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Use of trifluoro-acetate derivatives for GC-MS and GC-MS/MS quantification of trace amounts of stera-3β,5α,6β-triols (tracers of Δ^5-sterol autoxidation) in environmental samples

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Abstract: Stera-3β,5α,6β–triols make useful tracers of the autoxidation of Δ^5-sterols. These compounds are generally analysed by gas chromatography-mass spectrometry (GC-MS) after silylation. Unfortunately, the 5α hydroxyl group of these compounds, which is not derivatized by conventional silylation reagents, substantially alters the chromatographic properties of these derivatives, thus ruling out firm quantification of trace amounts. Here we developed a derivatization method (trifluoroacetylation) that enables derivatization of the three hydroxyl groups of 3β,5α,6β–steratriols. The derivatives thus formed present several advantages over silyl ethers: (i) better stability, (ii) shorter retention times, (iii) better chromatographic properties, and (iv) mass spectra featuring specific ions or transitions that enable a very low limit of detection in selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes. This method, validated with cholesta-3β,5α,6β-triol, was applied on several environmental samples (desert dusts, marine sediments and particulate matter) and was able to quantify trace amounts of 3β,5α,6β–steratriols corresponding to several sterols: not only classical monounsaturated sterols (e.g. cholesterol, campesterol and sitosterol) but also, and for the first time, di-unsaturated sterols (e.g. stigmasterol, dehydrocholesterol, brassicasterol).

Keywords: Stera-3β,5α,6β–triols; Autoxidation tracers; Derivatization; Trifluoroacetylation; GC-MS; GC-MS/MS; Environmental samples.

1. Introduction

Autoxidation (free radical oxidation) of Δ^5-sterols mainly affords 7α- and 7β-hydroperoxides and, to a lesser extent, 5α/β,6α/β-epoxysterols and 3β,5α,6β-trihydroxysterols [1]. The 7α- and 7β-hydroperoxides have been ruled out as possible markers of autoxidation processes in the environment due to their instability and lack of specificity [2]. Unfortunately, the highly-specific 5α/β,6α/β-epoxysterols are also ruled out as they are too unstable under environmental conditions: they get quickly hydrolyzed to the corresponding triol by epoxide hydrolase [3] and under acidic conditions [4]. 3β,5α,6β-trihydroxysterols, which are stable and only produced during autoxidation processes, were thus proposed as tracers of sterol autoxidation in the environment [2,5].

Electron ionization (EI) provides more structural information than the soft ionization techniques such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) employed in HPLC-MS analyses [6], and so quantification of Δ^5-sterols and their oxidation products in environmental samples is most often performed using gas chromatography–electron ionization mass spectrometry (GC-EIMS). GC-EIMS analyses are generally carried out on a nonpolar silicone stationary phase after silylation [2,5,7-9].

Silylation of sterol involves the replacement of the hydrogen of the OH group with an alkylsilyl (often trimethylsilyl) group. Trimethylsilyl (TMS) derivatives are highly volatile, thermally stable and present outstanding gas chromatographic characteristics. Moreover, fragmentations of these derivatives are also very informative for structural elucidations [10,11]. However, since TMS derivatives can lose easily trimethylsilanol molecules under the effect of moisture, a short delay between derivatization and injection is needed. Despite this drawback, silylation is a very popular derivatization method often employed during sterol quantification by GC-MS [12-16]. Unfortunately, steric hindrance makes complete silylation of 3β,5α,6β-trihydroxysterols difficult, and common silylation reagents (such as bis(trimethylsilyl)trifluoroacetamide (BSTFA)/pyridine) afford derivatives that are only silylated at C-3 and C-6 [17]. Trisilylated derivatives can be obtained after treatment with BSTFA/dimethylsulfoxide [18], but conversion is still not complete (yield close to 50%) and this treatment is still too complex to be applied for analysis of trace amounts in environmental samples. The presence of a polar non-derivatized hydroxyl group at C-5 in the disilylated derivative strongly alters its chromatographic characteristics and leads to the formation of tailing peaks that substantially limit the sensitivity of the analyses [19].

Here we set out to develop a trifluoroacetylation method able to derivatize the three hydroxyl groups of 3β,5α,6β-trihydroxysterols in order to reduce analyte adsorption in the GC system and improve detector response, peak separation and peak symmetry. We used trifluoroacetic anhydride, which is well-known to be highly reactive in the case of steric hindrance [20]. This derivatization technique was then validated on environmental samples (desert dusts, marine sediments and particulate matter) where it detected traces of several triols resulting from the oxidation of mono- and di-unsaturated sterols.

2. Results and Discussion

2.1. Formation and characterization of trifluoroacetate derivative of cholesta-3β,5α,6β-triol

Reaction of cholesta-3β,5α,6β-triol with trifluoroacetic anhydride in tetrahydrofurane (THF) under the conditions described in section 3.2 affords a trifluoroacetate derivative at high yield (>95 %). As expected, this derivative presents better chromatographic characteristics (shorter retention time and better peak shape) than the corresponding *bis*-trimethylsilyl ether (Figure 1).

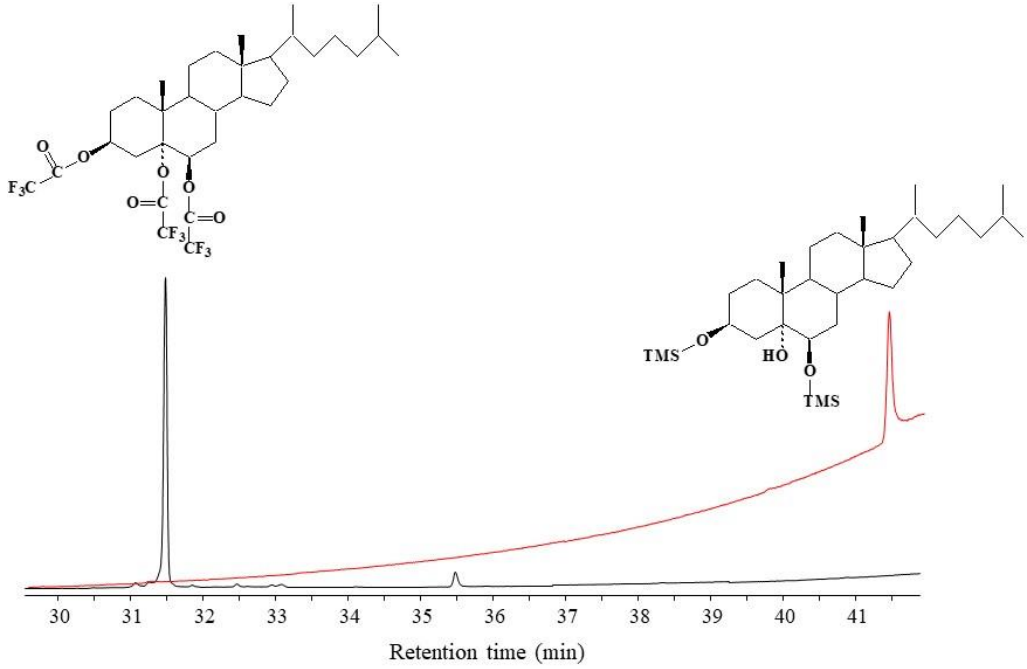

Figure 1. Total ion chromatograms of the same amount (25 ng) of trifluoroacetate (black) and trimethylsilyl (red) derivatives of cholesta-3β,5α,6β-triol.

It is well-known that the introduction of fluorine atoms strongly enhances analyte volatility and thus reduces analyte retention time [21]. Due to its high content of fluorine atoms (nine per molecule), the trifluoroacetate derivative of cholesta-3β,5α,6β-triol elutes 1.5 min before cholesterol trifluoroacetate. Although negative inductive effects of the fluorine atoms in the derivatized product may drive hydrolysis in the presence of moisture [22], here the trifluoroacetate derivative of cholesta-3β,5α,6β-triol was found to be highly stable. Indeed, in contrast to the corresponding TMS derivative, which is hydrolysed in a few days, it can be stored at 4°C for several months without significant alteration.

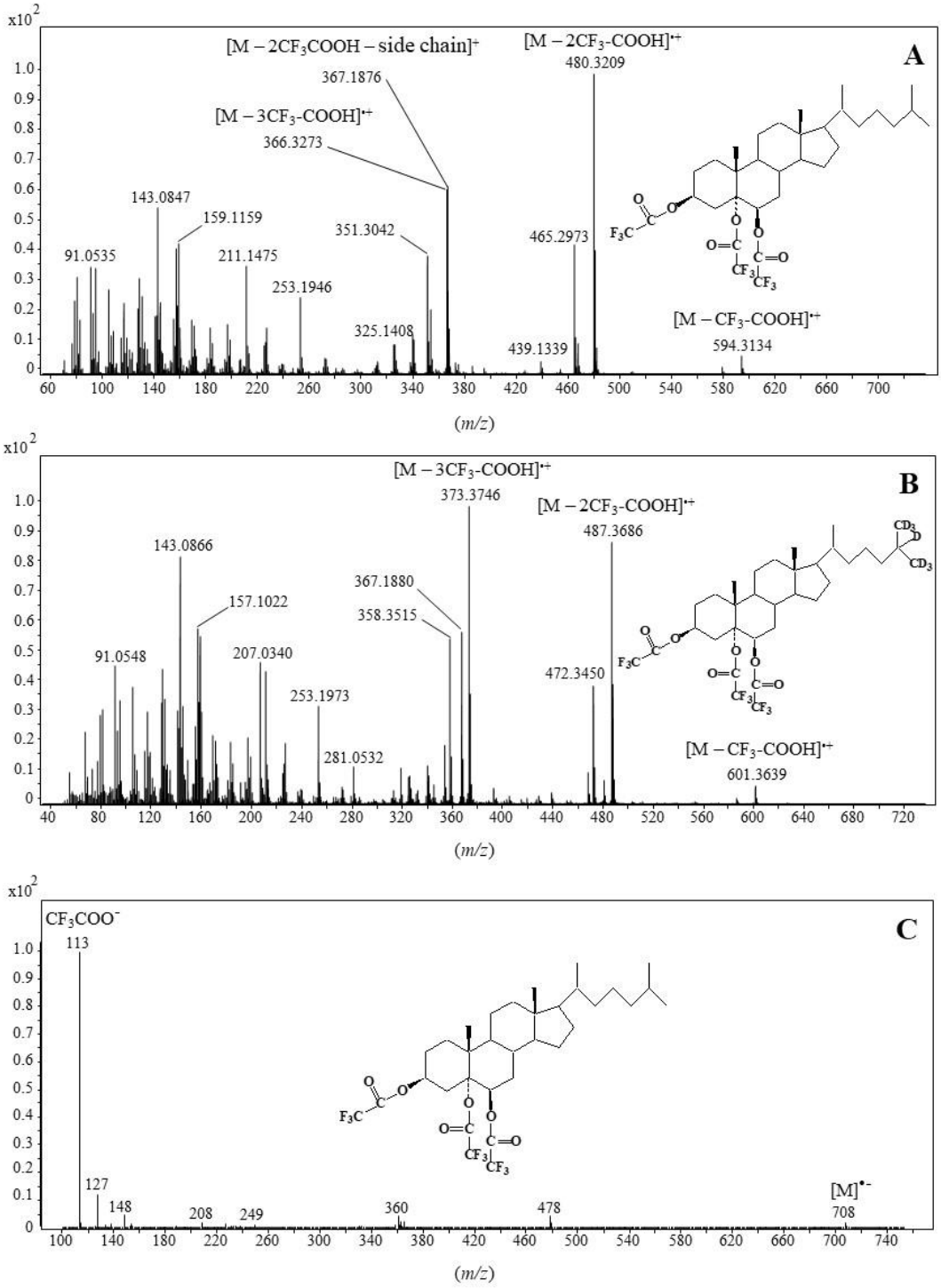

Figure 2. TOF mass spectra of cholesta-3β,5α,6β-triol (A) and cholesta-25,26,26,26,27,27,27-d7-3β,5α,6β-triol (B) trifluoroacetate derivatives and electron capture negative ionization (ECNI) mass spectrum of cholesta-3β,5α,6β-triol trifluoroacetate derivative (C).

The TOF mass spectrum of the cholesta-3β,5α,6β-triol trifluoroacetate derivative (Figure 2A) exhibits ions at *m/z* 594.3134 (**b**$^{+\bullet}$), 480.3209 (**c**$^{+\bullet}$) and 366.3273 (**d**$^{+\bullet}$) corresponding to the successive loss of 1, 2 and 3 neutral molecules of trifluoroacetic acid by the molecular ion (**a**$^{+\bullet}$), respectively (Figure 3). Note that the abundance of the **c**$^{+\bullet}$ ion results from the formation of a stable conjugated enol ester group. An ion at *m/z* 367.1876 (**e**$^{+}$) resulting from the loss of two molecules of trifluoroacetic acid and the side-chain is also formed. The shift of ions **b**$^{+\bullet}$, **c**$^{+\bullet}$ and **d**$^{+\bullet}$ by seven *m/z* units and the lack of shift of **e**$^{+}$ ion observed in the TOF mass spectrum of cholest-5-en-25,26,26,26,27,27,27-d$_7$-3β,5α,6β-triol trifluoroacetate derivative (Figure 2B) further supports these attributions. Unfortunately, the molecular peak of cholesta-3β,5α,6β-triol trifluoroacetate derivative was not observable in its TOF mass spectrum. We therefore used electron capture negative ionization (ECNI), which is generally considered as a soft ionization technique that yields a mass spectral pattern with less fragmentation than under EI ionization [23]. The ECNI mass spectrum of cholesta-3β,5α,6β-triol trifluoroacetate derivative (Figure 2C) appeared to be dominated by a peak at *m/z* 113 corresponding to the anion CF_3-COO^- and a smaller molecular peak at *m/z* 708 attesting that the derivative is well triacetylated could be observed.

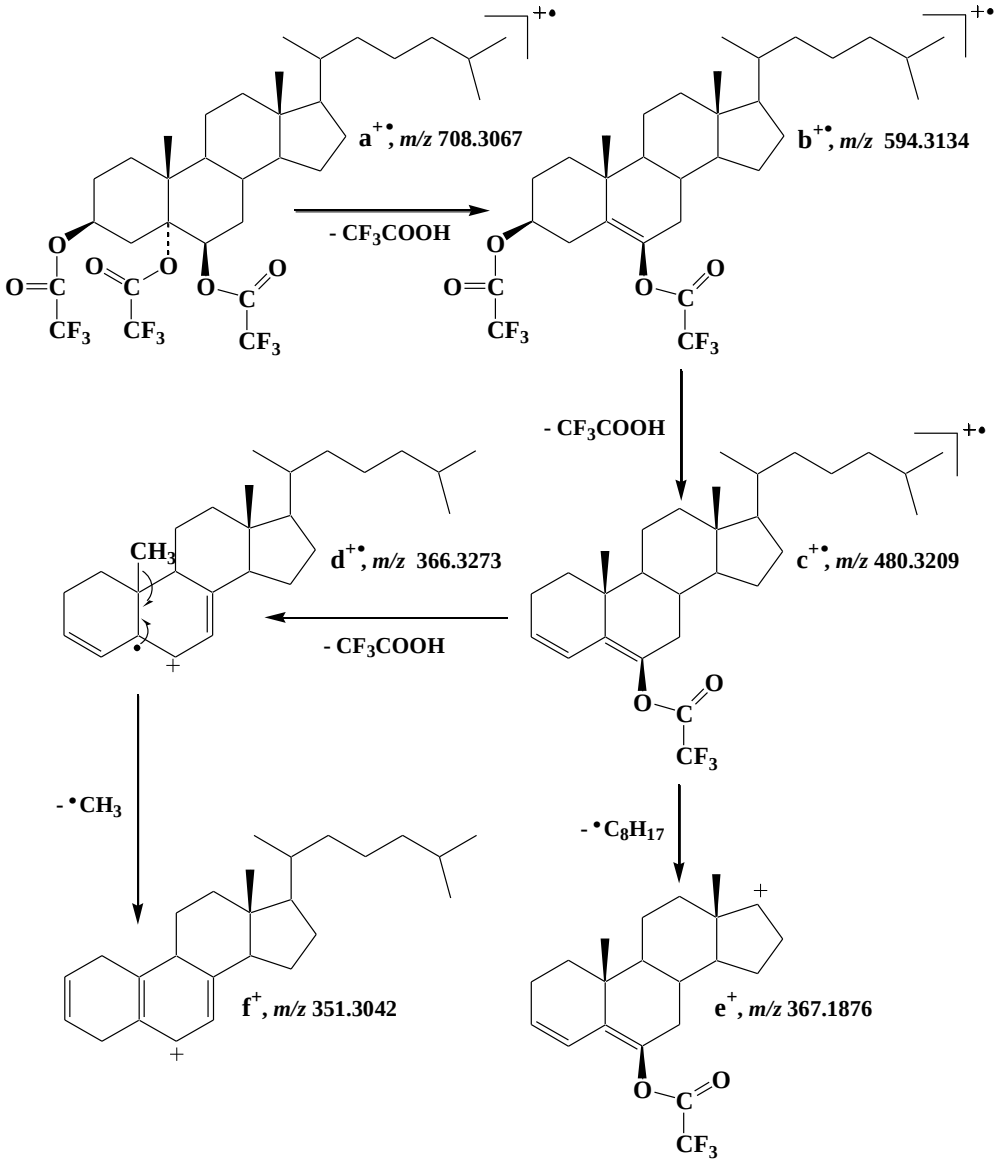

Figure 3. Proposed fragmentation of cholesta-3β,5α,6β-triol trifluoroacetate derivative. Note that another mechanism involving initial loss of the 3β- acyl group is also possible.

Based on its abundance (Figure 2A) and specificity, **c**$^{+\bullet}$ ion corresponding to [M – 2CF_3COOH]$^{+\bullet}$ was selected as target ion for selected ion monitoring (SIM)-based quantification of the main 3β,5α,6β-steratriol trifluoroacetate derivatives present in environmental samples. Due to its high specificity, the less abundant **b**$^{+\bullet}$ ion corresponding to [M – CF_3COOH]$^{+\bullet}$ constitutes a useful qualifier allowing to confirm the identifications. Collision-induced dissociation (CID) analyses (Figure 4) allowed to select the efficient transition **c**$^{+\bullet}$ → **f**$^{+}$ [M – 3CF_3COOH – CH_3]$^{+}$ corresponding to the loss of a neutral molecule of CF_3COOH and a methyl radical by the **c**$^{+\bullet}$ ion (Figure 3) for multiple reaction monitoring (MRM) analyses (Table 1).

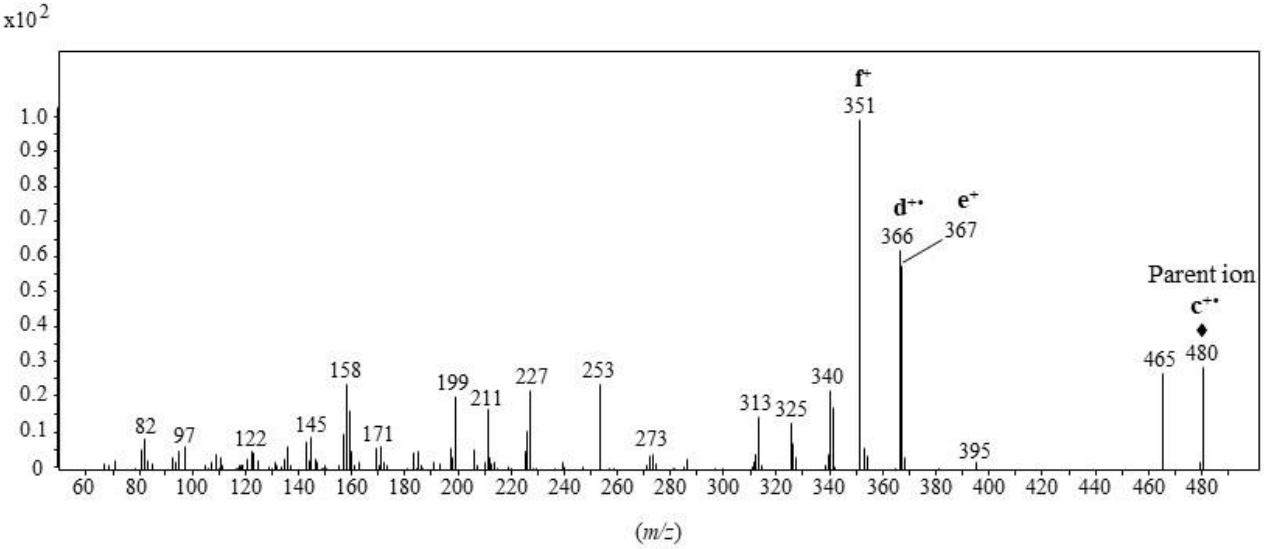

Figure 4. CID mass spectrum of the **c**$^{+\bullet}$ ion at *m/z* 480 (collision energy: 14 eV).

Table 1. MRM transitions employed for the quantification of 3β,5α,6β–steratriols originating from autoxidation of common Δ^5-sterols.

3β,5α,6β-steratriols	**c**$^{+\bullet}$ parent ion [M – 2CF_3COOH]$^{+\bullet}$	**f**$^{+}$ product ion [M – 2CF_3COOH – CH_3]$^{+}$	CE* (eV)
Cholesta-3β,5α,6β–triol	480	351	14
Cholest-22-en-3β,5α,6β–triol	478	349	14
24-Methylcholest-22-en-3β,5α,6β–triol	492	363	14
24-Methylcholest-24(28)-en-3β,5α,6β–triol	492	363	14
24-Methylcholesta-3β,5α,6β–triol	494	365	14
24-Ethylcholest-22-en-3β,5α,6β–triol	506	377	14
24-Ethylcholesta-3β,5α,6β-triol	508	379	14

* Collision energy

2.2. Validation of the derivatization method

Validation of the derivatization method was carried out using the cholesta-3β,5α,6β–triol trifluoroacetate derivative. Results of linearity tests in SIM and MRM modes are presented in Table 2. In the concentration range tested here (0.2325–46.5 ng/mL), the correlation coefficients of the linear regression curves were better than 0.995 and the intercepts did not differ significantly from 0.

Table 2. Linearity in SIM and MRM modes

Mode	Concentration range (ng/mL)	Equation of the linear regression	Correlation coefficient (R^2)
SIM			
Ion *m/z* 480	2.3-46.5[a]	y = 0.0315x - 0.0202	0.9952
Ion *m/z* 594	2.3-46.5	y = 0.0324x - 0.0029	0.9995
MRM			
m/z 480 → *m/z* 351	2.3-46.5	y = 0.0358x - 0.0194	0.9974

[a] (2.325, 4.65, 9.3, 18.6, 23.25, 46.5)

Table 3 reports the reproducibility of this derivatization technique. The precision (given by the standard deviation) and accuracy (defined as the difference between found concentration and expected concentration) were acceptable over the concentration range.

Table 3. Reproducibility in SIM and MRM modes.

Mode	Concentration (ng/mL) Added	Found	n	Relative standard deviation* (%)	Difference between found and added amount (%)
SIM					
Ion *m/z* 480	46.5	44.7	6	4.4	-3.9
	18.6	18.4	9	8.7	-1.1
	9.3	8.9	8	3.8	-4.3
	2.325	2.618	6	9.9	12.6
Ion *m/z* 594	46.5	44.4	6	5.4	-4.5
	18.6	18.3	9	8.5	-1.6
	9.3	9.2	8	3.0	-1.1
	2.325	2.696	6	4.2	15.9
MRM					
m/z 480 → *m/z* 351	46.5	45.6	6	3.1	-1.9
	18.6	18.63	9	4.1	0.2
	9.3	9.0	8	3.7	-3.2
	2.325	2.563	6	4.2	10.2

* 95% confidence

The limit of detection (LOD) (defined by a signal-to-noise ratio of 5) was about 25.8 and 0.78 pg injected in SIM and MRM modes, respectively. For comparison, the LOD obtained for the corresponding disilylated derivative was 0.62 ng in SIM mode with the target ion *m/z* 456 corresponding to $[M - TMSOH - H_2O]^{+\bullet}$.

2.3. Application to different environmental samples

In an application of the derivatization method, 3β,5α,6β–steratriol trifluoroacetate derivatives originating from the autoxidation of common Δ^5-sterols were quantified in several environmental samples (desert dusts, marine sediments and particulate matter). The results obtained are summarized in Tables 4 and 5. This method allowed precise

quantification of 3β,5α,6β–triols derived from classical Δ^5-sterols (cholest-5-en-3β-ol, 24-methylcholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol) (Table 4, Figure 5).

Table 4. Concentrations of saturated 3β,5α,6β–steratriols in several environmental samples, measured after trifluoroacetylation in SIM and MRM modes.

	Cholesta-3β,5α,6β-triol		24-Methylcholesta-3β,5α,6β-triols[d]		24-Ethylcholesta-3β,5α,6β-triol	
	SIM *m/z* 480	MRM *m/z* 480 → *m/z* 351	SIM *m/z* 494	MRM *m/z* 494 → *m/z* 365	SIM *m/z* 508	MRM *m/z* 508 → *m/z* 379
Negev loess sample 1[a]	0.21	0.21	0.09	0.06	0.24	0.17
Negev loess sample 2[a]	0.24	0.19	0.07	0.05	0.19	0.12
Negev loess sample 3[a]	0.23	0.22	0.12	0.09	0.35	0.25
Particles Antarctica st 4[b]	11.4	11.73	0.80	nd[c]	1.95	1.25
Particles Antarctica st 13[b]	7.11	8.10	0.85	1.00	2.00	1.25
Particles Antarctica st 28[b]	6.22	7.40	0.95	0.95	2.00	1.10
Particles Antarctica st 42[b]	6.08	6.45	1.00	nd	1.70	0.90
Particles Antarctica st 46[b]	11.73	11.88	1.05	1.00	1.85	1.15
Sediment Baffin Bay st 600[a]	20.30	21.05	5.63	4.73	20.27	17.91
Sediment Baffin Bay st 605[a]	22.98	23.56	8.67	5.33	37.67	24.11
Sediment Baffin Bay st 615[a]	19.32	19.32	4.11	3.47	26.68	17.21
Sediment Baffin Bay st 707[a]	45.60	49.28	2.20	2.20	37.60	40.00
Sediment Baffin Bay st 719[a]	18.19	17.87	5.00	3.27	24.33	13.5

[a] ng mg^{-1} [b] ng L^{-1} [c] Not detected [d] Sum of diastereoisomers

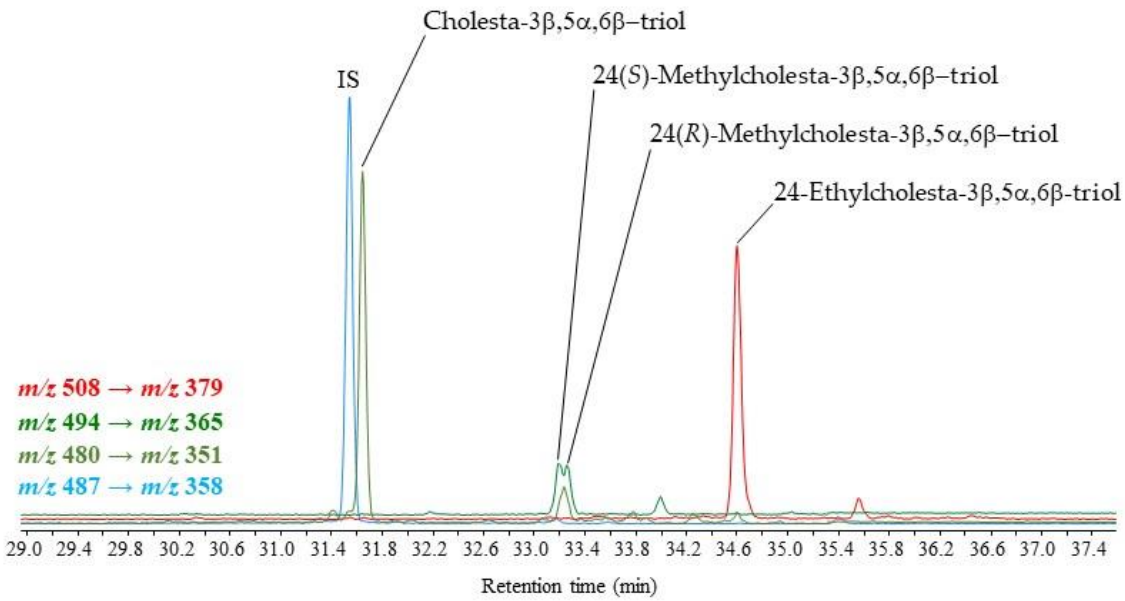

Figure 5. MRM chromatograms showing the presence of 3β,5α,6β–steratriol trifluoroacetate derivatives in a total lipid extract (TLE) of dusts collected in the Negev desert (Israel). (IS = internal standard)

Table 5. Concentrations of unsaturated 3β,5α,6β–steratriols in several environmental samples, measured after trifluoroacetylation in SIM and MRM modes.

	Cholest-22*E*-en-3β,5α,6β-triol		24-Ethylcholest-22*E*-en-3β,5α,6β-triol		24-Methylcholest-22*E*-en-3β,5α,6β-triol	
	SIM	MRM	SIM	MRM	SIM	MRM
	m/z 506	*m/z* 506 → *m/z* 377	*m/z* 478	*m/z* 478 → *m/z* 349	*m/z* 492	*m/z* 492 → *m/z* 363
Negev loess sample 1[a]	-[d]	0.11	nd	0.04	nd[c]	0.02
Negev loess sample 2[a]	-[d]	0.02	nd	nd	nd	nd
Negev loess sample 3[a]	-[d]	0.01	nd	nd	nd	nd
Particles Antarctica st 4[b]	-[d]	1.42	1.11	nd	1.11	1.80
Particles Antarctica st 13[b]	-[d]	1.50	nd	nd	nd	1.40
Particles Antarctica st 28[b]	-[d]	1.60	nd	nd	nd	3.70
Particles Antarctica st 42[b]	-[d]	1.85	nd	nd	nd	2.20
Particles Antarctica st 46[b]	-[d]	1.95	nd	nd	nd	1.70
Sediment Baffin Bay st 600[a]	-[d]	4.09	3.80	1.45	1.64	1.63
Sediment Baffin Bay st 605[a]	-[d]	6.22	4.67	1.78	1.78	1.78
Sediment Baffin Bay st 615[a]	-[d]	2.68	nd	1.26	1.84	1.84
Sediment Baffin Bay st 707[a]	-[d]	6.20	nd	3.40	nd	5.40
Sediment Baffin Bay st 719[a]	-[d]	1.07	nd	1.07	1.40	1.40

[a] ng mg^{-1} [b] ng L^{-1}
[c] Not detected [d] Quantification hindered by a strong coelution

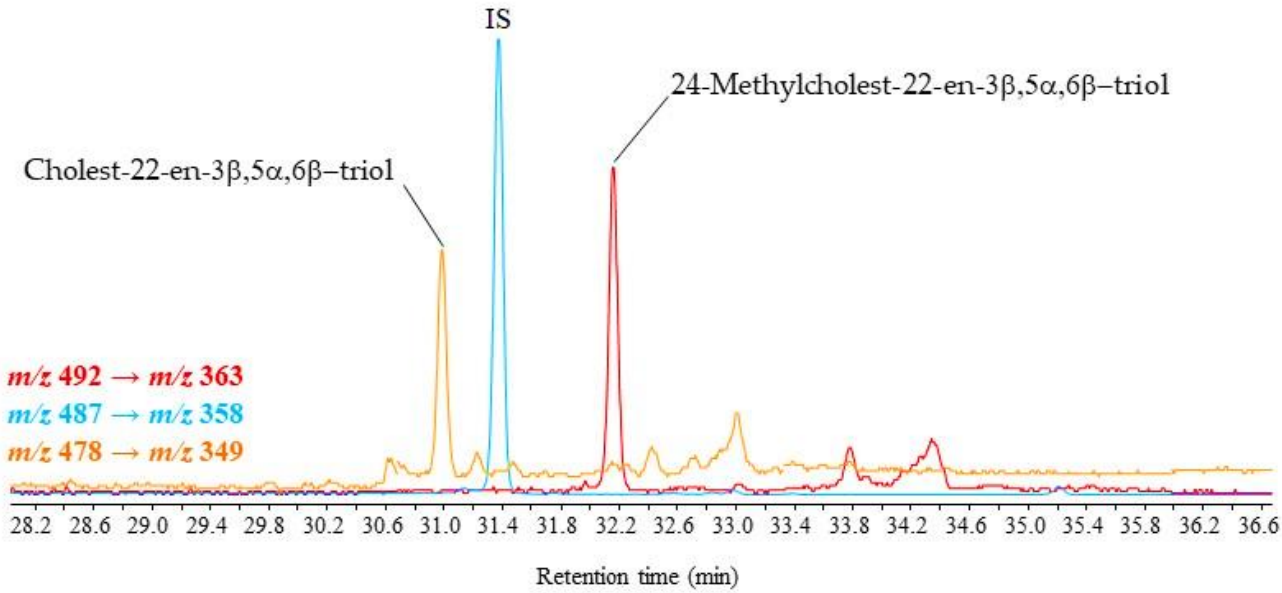

Figure 6. MRM chromatograms showing the presence of unsaturated 3β,5α,6β–steratriol trifluoroacetate derivatives in TLE of suspended particulate matter collected in the Amundsen Sea (Antarctica).

The double peak observed in the case of the transition *m*/*z* 494 → *m*/*z* 365 (Figure 5) results from the well-known production of a mixture of 24-methylcholesterol epimers by eukaryotic organisms, where campesterol (24(*R*)-methylcholest-5-en-3β-ol) and dihydrobrassicasterol (24(*S*)-methylcholest-5-en-3β-ol) are found in variable proportions [24,25]. Due to their excellent chromatographic properties, these 3β,5α,6β–steratriol trifluoroacetate derivatives are thus able to separate diastereoisomers.

Interestingly, we also detected unsaturated triols deriving from 5,22-di-unsaturated sterols (Table 5, Figure 6), which have never previously been described in the literature. Note that the mass spectra of these derivatives (Figure 7) are similar to the mass spectra of monounsaturated sterols (Figure 2A).

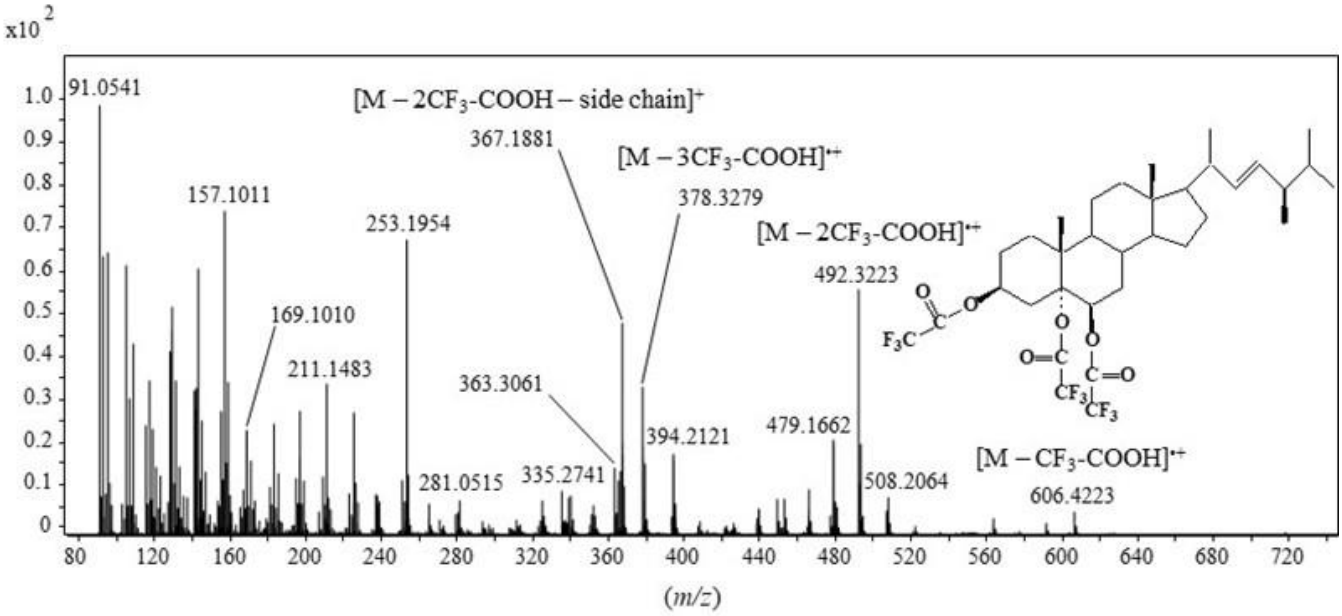

Figure 7. TOF mass spectrum of 24-methylcholest-22*E*-en-3β,5α,6β–triol trifluoroacetate derivative.

Sterols are commonly used as tracers for specific classes of organisms in environmental samples [26-28]. The quantification of 3β,5α,6β–steratriols (autoxidation tracers) [2,5] and their corresponding sterols in these samples thus provides valuable information on the oxidation state (and thus alteration) of specific organisms (e.g. higher plants, seagrasses, phytoplankton, zooplankton and fungi).

3. Materials and Methods

3.1. Chemicals

Trifluoroacetic anhydride (TFAA), cholesta-3β,5α,6β-triol, sterols, cholest-5-en-25,26,26,26,27,27,27-d_7-3β-ol, *meta*-chloroperoxybenzoic acid, H_2O_2, BSTFA and chemical reagents were obtained from Sigma-Aldrich. The synthesis of standards of the other stera-3β,5α,6β-triols involved epoxidation of the corresponding sterols with *meta*-chloroperoxybenzoic acid in dry methylene chloride, and subsequent acidic hydrolysis [29].

The internal standard used (cholesta-25,26,26,26,27,27,27-d_7-3β,5α,6β-triol) was synthesized by KI/H_2O_2 oxidation of the corresponding heptadeuterosterol [30]. Cholest-5-en-25,26,26,26,27,27,27-d_7-3β-ol (5 mg), KI (2.2 mg) and dioxane/water (0.9 mL, 2:1, v/v) were placed in a 20 mL flask, and then H_2SO_4 (98%, 5 μL) and H_2O_2 (30%, 10 μL) were added sequentially at room temperature under magnetic stirring. After stirring for 1 h at room temperature, the system was stirred 3 h at 60°C and the reaction mixture was then neutralized

with anhydrous Na_2CO_3 (2.2 mg) and treated with a saturated solution of Na_2SO_3 (4 mL). The crude triol was extracted twice (4 mL) with ethyl acetate and the organic extracts were evaporated to dryness under nitrogen at 50°C. The crude triol was then purified using column chromatography (silica, Kieselgel 60 with 55% water, 6 × 0.6 cm). The column was conditioned with CH_2Cl_2. After elimination of the residual sterol with CH_2Cl_2 (8 mL), the triol was eluted with CH_3CN (6 mL).

3.2. Environmental samples

Detailed descriptions of the collection and treatment of samples of desert dusts, marine particulate matter and sediments used for validation of the proposed 3β,5α,6β-steratriol derivatization method can be found elsewhere [31-34]. The different TLEs obtained were derivatized as described in the following section.

3.2. Trifluoroacetylation method

In an effort to optimize the derivatization reaction, we tested several parameters, including nature of the solvent (cyclohexane, THF, diethyl ether, dichloroethane, ethyl acetate and 1,4-dioxane), reaction temperature (50–100°C), heating time (1–24 h) and volume of TFAA (25–200 μL). The best reaction efficiency was obtained with the following conditions.

Samples to be derivatized (2–100 ng), anhydrous THF (200 μL) and TFAA (100 μL) were put in glass vials (4 mL) with PTFE-lined screw caps, and the mixture was maintained at 68–70°C in a heating block for 24 h. After evaporation to dryness under nitrogen at 50°C, the residue was dissolved in BSTFA to silylate the traces of trifluoroacetic acid formed during the reaction and avoid damaging the GC-column.

3.3. Silylation

3β,5α,6β-steratriols were silylated by dissolving them in 300 μL of a mixture of pyridine and BSTFA (2:1, v/v) and heating to 50 °C for 1 h. After evaporation to dryness under a stream of N_2, the derivatized residue was dissolved in BSTFA.

3.4. Gas chromatography-tandem electron ionization mass spectrometry (GC-EIMS/MS)

GC-EIMS and GC-EIMS/MS analyses were performed using an Agilent 7890A/7010A tandem quadrupole gas chromatograph system (Agilent Technologies, Les Ulis, France) with a cross-linked 5% phenyl-methylpolysiloxane capillary column (Agilent; HP-5MS ultra inert, 30 m × 0.25 mm, 0.25-μm film thickness). Analyses were performed with an injector operating in pulsed splitless mode (1.7×10^5 Pa during 0.5 min) set at 270°C. Oven temperature was ramped from 70°C to 130°C at 20°C min^{-1}, then to 250°C at 5°C min^{-1} and then to 300°C at 3°C min^{-1}. The pressure of the carrier gas (He) was held at 0.76×10^5 Pa until the end of the temperature program. The mass spectrometer conditions were as follows: electron energy, 70 eV; source temperature, 230°C; quadrupole 1 temperature, 150°C; quadrupole 2 temperature, 150°C; collision gas (N_2) flow, 1.5 mL min^{-1}; quench gas (He) flow, 2.25 mL min^{-1}; mass range, *m/z* 50–700; cycle time, 313 ms. Steratriol derivatives were quantified in SIM and MRM modes. Target and precursor ions were selected from the most intense and specific fragmentations observed in the electron ionization (EI) mass spectra. Collision-induced dissociation (CID) was optimized by using collision energies ranging from 0 to 20 eV. Quantification with Mass Hunter software (Agilent Technologies, Les Ulis, France) involved peak integration and quantitative determination using calibration curve and ratio between areas of triol and internal standard (cholesta-25,26,26,26,27,27,27-d_7-3β,5α,6β-triol).

ECNI analyses were carried out on the same apparatus with methane as reagent gas at 50 mA emission current and 195 eV electron energy. During the experiment, the temperature of the source was held at 150°C and reactant gas flow was 0.5–0.7 mL min^{-1}.

3.5. Gas chromatography–EI quadrupole time-of-flight mass spectrometry (GC-QTOF)

Accurate mass measurements were carried out in full scan mode using an Agilent 7890B/7200 GC/QTOF system (Agilent Technologies, Les Ulis, France) with a cross-linked 5% phenyl methylpolysiloxane capillary column (Agilent Technologies; HP-5MS Ultra inert, 30 m × 0.25 mm, 0.25 μm film thickness). Analyses were performed with an injector operating in pulsed splitless mode set at 270°C. Oven temperature was ramped from 70°C to 130°C at 20°C min^{-1} and then to 300°C at 5°C min^{-1}. Pressure of the carrier gas (He) was held at 0.76 x 10^5 Pa until the end of the temperature program. Instrument temperatures were 300°C for the transfer line and 230°C for the ion source. Nitrogen (1.5 mL min^{-1}) was used as collision gas. Accurate mass spectra were recorded across the range *m/z* 50–700 at 4 GHz with the collision gas opened. The QTOF-MS instrument provided a typical resolution ranging from 8009 to 12252 from *m/z* 68.9955 to 501.9706. Perfluorotributylamine (PFTBA) was used for daily MS calibration. Compounds were identified by comparing their TOF mass spectra, accurate masses and retention times against standards.

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