Supporting Information

Simultaneous Analysis of Fatty Alcohols, Fatty Aldehydes and Sterols in Thyroid Tissues by Electrospray ionization-ion mobility-mass spectrometry based on Charge Derivatization

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Experimental details

Chemicals

HPLC grade acetonitrile (ACN) and trifluoroacetic acid (TFA, ≥ 99%) were purchased from Merck KGaA (Darmstadt, Germany). Dichloromethane (CH_2Cl_2) was of HPLC grade and purchased from Adamas-bata (Shanghai, China). Ultrapure deionized water was produced by a Direct-Q water purification system (Millipore, El Paso, TX, USA). Standard fatty alcohols were purchased from Shanghai Aladdin Reagent Co., Ltd (Shanghai, China). Standard fatty aldehydes (C14-C18, > 98.0%, GC) were purchased from Tokyo Chemical Industry Co., Ltd. Standard sterols and anhydrous pyridine (d_0-pyridine) came from Sigma-Aldrich (St. Louis, Missouri, USA.). Deuterium pyridine (d_5-pyridine) was obtained from Cambridge Isotope Laboratories. Thionyl chloride ($SOCl_2$) was provided by Sinopharm Chemical Reagent CO., Ltd (Shanghai, China).

A mixed standard stock solution containing all the standard compounds was prepared by dissolving them in CH_2Cl_2, and the concentrations of these analytes were 10 μg/mL, except that octadecanol, octadecanal, and sterols were at 100 μg/mL. Then the mixed standard stock solution was diluted to provide working solutions with different concentrations.

Derivatization of fatty alcohols, fatty aldehydes and sterols in mixed standard solution

Solutions of d_0-pyridine (20%, v/v) in CH_2Cl_2 (40 μL) and $SOCl_2$ (50%, *v/v*) in CH_2Cl_2 (40 μL) were added sequentially to standard solutions in CH_2Cl_2 (50 μL). The solutions were incubated at 30°C with continuous shake (220 r/min) for 2 hours. Then 30 μL of deionized water was added to the samples to terminate the derivatization. The CH_2Cl_2 (bottom) layer was collected, evaporated by nitrogen, and dissolved in ACN–water mixture (120 μL, 1:1, *v/v*). Each sample was centrifuged at 12,000 rpm for 10 min and transferred to a HPLC vial for analysis.

HPLC-ESI-IM-MS analysis

The HPLC-ESI-IM-QTOF-MS system consisted of a 1290 HPLC system incorporated a drift tube coupled to a quadrupole time-of-flight mass spectrometer equipped with an ESI interface (6560, Agilent, USA). Agilent MassHunter workstation software was used to fully control the liquid chromatography, mass spectrometer, and to process the data. The chromatographic separation was performed on a shim-pack XR-ODS III column (1.6 μm, 2.0×50 mm, Shimadzu). An isocratic elution of a mixture of acetonitrile and water (75:25, *v/v*, 0.025% TFA in both acetonitrile and water) was used in 4 minutes. The LC solvent flow rate was maintained at 0.4 mL/min and injection volumes were 2 μL.

The mass spectrometer was operated in the positive mode. The operating parameters were optimized as follows: capillary voltage 3000 V, drying gas (N_2) 7 L/min, gas temperature 300 °C, nebulizer gas pressure 40 psi, sheath gas (N_2) 10 L/min, gas temperature 250 °C.

In the IM-MS operation, the three ion funnels were operated as follows: high-pressure funnel RF at 200 V DC; trapping funnel RF at 150 V DC; rear funnel RF at 100 V DC. Trap fill time of IM was 40000 μs. Drift tube entrance voltage was 1700 V. The pressure of the nitrogen used as the IM drift gas was maintained at ~4 Torr at 25 °C.

SPSS 20.0 software was used for statistical analysis. All statistical comparisons were made by means of a single sample t-test followed by a test value of 1. $p < 0.05$ was considered statistically significant.

Limits of Detection (LOD)

A series of mixed solutions from low to high concentrations were labeled by d_0-pyridine and analyzed by HPLC-ESI-IM-QTOF-MS.

LODs was defined as the concentrations at which the signal to noise ratio (S/N) was equal to or greater than 3, determined by the MassHunter workstation software.

Calibration curve

Solutions of d_0-pyridine labeled and d_5-pyridine derivatives were mixed at different ratios (0.1:1, 0.25:1, 0.5:1, 1:1, 2.5:1, 5:1 and 10:1; 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 μg/mL, except 18OH, 18CHO, and sterols at 0.2, 0.5, 1.0, 2.0, 5.0, and 10 μg/mL) in triplicate and then analyzed by HPLC-ESI-IM MS.

Biological sample collection and preparation

Twenty seven patients diagnosed with thyroid cancer were recruited under informed consent. Carcinoma and para-carcinoma (normal) tissues from these patients were provided by Fudan University Shanghai Cancer Center (FUSCC) with ethical approval by the ethics board of FUSCC. Carcinoma and para-carcinoma tissues were further diagnosed by an independent, blinded pathologist. Their recorded medical data including recipient gender, age, and other clinical information are listed in Table S1. Tissue samples were taken immediately after surgery. Then they were rinsed with cold deionized water, and subsequently frozen in liquid nitrogen. Each thyroid tissue was freeze-dried. After that, 10 mg of each tissue was transferred into tubes and homogenized in ACN (100 μL) with Precellys 24 homogenizer (Bertin Technologies). The samples were then stored at −80 °C till further analysis.

Matrix Effect

d_0-pyridine labeled octadecanol, hexadecanal, and cholesterol at three different concentrations were spiked with the extraction solutions of thyroid tissue homogenate (*v/v*, 1:1) in triplicates. Matrix effect was calculated as (peak area of derivatives spiked with extraction solutions / peak area of derivatives spiked with ACN–water mixture) ×100%.

Recovery

Thyroid tissue homogenate solutions were spiked with mixed standard of octadecanol, hexadecanal, and cholesterol at three different concentrations in triplicates each. Then, spiked solutions and thyroid tissue homogenate solutions were labeled and tested following the same protocol. The concentrations of octadecanol, hexadecanal, and cholesterol were calculated by calibration curves. Recovery was expressed as (increased concentration/ spiked concentration) ×100%.

Derivatization of fatty alcohols, fatty aldehydes and sterols in carcinoma and para-carcinoma tissue samples

The homogenate solutions in ACN (50 μL) of carcinoma and para-carcinoma tissues were evaporated by nitrogen and dissolved in CH_2Cl_2 (50 μL). Then solutions of d_0-/d_5- pyridine (20%, *v/v*) in CH_2Cl_2 (40 μL) and $SOCl_2$ (50%, *v/v*) in CH_2Cl_2 (40 μL) were added sequentially to the carcinoma and para-carcinoma tissue samples, respectively. The solutions were incubated at 30 °C with continuous shake (220 r /min) for 2 hours followed by addition of deionized water to the samples to terminate the derivatization. After that, the d_0 and d_5-labeled samples were mixed further. The CH_2Cl_2 (bottom) layer was collected, evaporated by nitrogen, and dissolved in ACN–water mixture (120 μL, 1:1, *v/v*). Each sample was centrifuged at 12,000 rpm for 10 min and transferred to a HPLC vial for analysis.

Supporting information Figure S1-S4

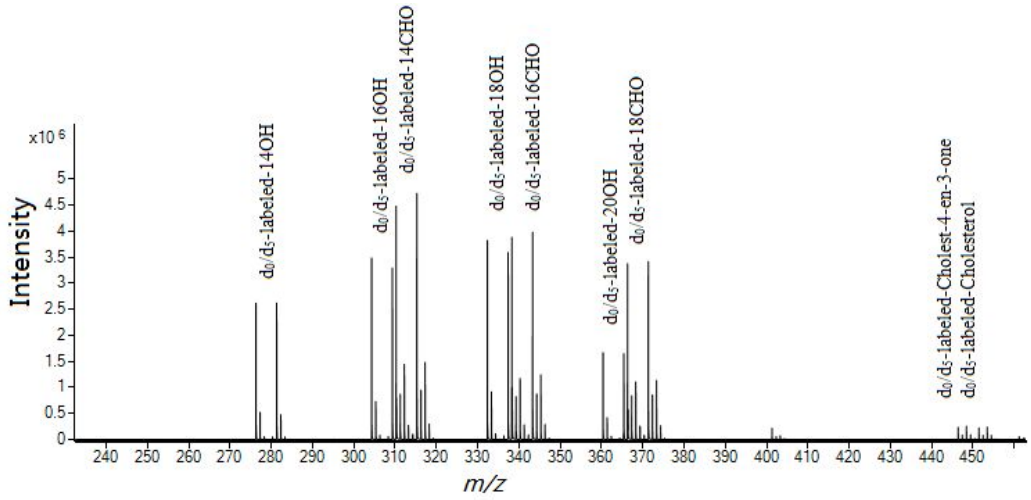

Figure S1. Representative mass spectrum of d_0/d_5-labeled standard fatty alcohols, fatty aldehydes and sterols.

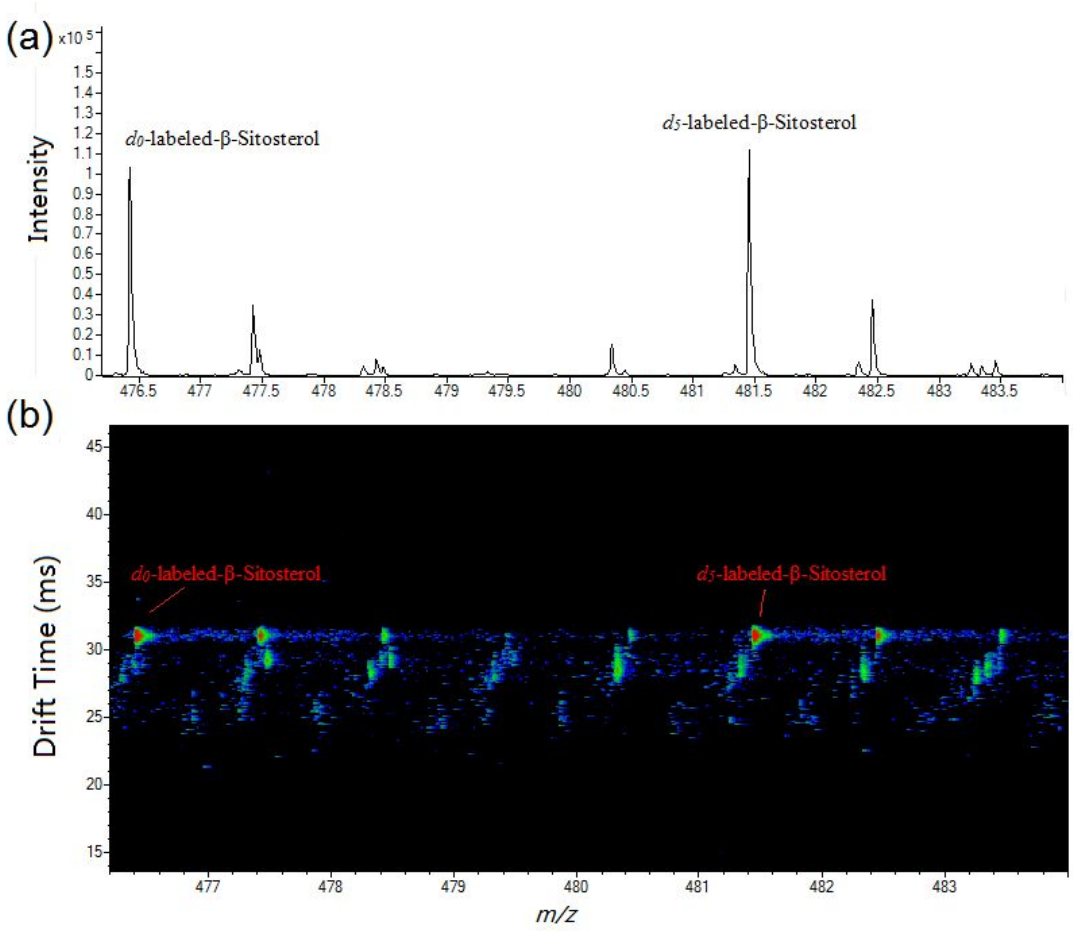

Figure S2. MS spectra (a) and drift time spectra (b) of d_0/d_5-labeled-β-Sitosterol in standard solution.

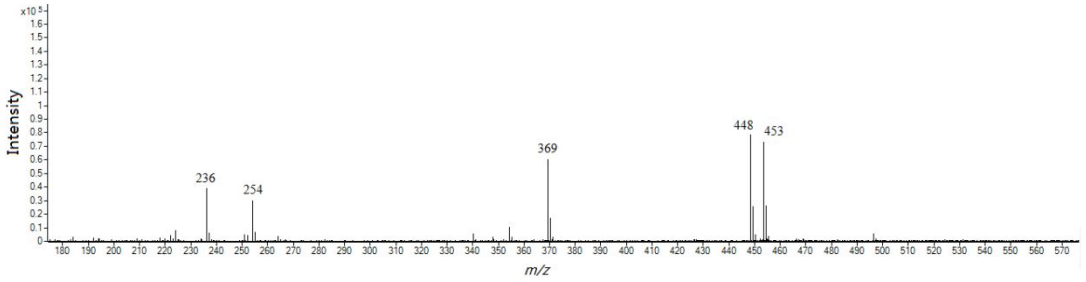

Figure S3. MS spectra of the tissue sample using direct infusion.

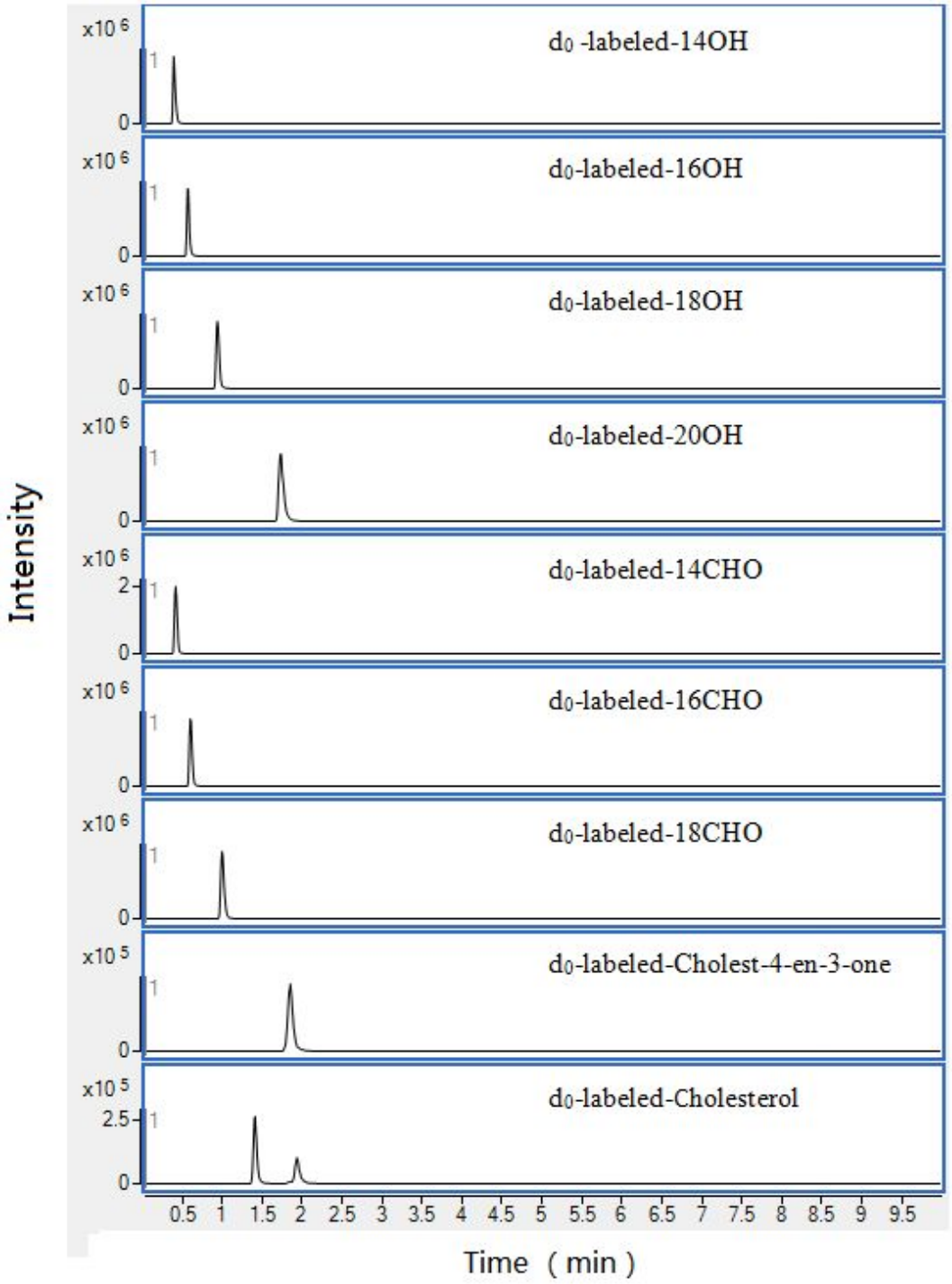

Figure S4. EIC spectra for d_0-labeled standards.

Supporting information Table S1-S6

Table S1. Drift time, relative error between theoretical value and experimental value of *m/z* for derivatives of the compounds.

Derivatives of the compounds	Molecular formula	Drift time (ms)	Theoretical value (*m/z*)	experimental value (*m/z*)	relative error (ppm)
14OH	$C_{19}H_{34}N^+$	23.1	276.2691	276.2687	1.4
	$C_{19}H_{29}D_5N^+$	23.08	281.3005	281.3	1.8
16OH	$C_{21}H_{38}N^+$	24.15	304.3004	304.3	1.3
	$C_{21}H_{33}D_5N^+$	24.2	309.3318	309.3314	1.3
18OH	$C_{23}H_{42}N^+$	25.18	332.3317	332.3311	1.8
	$C_{23}H_{37}D_5N^+$	25.22	337.3631	337.3624	2.1
20OH	$C_{25}H_{46}N^+$	26.19	360.363	360.3624	1.7
	$C_{25}H_{41}D_5N^+$	26.23	365.3944	365.3936	2.2
22OH	$C_{27}H_{50}N^+$	27.2	388.3943	388.3933	2.6
	$C_{27}H_{45}D_5N^+$	27.23	393.4257	393.4246	2.8
14CHO	$C_{19}H_{33}ClN^+$	23.62	310.2302	310.2298	1.3
	$C_{19}H_{28}D_5ClN^+$	23.64	315.2615	315.2607	2.5
15CHO	$C_{20}H_{35}ClN^+$	24.17	324.2458	324.245	2.5
	$C_{20}H_{30}D_5ClN^+$	24.19	329.2772	329.2761	3.3
16CHO	$C_{21}H_{37}ClN^+$	24.69	338.2615	338.2607	2.4
	$C_{21}H_{32}D_5ClN^+$	24.72	343.2928	343.2922	1.7
17CHO	$C_{22}H_{39}ClN^+$	25.18	352.2771	352.2762	2.6
	$C_{22}H_{34}D_5ClN^+$	25.19	357.3085	357.3075	2.8
18CHO	$C_{23}H_{41}ClN^+$	25.68	366.2928	366.2915	3.5
	$C_{23}H_{36}D_5ClN^+$	25.7	371.3241	371.3233	2.2
Cholest-4-en-3-one	$C_{32}H_{48}N^+$	29.6	446.3787	446.3782	1.1
	$C_{32}H_{43}D_5N^+$	29.62	451.4101	451.4096	1.1
Cholesterol	$C_{32}H_{50}N^+$	29.75	448.3943	448.3935	1.8
	$C_{32}H_{45}D_5N^+$	29.76	453.4257	453.4245	2.6
7α-hydroxy-4-cholesten-3-one	$C_{32}H_{48}NO^+$	29.89	462.3736	462.3731	1.1
	$C_{32}H_{43}D_5NO^+$	29.93	467.405	467.4044	1.3
Campesterol	$C_{33}H_{52}N^+$	30.14	462.41	462.4086	3.0
	$C_{33}H_{47}D_5N^+$	30.16	467.4414	467.4401	2.8
β-Sitosterol	$C_{34}H_{54}N^+$	30.98	476.4256	476.4249	1.5
	$C_{34}H_{49}D_5N^+$	30.98	481.457	481.4563	1.5

Tabel S2. Reproducibility of the reaction and LOD of the labeled and unlabeled analytes

Analytes	RSD/%[a]			Peak intensity ratio[b]	LOD[c] (ng/mL)	LOD[d] (ng/mL)
	low	middle	high			
14OH	1.9	2.6	5.6	0.967	0.02	1500
16OH	4.7	3.1	4.8	0.988	0.02	1000
18OH	4.6	2.3	2.1	0.977	0.02	1500
20OH	6.3	0.8	4.7	1.042	0.02	2000
22OH	3.8	2.9	3.7	1.022	0.02	2000
14CHO	3.9	2.8	1.1	0.974	0.50	5000
15CHO	1.3	2.2	8.1	0.983	0.05	7500
16CHO	1.5	2.9	1.8	0.986	0.02	5000
17CHO	2.1	0.8	1.1	0.963	0.02	2500
18CHO	2.5	1.9	2.4	0.978	0.02	2500
Cholest-4-en-3-one	4.1	2.7	2.1	1.02	20	1500
Cholesterol	2.4	4.4	1.8	1.011	10	1000
7α-hydroxy-4-cholesten-3-one	3.3	4.9	4.6	0.996	10	1500
Campesterol	4	3.9	4.2	0.991	20	1000
β-Sitosterol	1.1	1	2.6	1.108	10	1000

[a]Two aliquots of standards at low, middle and high concentration ratios (0.25:1, 1:1, 5:1, each in triplicates) were labeled by d_0 and d_5-pyridine and then each two aliquots were mixed and analyzed by HPLC-ESI-IM MS.

[b]Peak intensity ratios of d_0-/d_5-pyridine labeled analytes when reacting with equal amount standards.

[c]LOD of the labeled analytes.

[d]LOD of the unlabeled analytes.

Table S3 Matrix Effect and Recovery for the analysis of octadecanol, hexadecanal, and cholesterol.

Analytes	Matrix Effect (%, n = 3)						Recovery (%, n = 3)					
	Low[a]		Middle		High		Low		Middle		High	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
Octadecanol (18OH)	92.8	8.8	91.5	5.6	91.0	8.5	80.5	4.7	86.7	5.3	89.9	3.8
Hexadecanal (16CHO)	89.2	6.3	91.4	1.9	91.4	3.1	90.4	5.0	87.8	6.9	93.2	1.8
Cholesterol	87.9	6.7	92.5	3.0	92.8	0.5	76.8	8.6	79.0	2.3	88.5	3.4

[a]For hexadecanal, the low, middle, and high concentrations were 0.05, 0.20, and 1.0 μg/mL, respectively; for octadecanol and cholesterol, the low, middle, and high concentrations were 0.5, 2.0, and 10 μg/mL, respectively.

Table S4. Recorded medical data of twenty-seven patients diagnosed with thyroid carcinoma.

	Gender [a]	Age	FT3 [b]	FT4 [c]	TSH [d]	HTG [e]	CT [f]
Mean ± SD	Female (n=18)	39 ± 12	4.60 ± 1.77	13.27 ± 1.41	2.30 ± 1.42	28.69 ± 23.61	17.78± 6.01

[a] total number of patients was 27. [b] Free triiodothyronine. [c] Free tetraiodothyronine.
[d] Thyroid-stimulating hormone. [e] Thyroglobulin. [f] Calcitonin.

Table S5. Correlation coefficients between fatty alcohols, fatty aldehydes and sterols in carcinoma calculated by Spearman correlation analysis.

	14OH	16OH	18OH	20OH	22OH	14CHO	15CHO	16CHO	17CHO	18CHO	Cholesterol	7α-hydroxy-4-cholesten-3-one	Campesterol	β-Sitosterol
14OH	1	0.926	0.858	0.722	0.861	0.903	0.932	0.897	0.761	0.677	0.642	0.114	0.824	0.843
16OH	0.926	1	0.916	0.819	0.826	0.817	0.856	0.874	0.761	0.686	0.631	0.076	0.769	0.782
18OH	0.858	0.916	1	0.875	0.813	0.765	0.754	0.757	0.684	0.639	0.644	0.129	0.628	0.667
20OH	0.722	0.819	0.875	1	0.781	0.646	0.638	0.689	0.698	0.676	0.603	0.103	0.571	0.584
22OH	0.861	0.826	0.813	0.781	1	0.846	0.888	0.892	0.841	0.687	0.554	0.198	0.796	0.829
14CHO	0.903	0.817	0.765	0.646	0.846	1	0.899	0.859	0.714	0.679	0.604	0.149	0.705	0.725
15CHO	0.932	0.856	0.754	0.638	0.888	0.899	1	0.916	0.861	0.692	0.578	0.23	0.821	0.829
16CHO	0.897	0.874	0.757	0.689	0.892	0.859	0.916	1	0.791	0.794	0.529	0.123	0.838	0.827
17CHO	0.761	0.761	0.684	0.698	0.841	0.714	0.861	0.791	1	0.701	0.628	0.352	0.753	0.752
18CHO	0.677	0.686	0.639	0.676	0.687	0.679	0.692	0.794	0.701	1	0.694	0.402	0.631	0.596
Cholesterol	0.642	0.631	0.644	0.603	0.554	0.604	0.578	0.529	0.628	0.694	1	0.528	0.579	0.573
7α-hydroxy-4-cholesten-3-one	0.114	0.076	0.129	0.103	0.198	0.149	0.23	0.123	0.352	0.402	0.528	1	0.22	0.232
Campesterol	0.824	0.769	0.628	0.571	0.796	0.705	0.821	0.838	0.753	0.631	0.579	0.22	1	0.977
β-Sitosterol	0.843	0.782	0.667	0.584	0.829	0.725	0.829	0.827	0.752	0.596	0.573	0.232	0.977	1

Table S6. Correlation coefficients between fatty alcohols, fatty aldehydes and sterols in para-carcinoma calculated by Spearman correlation analysis.

	14OH	16OH	18OH	20OH	22OH	14CHO	15CHO	16CHO	17CHO	18CHO	Cholesterol	7α-hydroxy-4 -cholesten-3-one	Campesterol	β-Sitosterol
14OH	1	0.918	0.736	0.61	0.672	0.889	0.922	0.896	0.872	0.42	0.579	0.226	0.77	0.769
16OH	0.918	1	0.829	0.697	0.629	0.834	0.861	0.855	0.835	0.378	0.518	0.109	0.693	0.683
18OH	0.736	0.829	1	0.798	0.72	0.805	0.729	0.775	0.763	0.64	0.591	0.353	0.655	0.651
20OH	0.61	0.697	0.798	1	0.612	0.705	0.543	0.683	0.656	0.739	0.73	0.397	0.711	0.639
22OH	0.672	0.629	0.72	0.612	1	0.781	0.767	0.713	0.761	0.603	0.496	0.419	0.657	0.672
14CHO	0.889	0.834	0.805	0.705	0.781	1	0.907	0.892	0.899	0.603	0.583	0.28	0.711	0.735
15CHO	0.922	0.861	0.729	0.543	0.767	0.907	1	0.908	0.918	0.42	0.538	0.236	0.741	0.783
16CHO	0.896	0.855	0.775	0.683	0.713	0.892	0.908	1	0.859	0.567	0.59	0.247	0.784	0.764
17CHO	0.872	0.835	0.763	0.656	0.761	0.899	0.918	0.859	1	0.494	0.619	0.382	0.727	0.726
18CHO	0.42	0.378	0.64	0.739	0.603	0.603	0.42	0.567	0.494	1	0.716	0.542	0.626	0.573
Cholesterol	0.579	0.518	0.591	0.73	0.496	0.583	0.538	0.59	0.619	0.716	1	0.553	0.811	0.741
7α-hydroxy-4 -cholesten-3-one	0.226	0.109	0.353	0.397	0.419	0.28	0.236	0.247	0.382	0.542	0.553	1	0.52	0.505
Campesterol	0.77	0.693	0.655	0.711	0.657	0.711	0.741	0.784	0.727	0.626	0.811	0.52	1	0.965
β-Sitosterol	0.769	0.683	0.651	0.639	0.672	0.735	0.783	0.764	0.726	0.573	0.741	0.505	0.965	1