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Characterization of nonpolar lipids and selected steroids by using laser-induced acoustic desorption/chemical ionization, atmospheric pressure chemical ionization, and electrospray ionization mass spectrometry

Zhicheng Jin[a], Shivani Daiya[b], Hilkka I. Kenttämaa[a,*]

[a] Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907, United States
[b] School of Chemical Engineering, Purdue University, 480 Stadium Mall Drive, West Lafayette, IN 47907, United States

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Dedicated to esteemed Professor Michael Gross on the occasion of his 70th birthday.

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ABSTRACT

Laser-induced acoustic desorption (LIAD) combined with $ClMn(H_2O)^+$ chemical ionization (CI) was tested for the analysis of nonpolar lipids and selected steroids in a Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR). The nonpolar lipids studied, cholesterol, 5α-cholestane, cholesta-3,5-diene, squalene, and β-carotene, were found to solely form the desired water replacement product (adduct-H_2O) upon reaction with the $ClMn(H_2O)^+$ ions. The steroids, androsterone, dehydroepiandrosterone (DHEA), estrone, estradiol, and estriol, also form abundant adduct-H_2O ions, but less abundant adduct-$2H_2O$ ions were also observed. Neither (+)APCI nor (+)ESI can ionize the saturated hydrocarbon lipid, cholestane. APCI successfully ionizes the unsaturated hydrocarbon lipids to form exclusively the intact protonated analytes. However, it causes extensive fragmentation for cholesterol and the steroids. The worst case is cholesterol that does not produce any stable protonated molecules. On the other hand, ESI cannot ionize any of the hydrocarbon analytes, saturated or unsaturated. However, ESI can be used to protonate the oxygen-containing analytes with substantially less fragmentation than for APCI in all cases except for cholesterol and estrone. In conclusion, LIAD/$ClMn(H_2O)^+$ chemical ionization is superior over APCI and ESI for the mass spectrometric characterization of underivatized nonpolar lipids and steroids.

1. Introduction

Lipids play crucial roles in cell, tissue and organ physiology [1]. Many lipids are nonpolar. These lipids include triacylglyerols and cholesterol [2]. Some naturally occurring hydrocarbons, including squalene and hydrocarbon carotenoids, also belong to the nonpolar lipid category. Carotenoids have important physiological and biological functions, such as provitamin A activity and antioxidant ability [3]. Unfortunately, mass spectrometric analysis of naturally occurring nonpolar hydrocarbons remains challenging because of their structural similarity, their thermal lability, and the lack of easily ionizable functional groups [4].

Various mass spectrometric ionization and evaporation methods, including electron ionization (EI) [5–7], electrospray ionization (ESI) [8–10], atmospheric pressure chemical ionization (APCI) [11–14], and matrix-assisted laser desorption ionization (MALDI) [15–17], have been employed in characterizing naturally occurring hydrocarbons and sterol lipids (including cholesterol and steroid hormones). However, each method has severe limitations. EI produces extensive fragmentation [18]. ESI and APCI are soft evaporation/ionization methods, but ESI is selective for polar compounds [19]. Although the sensitivity of APCI is low for polar and ionic compounds [20], APCI is often used for the analysis of squalene and carotenoids [4,14]. Derivatization is usually necessary to increase the volatility and/or ionization efficiency, or to allow chromatographic separation of steroid hormones [10,21,22]. However, derivatization is time-consuming, and can be a source of inaccuracy in quantitative analysis. MALDI suffers from interference of abundant matrix-derived ions in the low mass range.

Laser-induced acoustic desorption (LIAD) was recently demonstrated to be able to evaporate nonvolatile and thermally labile analytes as intact neutral molecules into a Fourier-transform ion cyclotron resonance mass spectrometer [23,24]. Because the high-intensity laser pulses in these experiments do not have a direct contact with the analyte, the analyte molecules are not ionized. Instead, desorption of neutral analyte molecules with low kinetic and internal energies takes place [24,25]. The evaporated neutral

* Corresponding author. Tel.: +1 765 494 0882; fax: +1 765 494 0239.
E-mail address: hilkka@purdue.edu (H.I. Kenttämaa).

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molecules can be ionized by using EI [26], chemical ionization [23], or ESI [27].

Compared to other ionization methods, chemical ionization (CI) allows more control over the efficiency and selectivity of ionization and the degree of fragmentation via proper selection of the reagent ion. CI mass spectra of sterol hydrocarbons and steroid hormones have been obtained by using CH_4, i-C_4H_{10}, and NH_3 as the reagent gases [28]. The gases CH_4 and i-C_4H_{10} mainly produce $M+H^+$ and dehydrated fragment ions ($M+H^+-nH_2O$). When using NH_3 as the reagent gas, $M+NH_4^+$, $M+H^+$ and dehydrated fragment ions ($M+H^+-nH_2O$) are commonly formed. Although some nonpolar lipids (such as 5α-cholestane) and polar lipids (steroid hormones) can be analyzed by using these methods [28], the issue of forming many ions from one analyte is a problem for mixture analysis.

Recently a novel chemical ionization reagent ion, $ClMn(H_2O)^+$, was demonstrated to ionize saturated hydrocarbons with no fragmentation of the analyte [19]. The reaction involves a simple replacement of the water molecule in the reagent ion with the hydrocarbon. The objective of this study was to test the utility of LIAD coupled with chemical ionization by using $ClMn(H_2O)^+$ reagent ions in an FT-ICR mass spectrometer for the analysis of nonpolar and polar lipids without pretreatment. The performance of this technique is compared to electrospray ionization and atmospheric pressure chemical ionization (both in positive ion mode) in a linear quadrupole ion trap mass spectrometer.

2. Experimental

Most experiments were carried out in a Nicolet model FTMS 2000 dual-cell FT-ICR mass spectrometer equipped with LIAD [29,30]. The instrument contains a differentially pumped dual cell equipped with a 3-Tesla superconducting magnet. The dimensions of each cell are 1.875 in. × 1.875 in. × 1.875 in. The cells are separated by a conductance limit with a 2-mm hole in the center. This plate and the other trapping plates were maintained at +2 V unless otherwise stated. The nominal baseline pressure inside the cells was less than 10^{-9} Torr, as maintained with two Edwards Diffstak 160 diffusion pumps (700 L/s). Each diffusion pump was backed by an Alcatel rotary vane mechanical pump (3.2 L/s). The pressure in the vacuum chamber was measured by Bayard–Alpert ionization gauges located on each side of the dual cell. Samples may be introduced into either side of the instrument by various ways, including a heated solids probe, Varian leak valves, batch inlets equipped with leak valves, pulsed valves, and a LIAD probe.

Cholesterol and five steroids, androsterone, dehydroepiandrosterone, estriol, estrone, and estradiol, were dissolved in methanol (99.9% purity, HPLC grade) at a concentration of 1.0 mg/mL. 5α-Cholestane, cholesta-3,5-diene, squalene, and β-carotene were dissolved in a mixture of acetonitrile (99.9% purity, HPLC grade) and dichloromethane (99.9% purity) (1:1, v/v) at a concentration of 1.0 mg/mL. 100 μL of each solution was deposited on thin Titanium foils (12.5 μm) by electrospray deposition [31]. The solvent was allowed to evaporate after sample deposition, and the foil was transferred to the sample support stage of the LIAD probe. The LIAD probe (outer diameter 7/8 in.) employed in this study was described previously [32]. The side of the foil coated with sample was exposed to the dual cell. Laser pulses generated by a Nd:YAG laser (Minilite II, Continuum Lasers; 532 nm; 3 ns pulse width) were delivered through an optical fiber and focused to an area of about 10^{-3} cm^2 on the back side of the foil. The output energy of the laser pulse was 3.6 mJ/pulse, as measured by a pyroelectric meter (PE25-SH, OPHIR Laser Measurement), corresponding to a power density of about 8×10^8 W/cm^2 at the foil surface. The outer cylinder of the LIAD probe was rotated so that analytes were desorbed from multiple spots. The probe was rotated at a speed of one degree per laser pulse (10 Hz). Typically, 10–20 laser pulses were used for each experiment.

A ligated water cluster of Mn^+, $ClMn(H_2O)^+$, was generated by electron ionization of $ClMn(CO)_5$ [19]. The $ClMn(CO)_5$ precursor was synthesized from $Mn(CO)_{10}$ according to a literature procedure [33]. The precursor was introduced into one side of the dual cell by using a solids probe (without heating the probe). Water vapor was introduced into the same side of the dual cell via a batch inlet system equipped with a variable leak valve. The reagent ion, $ClMn(H_2O)^+$, was generated by electron ionization (ionization energy 25 eV, emission current 7 μA, and beam duration 0.05 s) of the $ClMn(CO)_5$ and H_2O mixture. The reagent ion was then transferred into the other side of the dual cell by grounding the conductance limit for about 100 μs while the other trapping plates were maintained at +2 V. The transferred ions were cooled by collisions with argon gas which was pulsed into the cell (peak nominal pressure of 1×10^{-5} Torr) via a pulsed valve assembly. The reagent ions were then isolated by ejecting unwanted ions via the use of a series of stored-waveform inverse Fourier transform (SWIFT) excitation pulses [34]. The isolated $ClMn(H_2O)^+$ ions were allowed to react with the lipid molecules desorbed into the same cell by LIAD using 10–15 laser shots. A broadband chirp excitation (1.9 kHz to 2.6 MHz, 200 V peak-to-peak, chirp rate 3200 Hz/μs) was used to excite all ions for detection. The spectra were obtained by collecting 64 k data points at an acquisition rate of 5333 kHz. All mass spectra were subjected to Hanning apodization followed by one zero-fill prior to Fourier transformation.

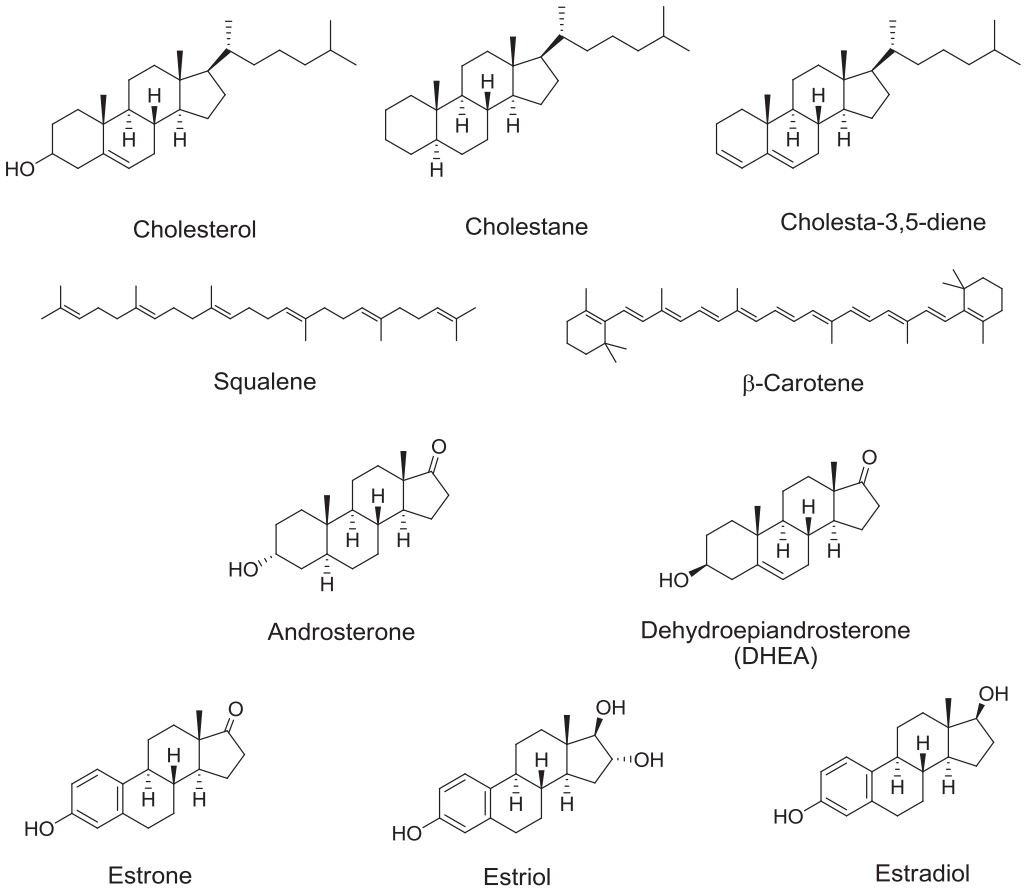
A linear quadrupole ion trap mass spectrometer (Thermo Fisher Scientific) was used to examine the same analytes by using ESI and APCI. Analyte solutions were prepared in a mixture of water and methanol at a concentration of 0.01–0.1 mg/mL for ESI and 0.1–0.5 mg/mL for APCI. For ESI, the solution was directly injected into the ionization source at 3–5 μL/min by using an integrated syringe drive. The ESI conditions were as follows: spray voltage, 4.5–5 kV; sheath gas flow, 10 (arbitrary units); capillary temperature 275 °C. For APCI, the analyte solution was mixed with a solution of methanol and water (50/50, v/v) through a union. The analyte solution was injected by using the integrated syringe drive at a flow rate of 10 μL/min and the solution of methanol and water (50/50, v/v) was delivered by a HPLC pump at a flow rate of 190 μL/min. The entire mixture was delivered into the mass spectrometer. The APCI conditions were as follows: vaporizer temperature, 450 °C; sheath gas flow rate, 50 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); capillary temperature, 275 °C; tube lens, 15 V.

3. Results and discussion

Five nonpolar lipids and five steroids (Scheme 1) were analyzed by using LIAD/$ClMn(H_2O)^+$ in an FT-ICR. All analytes were successfully evaporated into the mass spectrometer by using LIAD. Cholesterol, 5α-cholestane, cholesta-3,5-diene, squalene, and β-carotene reacted with the CI reagent ion by replacement of the water molecule, as expected. For the steroids studied, the water replacement product ion was the main ionic reaction product although fragment ions were also observed. Details of these results, as well as their comparison to the results obtained for the same analytes by using (+)APCI and (+)ESI in a linear quadrupole ion trap, are given below.

3.1. LIAD/CI, APCI, and ESI of nonpolar lipids

LIAD/$ClMn(H_2O)^+$ was found to ionize the saturated hydrocarbon lipid, 5α-cholestane, via water replacement and without fragmentation (Table 1), as expected [19,35]. Similarly, $ClMn(H_2O)^+$ reacts with the unsaturated hydrocarbons cholesta-3,5-diene,



Scheme 1. Chemical structures of the nonpolar lipids and steroid hormones used in this study.

squalene (Fig. 1) and β-carotene, as well as cholesterol, by exclusively forming the water replacement product with no fragmentation (Table 1).

In contrast, APCI did not yield any detectable ions for cholestane. However, this method produced stable protonated molecules for squalene, β-carotene, and cholesta-3,5-diene (but not for cholesterol), with no fragmentation. ESI proved to be useless for the characterization of these analytes. No ion signal was detected for any of the hydrocarbon analytes when subjected to ESI. For cholesterol, so extensive fragmentation took place that no protonated cholesterol was observed.

The differences between the results obtained by APCI and ESI are likely due to their quite different ionization mechanisms. Analytes are ionized by proton or metal cation transfer in highly charged droplets in ESI [36]. The low basicity of nonpolar lipids prevents this mode of ionization. In contrast, an APCI source initially forms nitrogen and solvent molecular ions upon corona discharge. These ions then produce protonated solvent and water cluster ions [37,38] that are the species usually responsible for ionization of the analyte molecules by proton transfer. However, these ions are unreactive toward nonpolar lipids due to their low basicity. Instead, the initially produced nitrogen and solvent radical cations react with the lipids, producing lipid radical cations. These ions yield protonated lipid molecules in secondary reactions with neutral lipid molecules.

3.2. LIAD/CI, APCI, and ESI of sterol lipids

$ClMn(H_2O)^+$ reacts with androsterone predominantly by forming the water elimination product, as expected. Some fragment ions

Table 1
Ions (with their branching ratios) formed upon LIAD/CI, APCI, and ESI of selected nonpolar lipids.

Lipids	LIAD/CI (+)	APCI (+)	ESI (+)
Cholesterol	Adduct-H_2O 100%	M−H, 2%, M+H–H_2O 98%	M+H–H_2O 100%
5α-Cholestane	Adduct-H_2O 100%	No ions detected	No ions detected
Cholesta-3,5-diene	Adduct-H_2O 100%	M+H 100%	No ions detected
β-Carotene	Adduct-H_2O 100%	M+H 100%	No ions detected
Squalene	Adduct-H_2O 100%	M+H 100%	No ions detected
Androsterone	Adduct-H_2O 79%, adduct-2H_2O 21%	M+H 14%, M+H–H_2O 60%, M+H–2H_2O 26%	M+H 61%, M+H–H_2O 32%, M+H–2H_2O 7%
Dehydroepiandrosterone (DHEA)	Adduct-H_2O 90%, adduct-2H_2O 10%	M+H 9%, M+H–H_2O 62%, M+H–2H_2O 29%	M+H 57%, M+H–H_2O 37%, M+H–2H_2O 6%
Estrone	Adduct-H_2O 64%, adduct-2H_2O 36%	M+H 91%, M+H–H_2O 9%	M+H 81%, M+H–H_2O 19%
Estradiol	Adduct-H_2O 65%, adduct-2H_2O 35%	M+H 13%,M−H 10%, M+H–H_2O 77%	M+H 37%, M−H 7%, M+H–H_2O 56%
Estriol	Adduct-H_2O 38%, adduct-2H_2O 19%, adduct-3H_2O 28%, adduct-3H_2O–acetylene 15%	M+H 4%, M−H 8%, M+H–H_2O 56%, M+H–2H_2O 25%, M+H–2H_2O–acetylene 7%	M+Na 14%, M+H 12%, M−H 8%, M+H–H_2O 51%, M+H–2H_2O 6%, M+H–2H_2O–acetylene 9%

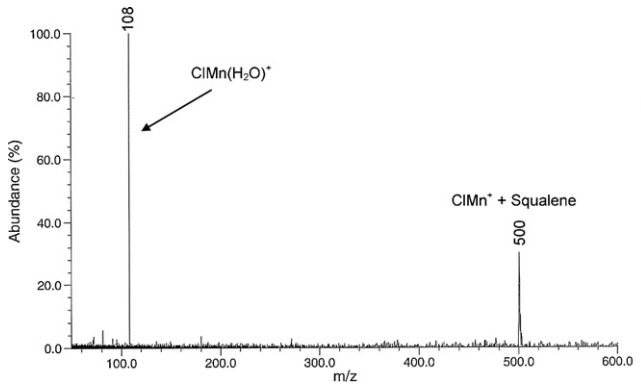


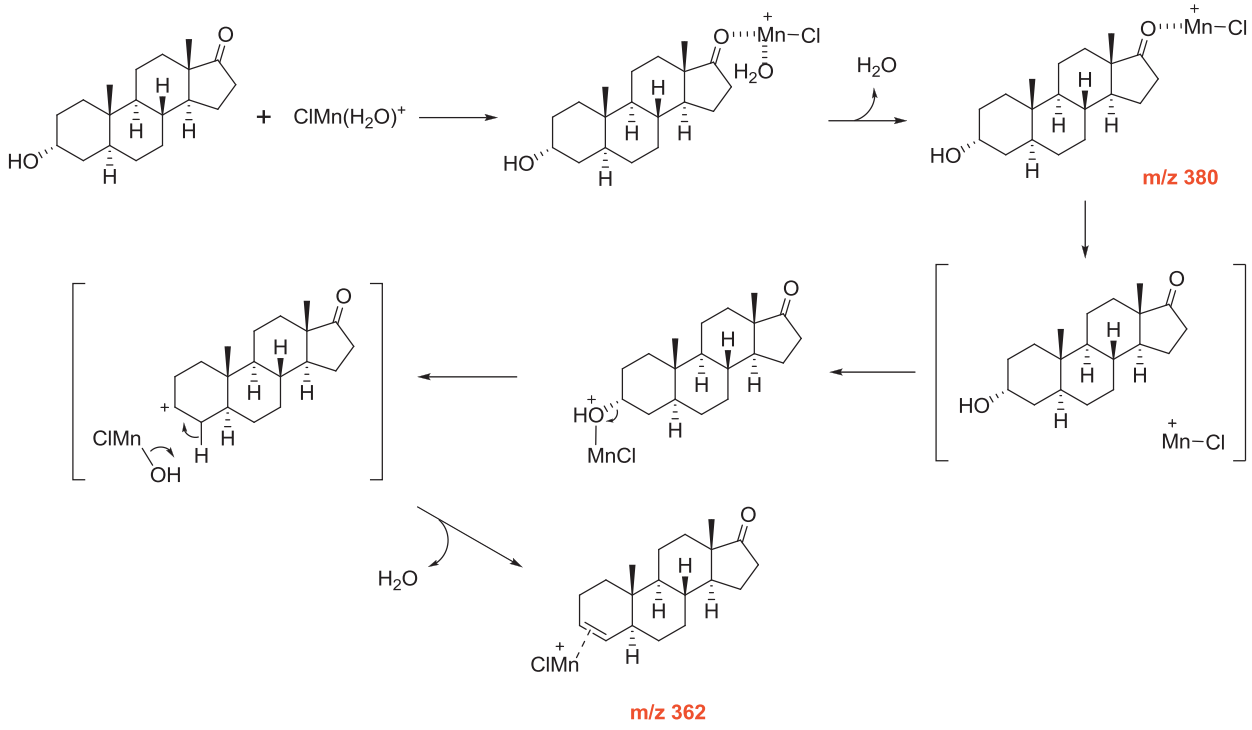
Fig. 1. LIAD/CI mass spectrum of squalene (MW 410).

formed by elimination of another water molecule (branching ratio 21%) were also observed (Table 1). Possible mechanisms for the reaction sequence are presented in Scheme 1. First, the water ligand of $ClMn(H_2O)^+$ is replaced by androsterone, forming an ion of m/z 380, wherein $MnCl^+$ is weakly bound to the carbonyl group (the most nucleophilic site in the molecule). Loss of the second water molecule may involve transfer of the $MnCl^+$ ion around androsterone to enable it to interact with the hydroxyl group. These sorts of reactions have been reported previously for ion-molecule complexes of steroids in mass spectrometers [39]. Alternatively, it is possible that interaction of some $ClMn(H_2O)^+$ ions with the carbonyl group leads to elimination of one water molecule, while interaction of other $ClMn(H_2O)^+$ ions with the less nucleophilic hydroxyl group leads to the consecutive elimination of two water molecules.

Dehydroepiandrosterone (DHEA), estrone, estradiol, and estriol react with $ClMn(H_2O)^+$ similarly as androsterone (Table 1). The phenol hydroxyl group is not eliminated as water from estrone and estradiol, as expected due to the strong phenyl-oxygen bond. The small branching ratio for the second water elimination product (10%) for DHEA, and the complete lack of this product for cholesterol, suggests that after the first water elimination, the $ClMn^+$ ion may be more strongly bound to the carbon–carbon double bond than to the carbonyl or hydroxyl group in these analytes. This prevents $ClMn^+$ from reaching the hydroxyl group, which would eventually result in elimination of the second water molecule. This finding provides further support for the mechanism presented in Scheme 2. Two additional fragment ions, formed by elimination of a total of three water molecules from the adduct and a further elimination of acetylene, were observed for estriol. The mechanism of the acetylene elimination is under investigation.

With the exception of estrone, ESI and APCI produce much more extensive fragmentation for the polar lipids (Table 1) than $LIAD/ClMn(H_2O)^+$. For example, a fragment ion of m/z of 369, which corresponds to water loss after protonation of cholesterol, dominates the ESI and APCI spectra of cholesterol. No stable protonated cholesterol molecules were observed in either the ESI or APCI spectra (Table 1). Apparently, the proton transfer reactions occurring in the ESI and APCI sources are exothermic enough to cause water elimination for all protonated cholesterol molecules. In the LIAD/CI experiment, the water ligand in the reagent ion is weakly bond and readily replaced. Further fragmentation is minor because the water loss lowers the energy of the system [19].

Protonated molecules dominate the ESI mass spectra of androsterone, DHEA and estrone (Table 1). The difference in the behavior of these analytes and cholesterol is the nature of the most basic site, a carbonyl group (that is stable after protonation) as opposed to a hydroxyl group (whose protonation readily results in the loss of water). APCI produces substantially more fragmentation than ESI for all these analytes with the exception of estrone, which suggests that the proton transfer reactions in this ion source are more exothermic than in the ESI source [20]. Estrone was found to predominantly form a stable protonated molecule upon ESI and APCI. This may be explained by the lack of non-aromatic hydroxyl groups in estrone.



Scheme 2. A possible reaction pathway for androsterone.

4. Conclusions

Laser-induced acoustic desorption coupled with chemical ionization by $ClMn(H_2O)^+$ allowed the evaporation and ionization of all the nonpolar and polar lipids studied. Adduct-H_2O is the exclusive product ion for cholesterol and all the hydrocarbons, including the saturated hydrocarbon, cholestane. Also for steroids, adduct-H_2O is the main product ion, but it is usually formed along with some adduct-$2H_2O$ ions due to the presence of hydroxyl or carbonyl groups in these analytes. This method is the only one studied that is capable of ionizing cholestane; no ions were observed for this analyte upon APCI or ESI.

APCI ionizes the unsaturated hydrocarbon lipids to form exclusively the intact protonated analytes. However, it causes extensive fragmentation for the oxygen-containing analytes. The worst case is cholesterol for which no stable protonated molecules were observed. On the other hand, ESI is not able to ionize any of the hydrocarbon analytes, saturated or unsaturated. However, ESI can be used to protonate the oxygen-containing analytes with substantially less fragmentation than for APCI in all cases except for cholesterol (extensive fragmentation) and estrone (no difference).

In conclusion, LIAD/$ClMn(H_2O)^+$ is the only method examined that is suitable for the mass spectrometric characterization of underivatized saturated hydrocarbon lipids. APCI (but not ESI) can be used to analyze unsaturated hydrocarbons. Both ESI and APCI are capable of ionizing the polar analytes but they both cause much more extensive fragmentation than $ClMn(H_2O)^+$ (the only exception being estrone).

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