-dihydroxy-3-oxocholest-4-en-26-oic acids (7α,25-diH,3O-CA) [25,95]. Avanti Polar Lipids have now synthesised these two molecules, and while identification of the peak eluting at 3.53 min as 7α,25-diH,3O-CA is correct, the peak eluting at 2.62 min is not 7α,24-diH,3O-CA. In fact, 7α,24-diH,3O-CA elutes a little earlier than the unknown peak, at 2.04 min. The MS^3 spectrum of the peak eluting at 2.62 min suggest that there is side-chain hydroxylation on the 7αH,3O-CA scaffold presumably at C-22 or C-23. Remaining peaks in the chromatograms shown in Fig. 11A are compatible, according to retention time and MS^3 spectra, with 7α,12α-diH,3O-CA, however, an authentic standard has yet to be synthesised. It should be noted that 7α,25-diH,3O-CA and 7α,12α-diH,3O-CA appear as *syn* and *anti* conformers, this is a consequence of derivatisation of an oxo group, and can be seen as a disadvantage, *i.e.* dilution of ion signal, or an advantage *i.e.* confirmation of Girard derivatisation.

6. Internal standards, method validation and quality assurance

Although a number of publications for oxysterol analysis describe validated methods [65,75], there are still questions about accuracy of the various assays employed. A critical factor is the purity of the standards used. Although a standard may be "pure" according to TLC, GC–MS, LC–MS or NMR, there may be contaminants which are not visible to the methods used, invalidating the supposed molar concentration of the standard. In collaboration with the Lipid Maps consortium Avanti Polar Lipids Inc have produced a range of high quality quantitative standards of defined concentrations of oxysterols in defined volumes of solvent whose use should reduce inter laboratory variations [69]. Other important factors for accurate quantification that must be considered are the isotopic purity of isotope labelled standards, the use of appropriate standard curves generated under the same conditions as for sample preparation and also the chromatographic resolution of isomers.

To-date there have been two inter-laboratory surveys organised by the European Network for Oxysterol Research (http://oxysterols.com/) where serum samples have been distributed to participating laboratories and oxysterol analysis made [105]. Twenty one laboratories participated in the second survey. Unfortunately, *ex vivo* cholesterol autoxidation was found to be a problem with highly variable concentrations of 7α-HC, 7β-HC and 7-OC (% coefficient of variation, %CV, 47–129%) returned. This highlights the danger of using any of these metabolites for diagnostic purposes. Even for side-chain oxysterols 24S-HC and (25R)26-HC the %CV for different laboratories was about 60%. Clearly more work is required to standardise analytical methods. The reader is also reminded that a validated method is not necessarily an accurate one and may not give the true answer.

7. Conclusions

To identify monogenetic disorders of cholesterol biosynthesis and metabolism current LC–MS and GC–MS methods are appropriate, for example CTX is readily diagnosed by an absence or very low levels of (25R)26-HC and an elevation in 7α,12α-diHCO in plasma, while oxysterol 7α-hydroxylase deficiency can be diagnosed by high levels of both (25R)26-HC and 3β-HCA in plasma. However, to compare plasma levels of 24S-HC from patients with AD and controls, where the differences are likely to be only small, methods with low CVs are required. This is particularly relevant where data generated by multiple laboratories is combined. Efforts to harmonise methodologies are ongoing spearheaded by the European Network for Oxysterol Research.

To-date most studies of oxysterols have adopted a targeted approach; however, a non-targeted "lipidomic" approach has been utilised by some. The idea of untargeted lipidomics is not new [106], but faster and more sensitive instruments now make this work-flow readily available. It is perhaps this aspect of oxysterol analysis where derivatisation strategies are most powerful. GC–MS analysis of TMS ethers using electron ionisation provide spectra rich in structural information allowing the determination of unknown compounds. Similarly, GP derivatisation and LC–MS exploiting MS^3 gives structural information and the identification of unexpected oxysterols.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.11.017.

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