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# Loss of H<sub>2</sub> and CO from protonated aldehydes in electrospray ionization mass spectrometry

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**RATIONALE:** Electrospray ionization mass spectrometry (ESI-MS) of many protonated aldehydes shows loss of CO as a major fragmentation pathway. However, we find that certain aldehydes undergo loss of H<sub>2</sub> followed by reaction with water in the collision cell. This complicates interpretation of tandem mass (MS/MS) spectra and affects multiple reaction monitoring (MRM) results.

**METHODS:** 3-Formylchromone and other aldehydes were dissolved in acetonitrile/water/formic acid and studied by ESI-MS to record their MS<sup>2</sup> and MS<sup>n</sup> spectra in several mass spectrometers (QqQ, QTOF, ion trap (IT), and Orbitrap HCD). Certain product ions were found to react with water and the rate of reaction was determined in the IT instrument using zero collision energy and variable activation times. Theoretical calculations were performed to help with the interpretation of the fragmentation mechanism.

**RESULTS:** Protonated 3-formylchromones and 3-formylcoumarins undergo loss of H<sub>2</sub> as a major fragmentation route to yield a ketene cation, which reacts with water to form a protonated carboxylic acid. In general, protonated aldehydes which contain a vicinal group that forms a hydrogen bridge with the formyl group undergo significant loss of H<sub>2</sub>. Subsequent losses of CO and C<sub>3</sub>O are also observed. Theoretical calculations suggest mechanistic details for these losses.

**CONCLUSIONS:** Loss of H<sub>2</sub> is a major fragmentation channel for protonated 3-formylchromones and certain other aldehydes and it is followed by reaction with water to produce a protonated carboxylic acid, which undergoes subsequent fragmentation. This presents a problem for reference libraries and raises concerns about MRM results. Published in 2014. This article is a U.S. Government work and is in the public domain in the USA.

In the course of expanding the NIST library of MS/MS spectra<sup>[1]</sup> for use in metabolomics, we recorded the spectra of various flavonoid compounds and their components, such as chromones and coumarins, using electrospray ionization in conjunction with several types of mass spectrometers. As for the NIST/EPA/NIH EI Library, collected spectra are manually evaluated<sup>[2]</sup> before they are added to the library. This process often involves comparison of spectra from different mass spectrometers and assignment of each peak in the spectrum to a reasonable product ion structure. Peak assignment is further enhanced by the use of instruments with very high mass accuracy. Recent measurements included those of several aldehydes derived from chromones and coumarins, and we noticed that some of them exhibit significant loss of H<sub>2</sub> from the protonated molecules while others undergo mainly loss of CO. We also noted the presence of unexpected product ions apparently arising from water adduction. We therefore undertook a more detailed study of this fragmentation process.

Aldehydes have been the subject of numerous mass spectrometric studies, where they were measured indirectly following derivatization with 2,4-dinitrophenylhydrazine or similar reagents.<sup>[3–8]</sup> The aim of some of those studies was to

determine the aldehydes formed in biological samples or in lipid peroxidation, and the aldehydes were derivatized because direct quantitation was difficult. Another complication arose when aromatic aldehydes were analyzed directly, without derivatization, by electrospray ionization mass spectrometry (ESI-MS) in methanol/water solutions.<sup>[9]</sup> A reaction of the aldehyde with methanol in the ESI source converted a large fraction of the initial protonated molecules (Ph-CHOH<sup>+</sup>) into a methylated derivative (Ph-CHOCH<sub>3</sub><sup>+</sup>) by addition of methanol and elimination of water. We confirmed this reaction with benzaldehyde, where the peak of [M+H]<sup>+</sup> at *m/z* 107 was accompanied by a peak at *m/z* 121, corresponding formally to [M+CH<sub>3</sub>]<sup>+</sup>. To avoid this complication we carried out most of our measurements in acetonitrile/water solutions, where the methylated derivative is not observed. We also compared the tandem mass (MS/MS) spectra of several [M+H]<sup>+</sup> ions observed in the two solvent mixtures and found them to be identical.

In this study we analyze the MS/MS spectra of the [M+H]<sup>+</sup> ions formed from various aldehydes, to examine whether loss of H<sub>2</sub> or CO predominates and how the structure of the aldehyde determines the relative contributions of these two dissociation reactions. We use mostly acetonitrile/water/formic acid (50/50/0.1) as the solvent mixture, to avoid the above complication with methanol, but in some cases we also use methanol instead of acetonitrile to compare the results. All the aldehydes were examined first in a triple quadrupole mass spectrometer and selected compounds were then examined in different instruments.

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## EXPERIMENTAL<sup>a</sup>

3-Formylchromone, several of its alkylated, methoxylated, and fluorinated derivatives, 7-diethylamino-3-formylcoumarin, and 7-formyl-6-nitrocoumarin (98–99%) were obtained from Indofine Chemical Company (Hillborough, NJ, USA). The other aldehydes were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) in the purest grade available. The solvents were LC/MS grade. D<sub>2</sub>O (99.9% D) was obtained from Sigma. The aldehydes were dissolved in micromolar concentrations in acetonitrile/water/formic acid or methanol/water/formic acid (50/50/0.1, v/v/v) mixtures. Experiments were also carried out using D<sub>2</sub>O instead of H<sub>2</sub>O. ESI-MS was carried out with a Micromass Quattro Micro triple quadrupole instrument (QqQ) (Waters Corp., Milford, MA, USA). First the mass spectra were recorded at different cone voltages to optimize the abundances of the precursor ions. The precursor ion at the optimal cone voltage was then selected for fragmentation in the collision cell [with 0.21 Pa (1.6 mTorr) Ar as collision gas] and the MS/MS spectrum was recorded at 20 different collision voltages. The range of collision voltage spanned from near zero V up to a value where no precursor ion remained. The peak intensities of all the significant product ion peaks were calculated as a fraction from the total ion current and plotted as a function of collision voltage. Pseudo-MS<sup>3</sup> spectra were measured by using a high cone voltage so as to produce the product ion in the cone region and this ion was then selected for MS/MS measurement as above. Spectra were acquired in 'centroid' mode, whereby signals within each individual time interval in a given spectrum were centered and integrated by the instrument data system. Typically, *m/z* values were within 0.2 of the theoretical *m/z* values throughout the *m/z* range of interest.

To examine the influence of type of collision cell, MS/MS spectra of some of the ions were also measured by ion trap (IT) fragmentation (LTQ, Thermo Fisher Scientific, Waltham, MA, USA) with 0.35 mTorr He as collision gas, using another beam-type collision cell (QTOF instrument, model 6530; Agilent, Santa Clara, CA, USA) with 0.02 mTorr N<sub>2</sub>, and using HCD (Higher-energy C-trap dissociation) in orbital ion trap (OIT) instruments (Orbitrap Velos and Elite, and QExactive, Thermo Fisher Scientific) with 3.75 mTorr N<sub>2</sub>. Both MS<sup>2</sup> and MS<sup>3</sup> spectra were obtained with the LTQ.

DFT (density functional theory) calculations were performed using the hybrid density functional method B3LYP<sup>[10,11]</sup> in conjunction with Pople's basis set [6-311++g(d,p)] as implemented in Gaussian 09.<sup>[12]</sup> For all the optimized structures, frequency analysis at the same level of theory was used to identify them as real minima and transition structures on the potential energy surface. Intrinsic reaction coordinate calculations were performed to confirm that specific transition states were connected to the

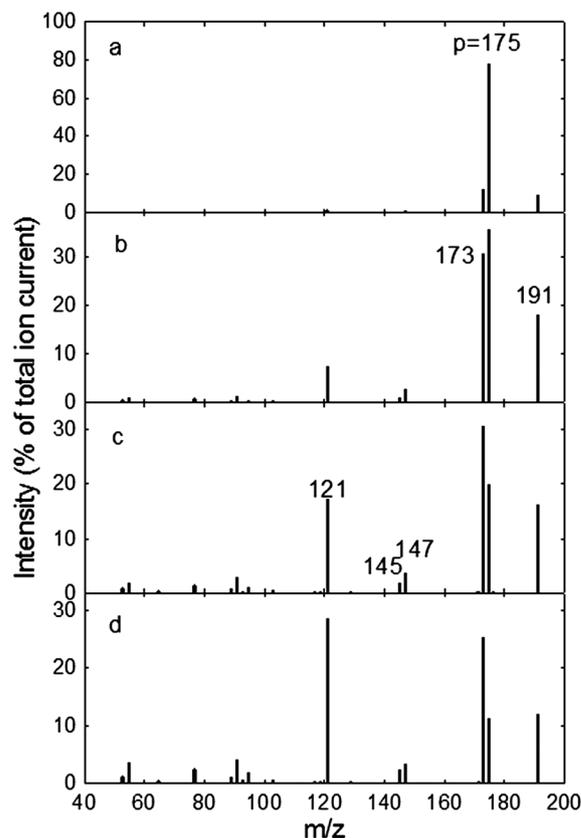
designated local minima. These calculations have been effectively used in previous studies of ion fragmentation under CID conditions.<sup>[13,14]</sup>

## RESULTS AND DISCUSSION

Tandem mass spectra of all the compounds were obtained first in the QqQ (Micromass) mass spectrometer and then selected compounds were compared in various instruments. We describe first the QqQ results.

### Tandem mass spectra in the QqQ mass spectrometer

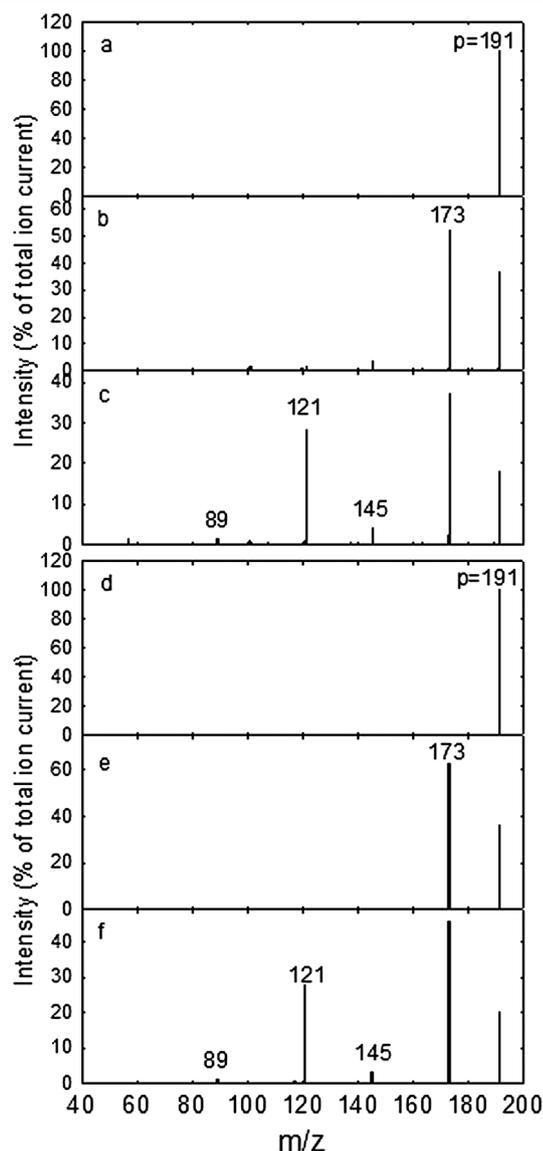
The first compound examined in detail was 3-formylchromone. The MS/MS spectrum of its [M+H]<sup>+</sup> ion (**1a**) at *m/z* 175 shows major product ion peaks at *m/z* 173, for [M+H-H<sub>2</sub>]<sup>+</sup> (**1b**), and at *m/z* 147, for [M+H-CO]<sup>+</sup> (**1c**). Additional peaks are detected at *m/z* 145, for [M+H-H<sub>2</sub>-CO]<sup>+</sup>, at *m/z* 121, for [M+H-H<sub>2</sub>-C<sub>3</sub>O]<sup>+</sup> or [M+H-C<sub>3</sub>H<sub>2</sub>O]<sup>+</sup>, and smaller fragments. However, a peak at *m/z* 191 is also observed, which is ascribed to the product of reaction of the [M+H-H<sub>2</sub>]<sup>+</sup> product ion with water to form [M+H-H<sub>2</sub>+H<sub>2</sub>O]<sup>+</sup> (formally [M+H+O]<sup>+</sup>) (**1d**) (Fig. 1). These assignments for 3-formylchromone and several other aldehydes were confirmed by exact mass measurements using the Orbitrap instrument (see Table 2 and related discussion).



**Figure 1.** MS/MS spectra of the [M+H]<sup>+</sup> ion of 3-formylchromone (precursor, *p*, *m/z* 175) at collision voltage 14 V (a), 18 V (b), 20 V (c), and 22 V (d) in the QqQ mass spectrometer. The intensity scale is the abundance of the ion as a percentage of the total ion current.

<sup>a</sup>Certain commercial equipment, instruments, or materials are identified in this document. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.





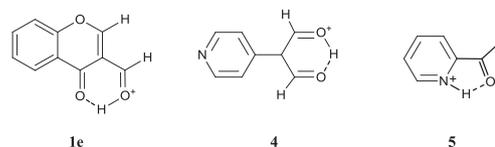
**Figure 4.** Comparison of the MS/MS spectra of the  $[M+H-H_2+H_2O]^+$  ion ( $m/z$  191), produced from 3-formylchromone in the ion source at cone voltage 60 V, at collision voltage 2 V (a), 14 V (b), and 20 V (c) in the QqQ mass spectrometer, with the MS/MS spectra of the  $[M+H]^+$  ion of authentic chromone-3-carboxylic acid under the same collision voltages (d, e, f, respectively).

To elucidate the reasons for this difference in behavior we examined several other aldehydes (Table 1). Protonated benzaldehyde ( $C_6H_5CHOH^+$ ) undergoes predominantly loss of CO (to form  $C_6H_7^+$ ), with no detectable loss of  $H_2$ , but the product ion  $C_6H_7^+$  undergoes loss of  $H_2$  to form  $C_6H_5^+$ , which then reacts with  $H_2O$  to form the protonated phenol,  $C_6H_5OH_2^+$ , i.e.  $[M+H-CO-H_2+H_2O]^+$ . The three isomeric pyridine aldehydes exhibited somewhat different behaviors. The protonated 3- and 4-isomers formed product ions equivalent to those formed from benzaldehyde, i.e. first loss of CO and only then loss of  $H_2$  and subsequent hydration. Protonated 2-pyridinecarboxaldehyde, however, exhibited loss of CO and also loss of  $H_2$  followed by addition of water to form the protonated carboxylic acid. This behavior is

similar to that observed with 3-formylchromone, but the loss of  $H_2$  is much less favorable in 2-pyridinecarboxaldehyde than in 3-formylchromone; the ratio of  $H_2$  to CO loss was only 0.16 compared with 9.4. By comparison with these results, the finding that loss of  $H_2$  was not observed with the 3- and 4-isomers indicates that the electron-withdrawing effects of the pyridyl nitrogen or the chromone carbonyl groups are not an important factor in determining whether the protonated molecule undergoes loss of  $H_2$ .

Experiments with other aldehydes support this conclusion. Benzaldehyde bearing electron-withdrawing groups, such as CN or  $CF_3$ , exhibited the same behavior as benzaldehyde, with no evidence for loss of  $H_2$  from the protonated molecule. 2-Furan- and 2-pyrrole-carboxaldehyde also behaved similarly. 2-Imidazolecarboxaldehyde exhibited no loss of  $H_2$ , but 4(5)-imidazolecarboxaldehyde clearly exhibited loss of  $H_2$ , almost to the same extent as the 2-pyridinecarboxaldehyde. The reason for the difference between the two isomeric imidazole aldehydes remains unclear.

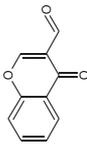
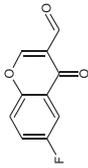
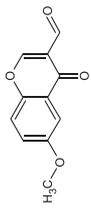
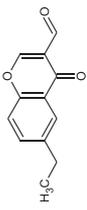
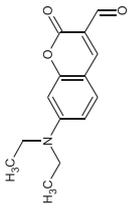
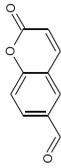
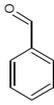
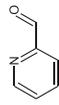
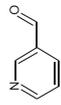
The above results suggest that the vicinity of other groups to the aldehyde group is an important factor in the fragmentation mechanism. To test this hypothesis we compared a compound that has two aldehyde groups on the same aliphatic carbon atom with a compound that has one aldehyde and one methyl group. The only compounds that were available for this comparison were (4-pyridyl)malonaldehyde (see structure 4) and 2-phenylpropionaldehyde (we could not procure two such compounds with the same ring). The latter compound exhibited no loss of  $H_2$ , whereas the former underwent  $H_2$  loss more than any other compound examined (Table 1); the ratio of  $H_2$  to CO loss was twice as high as that found with 3-formylchromone. Both 3-formylchromone and (4-pyridyl)malonaldehyde contain another carbonyl group at the  $\beta$ -position to the aldehyde group. Such compounds are known to form stable hydrogen-bridged structures and may do so when protonated (structures 1e and 4).



A similar, though more strained, hydrogen bridge may form in protonated 2-pyridinecarboxaldehyde (5). The stability of these hydrogen-bridged dicarbonyl structures may enhance loss of  $H_2$ . Further details of the mechanism will be discussed below.

By comparison with these stabilized ring structures, positioning an aldehyde group *ortho* to a hydroxyl group, such as in salicylaldehyde, does not provide sufficient stabilization and results in loss of  $H_2$  of only 0.8%, with most of the initial fragmentation leading to loss of CO (53% of total ions at the maximum level). However, positioning two aldehyde groups *ortho* to each other, such as in 2,3-naphthalenedicarboxaldehyde (Table 1), results in a ring structure of intermediate stability and loss of  $H_2$  from the protonated molecule, reaching 4.6% at its maximum level. In this compound, loss of one CO was also a minor process, reaching only 1.9%, but loss of two CO molecules is predominant, reaching 56% of total ions.

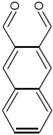
**Table 1.** Product ions observed in the MS/MS spectra of several protonated aldehydes (precursor = [M+H]<sup>+</sup>)<sup>a,b</sup>

Compound (M)	Structure	[M+H-H <sub>2</sub> ] <sup>+</sup>	[M+H-H <sub>2</sub> +H <sub>2</sub> O] <sup>+</sup>	[M+H-CO] <sup>+</sup>	[M+H-H <sub>2</sub> -CO] <sup>+</sup> <sup>c</sup>	Other product ions
3-Formylchromone		32	15	3.4	2.3	42 [M+H-H <sub>2</sub> -C <sub>3</sub> O] <sup>+</sup>
6-Fluoro-3-formylchromone		33	14	4	2.3	42 [M+H-H <sub>2</sub> -C <sub>3</sub> O] <sup>+</sup>
6-Methoxy-3-formylchromone		33	14	2	2.4	26 [M+H-H <sub>2</sub> -C <sub>3</sub> O] <sup>+</sup>
6-Ethyl-3-formylchromone		27	11	6.8 <sup>d</sup>	6.7 <sup>d</sup>	34 [M+H-H <sub>2</sub> -C <sub>3</sub> O] <sup>+</sup>
7-Diethylamino-3-formylcoumarin		29	1.3	8.3 <sup>d</sup>	1.3 <sup>d</sup>	16 [M+H-44] <sup>+</sup>
6-Formylcoumarin		1.4	0.24	2.8	0.28	9 [M+H-44] <sup>+</sup>
Benzaldehyde				85	49	16 [M+H-CO-H <sub>2</sub> +H <sub>2</sub> O] <sup>+</sup>
2-Pyridinecarboxaldehyde		6.3	3.7	40	26	9 [M+H-CO-H <sub>2</sub> +H <sub>2</sub> O] <sup>+</sup>
3-Pyridinecarboxaldehyde				61	5	3 [M+H-CO-H <sub>2</sub> +H <sub>2</sub> O] <sup>+</sup> , 7 [M+H-CHO] <sup>+</sup>

(Continues)

Table 1. (Continued)

Compound (M)	Structure	[M+H-H <sub>2</sub> ] <sup>+</sup>	[M+H-H <sub>2</sub> +H <sub>2</sub> O] <sup>+</sup>	[M+H-CO] <sup>+</sup>	[M+H-H <sub>2</sub> -CO] <sup>+</sup> c	Other product ions
4-Pyridinecarboxaldehyde				76	5	1.5 [M+H-CO-H <sub>2</sub> +H <sub>2</sub> O] <sup>+</sup> , 19 [M+H-CHO] <sup>+</sup>
2-(Trifluoromethyl)benzaldehyde						97 [M+H-HF] <sup>+</sup> , 80 [M+H-HF-CO] <sup>+</sup>
4-Cyanobenzaldehyde				52		52 [M+H-CO-HCN] <sup>+</sup> , 5 [M+H+H <sub>2</sub> O] <sup>+</sup> , 11 [M+H-CO-HCN+H <sub>2</sub> O] <sup>+</sup>
2-Furancarboxaldehyde				40		
2-Pyrrolecarboxaldehyde				78		
2-Imidazolecarboxaldehyde				72		
4-Imidazolecarboxaldehyde				58	2.8	
2-Phenylpropionaldehyde					1.5	
(4-Pyridyl)malonaldehyde					5.6	
				1.9	52	33 [M+H-H <sub>2</sub> O] <sup>+</sup> , 24 [M+H-H <sub>2</sub> O-H <sub>2</sub> ] <sup>+</sup>
						12 [M+H-CO-H <sub>2</sub> O] <sup>+</sup> , 12 [M+H-H <sub>2</sub> -2CO] <sup>+</sup>

Salicylaldehyde		0.8	53	65 [M+H-CO-H <sub>2</sub> O] <sup>+</sup>
2,3-Naphthalenedicarboxaldehyde		4.6	1.9	56 [M+H-2CO] <sup>+</sup>

<sup>a</sup>The relative abundances of the peaks due to loss of H<sub>2</sub>, loss of CO, and sequential loss of H<sub>2</sub> and CO are indicated for all the aldehydes, if observable.

<sup>b</sup>The values in columns 3 to 7 are percentage intensity of the ion, compared with total ion current, at the collision voltage where that ion reached its maximum abundance. In the last column other product ions are listed for various aldehydes to indicate significant peaks in the MS/MS spectra.

<sup>c</sup>This product ion is formed by loss of H<sub>2</sub> and CO in two steps; the sequence of losses varies with structure and depends on the dominant first loss.

<sup>d</sup>These values are probably too high and represent the combined losses of CO and C<sub>2</sub>H<sub>4</sub>.

In summary, the proximity of an electronegative group to the aldehyde group is critical in determining whether the ion loses H<sub>2</sub>. The electronegative neighbor has to be situated in a position that promotes formation of a stable hydrogen-bridged structure which allows it to undergo significant loss of H<sub>2</sub>. The above reactions explain some of the unexpected peaks in the MS/MS spectra of protonated aldehydes. In those cases where the protonated aldehyde undergoes significant loss of H<sub>2</sub>, the resulting product ion is converted into the protonated carboxylic acid, which is manifested in a product ion equivalent to an unexpected [M+H+O]<sup>+</sup>, which is in reality [M+H-H<sub>2</sub>+H<sub>2</sub>O]<sup>+</sup>, and to formation of product ions from the protonated carboxylic acid, not formed directly from the original aldehyde. In benzaldehyde and other compounds, which do not undergo direct loss of H<sub>2</sub> from the protonated molecules, but do so after loss of CO, subsequent hydration leads to a product ion equivalent overall to [M+H-C]<sup>+</sup>, which is not expected by any direct mechanism but is in reality [M+H-CO-H<sub>2</sub>+H<sub>2</sub>O]<sup>+</sup>. These complications must be taken into account when examining the MS/MS spectra of protonated aldehydes and may greatly influence results of MRM measurements.

Loss of H<sub>2</sub> from protonated molecules has been reported before for ions of small molecules, such as CH<sub>2</sub>O and CH<sub>2</sub>NH<sup>[17]</sup> and benzene and toluene.<sup>[18,19]</sup> The relative contribution of H<sub>2</sub> loss, however, is diminished in larger molecules as alternative dissociation pathways become more likely. In fact, a search among the 6000 compounds included in the latest release of the NIST MS/MS library (NIST, Gaithersburg, MD, USA) for losses of even minimal (>0.1% of total ion current) amounts of H<sub>2</sub> from [M+H]<sup>+</sup> ions found very few cases and with only small contributions of H<sub>2</sub> loss. Some of these are tertiary amines with at least one  $\alpha$ -hydrogen, enabling loss of H<sub>2</sub> between the  $\alpha$ -hydrogen and the proton attached to the amine nitrogen (as in Williams and Hvistend<sup>[17]</sup> for CH<sub>2</sub>NH<sub>2</sub><sup>+</sup>). However, the extent of H<sub>2</sub> loss is much smaller than that observed with the aldehyde discussed above and leads us to conclude that the high relative contributions for H<sub>2</sub> loss observed with certain aldehydes remain specific to these types of compounds.

### Comparison of results in different mass spectrometers

The orbital ion trap mass spectrometers operate at higher resolution than the other instruments used in this study (listed in Table 3 below) and provide accurate mass measurements. This capability was utilized to confirm the assignments of the various peaks in the MS/MS spectra of several protonated aldehydes. Table 2 lists the experimental *m/z* values for the main peaks and the difference from the theoretical values. In most cases the difference is less than 5 ppm.

The loss of H<sub>2</sub> vs CO from protonated aldehydes is a property of the energetics of the ion, which depends on the collision energy, but is expected to take place at similar rates in different mass spectrometers under similar conditions. However, these reactions are followed by secondary processes, which include additional fragmentations or reaction with water. These latter processes occur at increased collision energy and can affect the relative peak intensities in the MS/MS spectrum. Because the reaction of the [M+H-H<sub>2</sub>]<sup>+</sup> ion with water is expected to depend on the concentration of water in the vicinity of the product ion and the stability

**Table 2.** Experimental exact mass values for the precursor and several product ions in the MS/MS spectra of several aldehydes recorded with the Orbitrap mass spectrometer and the difference from the theoretical values

Compound	[M+H] <sup>+</sup>		[M+H-H <sub>2</sub> ] <sup>+</sup>		[M+H-CO] <sup>+</sup>		[M+H-H <sub>2</sub> -CO] <sup>+</sup>		[M+H-H <sub>2</sub> -C <sub>3</sub> O] <sup>+</sup>		Δ <i>m/z</i> (ppm)
	exp	Δ <i>m/z</i> (ppm)	exp	Δ <i>m/z</i> (ppm)	exp	Δ <i>m/z</i> (ppm)	exp	Δ <i>m/z</i> (ppm)	exp	Δ <i>m/z</i> (ppm)	
3-Formylchromone	175.0388	1.14	173.0240	-3.93	147.0445	-2.99	145.0289	-3.38	121.0289	-4.05	
3-Formyl-6-methylchromone	189.0546	0.00	187.0397	-3.90	161.0603	-3.66	159.0446	-3.41	135.0446	-4.00	
3-Formyl-6-ethylchromone	203.0702	0.49	201.0555	-4.38	175.0759	-3.08	173.0603	-3.41	149.0602	-3.29	
3-Formyl-6-isopropylchromone	217.0860	-0.46	215.0712	-4.32	165.0351	-3.03	187.0762	-4.49	163.0760	-3.92	
3-Formyl-6-fluorochromone	193.0292	1.55	191.0145	-3.14	177.0549	-1.58	163.0194	-2.45	139.0193	-2.16	
3-Formyl-6-methoxychromone	205.0490	2.44	203.0343	-2.02	177.0553	-3.84	175.0396	-3.60	151.0391	-0.86	
3-Formyl-7-methoxychromone	205.0497	-0.98	203.0349	-4.97	177.0552	-3.28	175.0396	-3.60	151.0394	-2.85	
3-Formyl-8-methoxychromone	205.0495	0.00	203.0351	-5.96	177.0552	-2.93	175.0396	-3.60	151.0394	-2.85	
7-Diethylamino-3-formylcoumarin	246.1120	2.03	244.0975	-2.79	218.1182	-2.93	216.1025	-2.73	151.0394	-2.85	
2-Pyridinecarboxaldehyde	108.0442	1.85	106.0291	-3.40	80.0496	-1.50	78.0339	-0.90	96.0445	-1.15	
(4-Pyridyl)malonaldehyde	150.0543	4.66	148.0396	-2.03	122.0602	-1.31	120.0446	-1.75	131.0488	2.59	
2,3-Naphthalenedicarboxaldehyde	185.0594	1.62	183.0439	0.87	157.0645	1.85	155.0489	1.55			

of the product, it is likely to vary in different mass spectrometers. We examined these variations for 3-formylchromone in several mass spectrometers: with fragmentation in beam-type collision cells (QqQ, QTOF, and HCD in an Orbitrap Velos and an Orbitrap Elite and QExactive) and in an ion trap (LTQ). The instruments contained different collision gases at different pressures (see Experimental section).

The relative abundance of [M+H-H<sub>2</sub>]<sup>+</sup> at low collision energies is about two orders of magnitude greater than that of [M+H-CO]<sup>+</sup> in beam-type collision cells. However, the ratio of the maximum levels of these ions, reached at higher collision energies, is smaller due to subsequent reactions. Table 3 shows the abundances of different product ions as a percentage of the total ion counts, at the collision energies which correspond, approximately, to the maximum levels of [M+H-H<sub>2</sub>]<sup>+</sup> and [M+H-CO]<sup>+</sup>. The reaction of [M+H-H<sub>2</sub>]<sup>+</sup> with water is pronounced in the Micromass, HCD/Orbitrap, and HCD/QExactive, and this results in the decreased ratio of peak intensities of [M+H-H<sub>2</sub>]<sup>+</sup>/[M+H-CO]<sup>+</sup>. This ratio decreases from a value of two orders of magnitude at low collision voltage to between 2 and 10 when the abundances of these ions reached their maximum values in the different instruments (Table 3). In the QTOF, however, the reaction with water is negligible (since the pressure is much lower) and that ratio remains higher.<sup>[18]</sup> The ratio of [M+H-H<sub>2</sub>]<sup>+</sup>/[M+H-CO]<sup>+</sup> is found to decrease as the contribution of the [M+H-H<sub>2</sub>+H<sub>2</sub>O]<sup>+</sup> ion increases, indicating that the reaction of [M+H-H<sub>2</sub>]<sup>+</sup> with water is the main cause for this decrease. In the ion trap instrument (LTQ), the reaction with water is also observed but to a lower extent than in the Micromass and Orbitrap, again due to the lower pressure in the LTQ. The pressure of the collision gas appears to be related to the amount of water in the mass spectrometer, as mentioned above, but the nature of the gas probably has little effect. Helium is used only in the LTQ while all the other instruments listed in Table 3 use heavier gases. The difference between the results in the QTOF and the Orbitrap instruments was pointed out above and explained by the difference in N<sub>2</sub> gas pressure. In the QqQ instrument, however, we can change the gas from the usually used Ar to N<sub>2</sub>. The results show that the relative abundances of the various product ions formed from protonated 3-formylchromone are the same with the two gases; the only difference being that with N<sub>2</sub> a higher voltage is required to achieve the same degree of fragmentation as is observed with the heavier Ar at the same gas pressure.

The relative abundance of [M+H-H<sub>2</sub>-CO]<sup>+</sup> in the LTQ (Table 3) is higher than in the other instruments, probably due to the relatively long activation time. In fact, decreasing the activation time from 30 ms (the standard value) to 15 ms decreases the abundance of this ion by a factor of five, and increasing the activation time to 45 ms increases the abundance by a factor of two. Variations in the relative abundances of the different ions were also observed when the normalized collision energy was varied between 25% and 50%. The commonly used value of 35% gave the highest abundances of [M+H-H<sub>2</sub>]<sup>+</sup> and [M+H-H<sub>2</sub>+H<sub>2</sub>O]<sup>+</sup> but the other ions continued to increase with increasing energy. This is due to further fragmentation of [M+H-H<sub>2</sub>]<sup>+</sup> and the absence of the precursor ion under these conditions.

The ion trap instrument also permits us to obtain multistage MS<sup>3</sup> and MS<sup>4</sup> spectra. The MS<sup>3</sup> results with the IT generally confirm the above pseudo-MS<sup>3</sup> observations

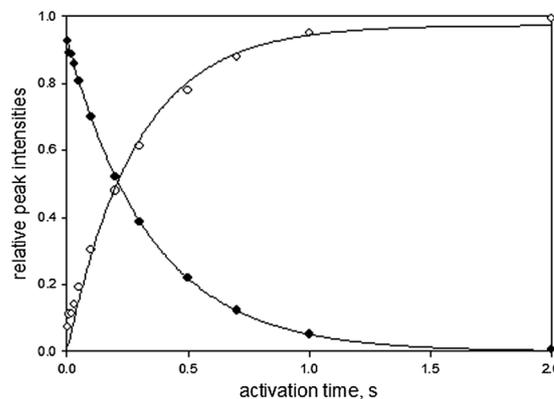
**Table 3.** MS/MS spectra of protonated 3-formylchromone in different mass spectrometers. Ion count contributions (%) of different product ions at the collision voltages \* which correspond, approximately, to the maximum levels of [M+H-H<sub>2</sub>]<sup>+</sup> and [M+H-CO]<sup>+</sup>

Instrument	Collision type	Collision voltage	Collision gas	Pressure (mTorr)	[M+H-H <sub>2</sub> ] <sup>+</sup> ( <i>m/z</i> 173)	[M+H-CO] <sup>+</sup> ( <i>m/z</i> 147)	Ratio	
							173/147	[M+H-H <sub>2</sub> +H <sub>2</sub> O] <sup>+</sup> ( <i>m/z</i> 191)
Quattro Micro Orbitrap Velos	QqQ	20 V	Ar	1.6	34.9	3.4	10	2.3
	HCD	22 eV*	N <sub>2</sub>	3.75	17.2	4.3	4	1.1
Orbitrap Elite QExactive	HCD	22 eV*	N <sub>2</sub>	3.75	15.2	3.3	5	0.8
	HCD	20 eV*	N <sub>2</sub>	3.75	7.2	3.2	2	3.0
QTOF 6530	QTOF	20 V	N <sub>2</sub>	0.02	63.8	3.6	18	2.0
LTQ	IT	35%	He	0.35	62.1	2.4	26	9.5

\*Normalized collision energy setting of 60 or 65 corresponded to HCD collision energy of 20 eV or 22 eV, respectively, as reported by the software for these experiments.

with the QqQ instrument. MS<sup>3</sup> of the product ion with *m/z* 173 from protonated 3-formylchromone shows significant production of the *m/z* 191 peak due to reaction with water molecules in the ion trap. The LTQ also permits us to follow this reaction by isolating the *m/z* 173 ion in the absence of collision energy and gradually increasing the activation time. Varying the activation time from 5 ms to 2000 ms leads to gradual and complete conversion of the *m/z* 173 ion into the *m/z* 191 ion in a time-dependent manner. From the plots in Fig. 5 we calculate a rate constant of  $(2.9 \pm 0.3) \text{ s}^{-1}$  for the reaction of [M+H-H<sub>2</sub>]<sup>+</sup> with H<sub>2</sub>O. If we assume that the reaction occurs on every collision, i.e. with a second-order rate constant close to  $1 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ ,<sup>[20]</sup> we estimate the concentration of water in the LTQ ion trap to be at least  $2.9 \times 10^9 \text{ molecules cm}^{-3}$ , or about  $1 \times 10^{-4} \text{ mTorr}$ . The pressure of He in the ion trap is 0.35 mTorr and it is specified to contain about 1 ppm water, i.e. only about 1% of the expected water concentration. The CID spectrum of the *m/z* 191 product ion obtained from 3-formylchromone in the QqQ ion source was similar to the MS/MS spectrum of authentic 3-carboxychromone, as discussed above (Fig. 4). The actual MS<sup>4</sup> spectrum of the *m/z* 191 ion in the LTQ also was identical to the MS<sup>2</sup> spectrum obtained with the authentic carboxylic acid, indicating that the added water molecule is covalently bonded to form the carboxylic acid. It should be pointed out, however, that the MS/MS spectrum of the [M+H]<sup>+</sup> ion of 3-carboxychromone (*m/z* 191) in the LTQ shows a dominant *m/z* 173 peak (loss of water) while the QqQ spectrum shows several intense peaks (Fig. 4) with increasing collision energy.

In the above discussion we assumed that loss of H<sub>2</sub> from protonated 3-formylchromone involves the loss of the proton added to the molecule to form the precursor ion. This was confirmed in both the QqQ and IT mass spectrometers by using a solution of the compound in acetonitrile/D<sub>2</sub>O/formic acid (instead of acetonitrile/H<sub>2</sub>O/formic acid). The precursor ion peak appeared at *m/z* 176 instead of 175, indicating formation of the [M+D]<sup>+</sup> ion. This ion then lost HD to form the *m/z* 173 peak predominantly. An attempt to observe reaction of the *m/z* 173 peak with D<sub>2</sub>O failed, even after flowing D<sub>2</sub>O solutions for several hours. Only reaction with H<sub>2</sub>O was observed. This result indicates that the source of water is probably moisture inside the mass spectrometer and not directly from the injected solution.



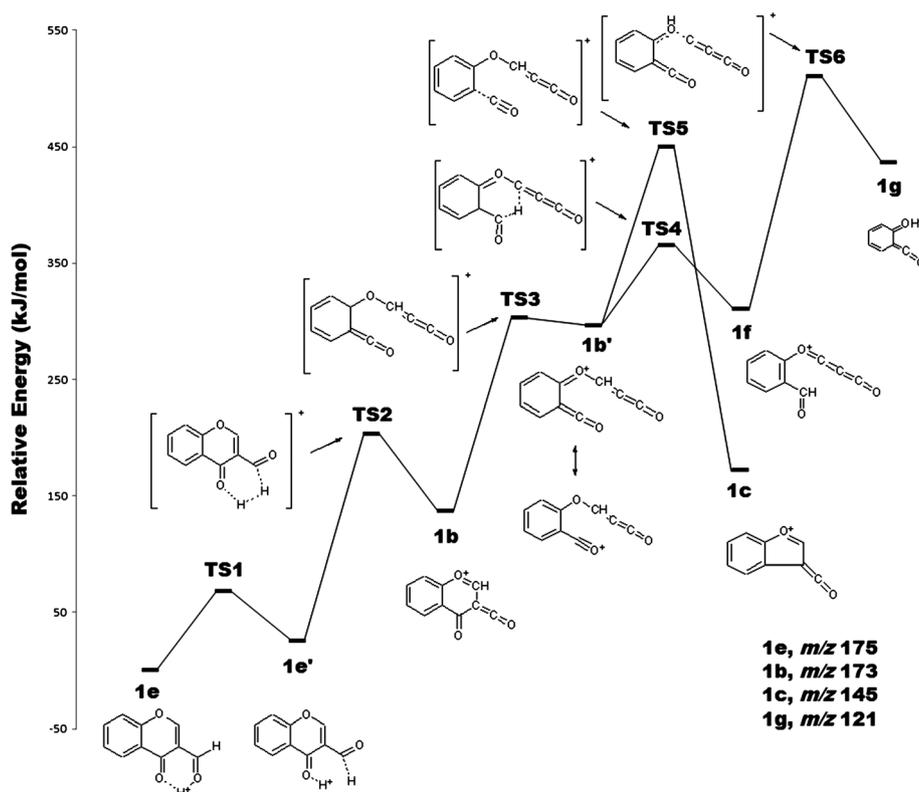
**Figure 5.** Conversion of the *m/z* 173 ion (solid circles) into the *m/z* 191 ion (open circles) by reaction with water in the ion trap of the LTQ mass spectrometer as a function of activation time with relative collision energy set at zero.

## Mechanism of reaction

For further insight into the mechanism, DFT calculations were performed. We explored the potential energy surfaces of a representative aldehyde (3-formylchromone) for the losses of H<sub>2</sub>, CO, and C<sub>3</sub>O from the protonated molecule. For the most stable protonated form of 3-formylchromone (**1e**) the proton bridges between the ketone and the aldehyde oxygen and appears closer to the aldehyde oxygen. The calculated proton affinity of 3-formylchromone at this protonation site is 866 kJ mol<sup>-1</sup>, slightly higher than the experimental value for ammonia, 853 kJ mol<sup>-1</sup>.<sup>[21]</sup> This structure is far more stable than any other 3-formylchromone structure with a different protonation site. Figure 6 shows in a reaction coordinate diagram the main steps for the fragmentation of this ion under CID conditions. The loss of H<sub>2</sub> from the protonated 3-formylchromone is a two-step reaction; first it involves the rotation of the aldehyde group, bringing the aldehyde hydrogen closer to the proton, and second the loss of H<sub>2</sub> producing a ketene cation. The rotation (**TS1**) and H<sub>2</sub> loss (**TS2**) barriers are 68 kJ mol<sup>-1</sup> and 180 kJ mol<sup>-1</sup>, respectively. The calculations show that the product of the H<sub>2</sub> loss, **1b**, is a ketene cation (R<sup>+</sup>=C=O) (Fig. 6), where the positive charge is distributed over several ring positions. This is in line with previous conclusions on the structure of benzoyl cations from nuclear magnetic resonance (NMR) studies.<sup>[22]</sup> Further fragmentation of the ketene structure follows mainly two channels, a major loss of C<sub>3</sub>O and a minor loss of CO. Both channels are preceded by a C–C bond breaking between carbons 3 and 4 (see structure **1a** for the numbering of the atoms) to form

an open structure (structure **1b'** of Fig. 6) passing through a barrier (**TS3**) of 166 kJ mol<sup>-1</sup>. The open structure is reached from **TS3** with a very small barrier (~7 kJ mol<sup>-1</sup>), but it is known that B3LYP underestimates barrier heights. MP2/6-31g optimization shows a similar small barrier. Two resonance forms of the open structure are shown in Fig. 6. The loss of CO occurs directly from this open form of the protonated molecule (*m/z* 173) with a barrier (**TS5**) of 154 kJ mol<sup>-1</sup>. The loss of C<sub>3</sub>O is preceded by a hydrogen transfer to the original aldehyde carbon with a barrier (**TS4**) of 70 kJ mol<sup>-1</sup>, then the C(2)–O(1) bond breaks (**TS6**, 200 kJ mol<sup>-1</sup>, see Supporting Information for further details) to form a ketene and C<sub>3</sub>O (Fig. 6). The generated ketene ion is relatively stable and shows up as a prominent peak in the spectrum (*m/z* 121).

The neutral loss of C<sub>3</sub>O has been experimentally verified in this work using accurate mass measurements in the Orbitrap mass spectrometer. It is worth mentioning that the existence of the neutral molecule C<sub>3</sub>O has been reported and studied in the past.<sup>[23,24]</sup> Also, some of these observations resemble earlier reports on the fragmentation pattern of chromone and coumarins under ESI conditions.<sup>[25]</sup> We have also considered the possibility of loss of propionaldehyde (HC≡CH=O) directly from the protonated aldehyde through a retro-Diels-Alder mechanism, but the calculation and the experimental evidence suggest that this pathway is unlikely. The calculations show that the reaction is better considered a multistage (non-concerted) and asynchronous mechanism, as shown in Fig. 6. Another possible mechanism for the production of the *m/z* 121 ion is from the hydrated *m/z* 173



**Figure 6.** Energy diagram for the consecutive losses of H<sub>2</sub>, CO, and C<sub>3</sub>O from protonated 3-formylchromone (**1e**). Energies are in kJ mol<sup>-1</sup> and include zero-point corrections.

ion by direct loss of propiolic acid from the  $m/z$  191 ion. Although this route seems thermodynamically feasible, results indicate that the  $m/z$  191 ion instead predominantly undergoes loss of water. The QqQ results in Fig. 4 show that the  $m/z$  121 ion appears only at higher collision energies while at lower energies only loss of water is observed. Moreover, the IT product ion spectra of the  $m/z$  191 ion, produced both indirectly from the protonated aldehyde in an MS<sup>4</sup> experiment and directly from the authentic carboxylic acid, show only loss of water. These results are also supported by calculations. Relaxed scans on the potential surface of the most stable protonated form of the 3-carboxychromone ( $m/z$  191) suggest that the loss of water has an activation barrier of approximately 130 kJ mol<sup>-1</sup> while the barrier for the retro-Diels-Alder reaction is at least 360 kJ mol<sup>-1</sup>.

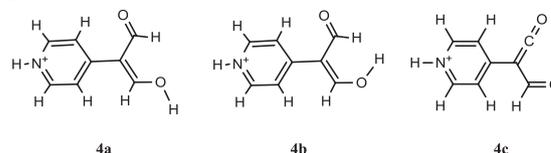
As discussed above, residual water in the collision cell can react with certain ions. It is possible also that interaction with water can facilitate (or catalyze) some neutral losses, such as the loss of C<sub>3</sub>O reported above. DFT calculations where a water molecule is added to structures **1f** and **TS6** suggest a substantial decrease in the activation barrier for such a process and the formation of more thermodynamically stable products, such as 3-oxoacrylaldehyde or propiolaldehyde (depending on the initial positioning of the water molecule). However, there is no experimental evidence for this hypothesis and theoretical calculations for all the hydrated versions of the proposed mechanism would be very extensive and beyond the scope of this paper.

As shown before, the pressure and nature of the buffer gas have an effect on fragmentation, but this effect is complicated by additional factors. In most cases, different gases are used with different mass analyzers (MAs) and probably the MAs have the major effects on the spectra (because of differences in distribution of deposited energy, time scale, CID efficiency, dissociation or rearrangement, etc.). On the other hand, reaction with residual water in the collision cell or ion trap significantly affects the spectra and occurs differently in different MAs (or even in similar MAs with different designs). In fact, the energy profiles for some product ions in Fig. 2 are influenced by the reaction with water. It may be difficult to account and separate all effects arising from this reaction and the differences between MAs. The same argument is valid for any kind of kinetic modeling of the process. The 'thermodynamic' model shown in Fig. 6 is only intended to find a reasonable pathway to explain the product ions observed, but the given energetic barriers are not by themselves sufficient to explain the observations because of the complex kinetics involved.

In general, protonated aldehydes with electronegative groups in the immediate vicinity of the aldehyde group, which form a hydrogen-bridged structure, behave in a similar manner to 3-formylchromone and exhibit a significant loss of H<sub>2</sub>. However, such aldehydes with a remote protonation site may exhibit a different reaction pathway for the loss of H<sub>2</sub>. We have also examined the loss of H<sub>2</sub> from (4-pyridyl) malonaldehyde. In contrast to 3-formylchromone, the most stable protonated form bears a proton on the pyridyl nitrogen, which has a calculated proton affinity of 923 kJ mol<sup>-1</sup>, close to the experimental value for pyridine, 930 kJ mol<sup>-1</sup>.<sup>[21]</sup> The most probable mechanism for the loss of H<sub>2</sub> from this species is a charge-remote process (i.e. the proton is not involved directly). This remote fragmentation mechanism is supported

by the following findings. An experiment with D<sub>2</sub>O leads to production of the deuterated species which undergoes loss of H<sub>2</sub>, indicating that the added deuterium remains on the pyridyl nitrogen. Also, the fixed charge quaternary ion, formed by butylation of the pyridyl nitrogen using butyl iodide, exhibits significant loss of H<sub>2</sub> under CID conditions. The loss of H<sub>2</sub> reaches a level of 10% of the total ion current, much less than in the case of the non-butylated ion, due to a major competing process involving the loss of butene.

The remote mechanism is via elimination of H<sub>2</sub> between the enolic hydrogen and the aldehyde hydrogen (**4a**→**4b**) and producing a stable ketene structure (**4c**).



It is a two-step process involving the rotation of the hydroxyl group (**4a**→**4b**), with a rotation barrier of approximately 42 kJ mol<sup>-1</sup>, and then the elimination reaction with an activation barrier of approximately 150 kJ mol<sup>-1</sup>. These barriers are smaller than the activation barriers for the loss of H<sub>2</sub> from protonated 3-formylchromone. This result supports the experimental findings of a higher relative rate of H<sub>2</sub> loss and lower relative rate of CO loss in (4-pyridyl) malonaldehyde than in 3-formylchromone.

## CONCLUSIONS

The loss of H<sub>2</sub> is a major fragmentation channel for protonated 3-formylchromones, 3-formylcoumarins, and other aldehydes which contain vicinal groups that can form a hydrogen bridge with the aldehyde group. Loss of CO from the precursor ions is also observed as a minor route. The most stable form of protonated 3-formylchromone bears the proton between the ketone and the aldehyde group and undergoes the loss reaction in two steps, first rotation of the aldehyde group and then loss of H<sub>2</sub>, leaving behind a relatively stable ketene cation. An experiment conducted using deuterated water shows that the added proton is involved in the loss of H<sub>2</sub>. These ketene cations subsequently experience an abundant loss of C<sub>3</sub>O. Aldehydes with a remote protonation site and with electronegative groups in the vicinity of the formyl group capable of forming a H-bridged structure form an even more stable ketene that further inhibits the loss of CO. Depending on instrument design, the observed loss of H<sub>2</sub> vs CO can be significantly affected by subsequent reactions, specifically the reaction of the ketene cation with water and the consecutive losses of H<sub>2</sub> and CO. The reaction of the ketene cation with water produces a protonated carboxylic acid, which undergoes subsequent fragmentation. As a result, the MS/MS spectrum of the protonated aldehyde may contain peaks which result from the corresponding carboxylic acid. In general, reaction of product ions with water may lead to the formation of ions that are not expected to be formed from the precursor ion, thus generating a puzzling MS/MS spectrum. These also present a problem for reference libraries, since major peaks can depend on conditions not under the full control of the analyst (the degree of H<sub>2</sub>O adduction). This will be handled by

including multiple spectra in the library and possibly by labeling peaks that are inherently variable. Further studies of these reactions and recording them in libraries are currently under way in our laboratory.

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