An electrospray ionization (ESI) ambient pressure ion-mobility spectrometer (APIMS) interfaced to an orthogonal reflector time-of-flight mass spectrometer (TOFMS) was evaluated for the first time as a detector for the identification of phenylthiohydantoin (PTH)-derivated amino acids, the final products in the Edman sequencing process of peptides and proteins. The drift and flight times of the twenty common PTH amino acids were characterized by a well-defined 2-D mobility/mass spectral pattern. The combination of mobility/mass modes of analysis gave rise to a unique trend-line formation for the series of PTH amino acids. In addition, each PTH amino acid had a unique reduced mobility constant \( K_o \), thus enabling the differentiation of all the amino acid derivatives including the PTH-leucine and PTH-isoleucine isomers. More importantly it was shown that it was possible to resolve a complete reference mixture of PTH amino acids in a single experimental run in less than 1 min. Detection limits for the PTH amino acids were found to range from 1.04 to 3.52 ng; indicating that the limits of detection were less than 17.0 pmol for all of the PTH amino acids.

**Keywords**  
Ion-mobility spectrometry · Time-of-flight mass spectrometry · Phenylthiohydantoin amino acid derivatives · Edman sequencing

**Introduction**

The field of proteomics – the simultaneous analysis of total gene expression at the protein level – has rapidly become one of the premiere strategies for studying biological systems and understanding the relationship between various expressed genes and gene products. Proteomic research, however depends upon the ability to accurately identify the proteins that are present in a selected sample in a timely fashion. In the early 1950s a powerful tool – called the Edman degradation process – was established for protein sequencing [1, 2]. This enabled many laboratories from around the world to systematically sequence proteins for their primary structures. Since then this method has become one of the principal methods of choice in bioanalytical research for the exploration of protein characterization. Currently most commercial protein sequencing systems employing the Edman degradation method use high-performance liquid chromatography (HPLC) with ultra-violet (UV) absorbance detection for the analysis of the phenylthiohydantoin (PTH) amino acids products that are produced from this process. Although systematic improvements over the years have been made to this novel method of protein sequencing [3, 4, 5, 6, 7], the choice of detection still suffers markedly from lack of speed, sensitivity, and structural information of nonstandard amino acids. Due to these limitations in detection there have been a number of alternative methodologies – mainly involving mass spectrometry (MS) – developed for the analysis of PTH amino acid derivatives. Especially, during the last several decades there has been an explosion of mass spectrometric methods for the characterization of PTH amino acids by electron ionization (EI) [8, 9], chemical ionization (CI) [10], thermospray ionization (TI) [11], and electrospray ionization (ESI) [12, 13]. There have even been several studies on the use of novel reagents yielding nonstandard amino acid derivatives [14, 15, 16] for which ESI–MS detection was sensitive.

Although all of these alternatives to improve the speed, sensitivity, and specificity of PTH amino acid detection have met with some degree of success they have in the end still been temporally limited by the principle separation mode (HPLC) of analysis. The importance, however, of a separation mode prior to detection should not be overlooked. Liang et al. have shown that without a purification step to remove or eliminate interfering species, aliphatic amines used in the actual Edman degradation process will completely suppress the PTH amino acid signals [13]. Nevertheless, there have been a number of publications describing post source separations that remove...
interfering ion species using high-resolution ion-mobility spectrometers (IMS) fitted with ESI sources [17, 18, 19, 20]; these have shown to give results similar to or better than typical HPLC separations in less than a second. Most importantly, preliminary and ongoing work with the coupling of ESI–IMS with MS has demonstrated the potential for the characterization of aqueous phase samples by two-dimensional (2-D) ion-mobility mass spectrometry (IMMS) [21, 22, 23, 24, 25, 26]. In this paper the initial feasibility of using an electrospray ionization ambient pressure ion-mobility spectrometer interfaced to an orthogonal reflector time-of-flight mass spectrometer (ESI/APIMS/TOFMS or IMMS) for the rapid detection of PTH amino acids from aqueous reference samples was explored for the first time. Several factors related to the detection of aqueous phase PTH amino acids that were investigated in this study included:

1. instrumental scan time,
2. ion drift times,
3. reduced mobility constants,
4. product ion masses,
5. limits of detection, and
6. reproducibility.

Interestingly it was found that all PTH amino acids had different drift times – thus enabling the distinction between PTH-leucine and PTH-isoleucine derivatives – and that limits of detection were in the low pmol range. This demonstrated the initial viability of using IMMS to accurately separate and detect reference PTH amino acid solutions in less than 1 min.

### Experimental

#### Instrumentation

The IMMS instrument used in this study was constructed at Washington State University where the fundamental components (ESI source; APIMS drift tube; high-pressure interface; TOF m/z analyzer; and data acquisition system) and modes of operation have been previously described in considerable detail [26, 27]. Due to this, only a brief outline of the experimental sequence is provided. A continuous flow ($5.0 \mu L \text{ min}^{-1}$) of solvent was sprayed in the positive ion mode with a needle voltage of $3.5 \text{kV}$ with respect to the target screen of the APIMS. The APIMS was divided into two regions – the desolvation (11.0 cm in length) and the drift (15.0 cm in length) regions – that were maintained at a temperature of 200°C. Desolvated ions from the electrospray process drifted through the target screen of the APIMS. The APIMS was divided into two regions – the desolvation (11.0 cm in length) and the drift (15.0 cm in length) regions – that were maintained at a temperature of 200°C. Desolvated ions from the electrospray process drifted through the drift tube is defined as the time required for ions to travel through the length of the drift cell space, $L$, in centimeters, as given by:

$$t_d = \frac{L^2}{KV}$$

where the mobility of the ions, $K$, in $\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$, is inversely related to the potential drop, $V$, in volts they experience. To correct for varying environmental and experimental conditions, it is practical to report ion drift times in terms of reduced mobility constants ($K_r$) [18] which are defined by:

$$K_r = \left( \frac{L^2}{V \tau_d} \right) \left( \frac{273.5}{T} \right) \left( \frac{P}{760} \right)$$

where $L$ is the drift region length (15.0 cm), $V$ is the drift voltage (6550 V), $T$ is the effective temperature in the drift region (200°C), and $P$ is the pressure (714 torr).

The term flight time, $t_f$, of an ion species refers to the time required for ions to traverse the length, $l$, from the high voltage extractor to the microchannel detection plates in the TOFMS flight chamber as shown by:

$$t_f = \left( \frac{l}{2E_{\text{kin}}} \right)^{1/2}$$

where $m$ is the ion mass, and $E_{\text{kin}}$ is the kinetic energy of the ions.

#### Results and discussion

IMMS 2-D spectra of PTC/PTH amino acids

The PTC/PTH amino acids were sprayed into the IMMS instrument to determine their respective drift and flight times. Figure 1 shows the 2-D separation of a rapid acquisition (<1 min) of a reference solution (1.0 µg mL$^{-1}$) of all 20 PTC/PTH amino acids, were the respective drift (mobility) and flight (mass) times of each ion can be seen. The PTC/PTH products were identified as 1: PTH glycine (M+H)$^+$, 2: PTH alanine (M+H)$^+$, 3: PTH serine (M+H)$^+$, 4: PTH proline (M+H)$^+$, 5: PTH threonine (M+H)$^+$, 6: PTH valine (M+H)$^+$, 7: PTH aspartic acid (M+H)$^+$, 8: PTH as...
paragine (M+H)+, 9: PTH isoleucine (M+H)+, 10: PTH glutamic acid (M+H)+, 11: PTH glutamine (M+H)+, 12: PTH leucine (M+H)+, 13: PTH methionine (M+H)+, 14: PTH phenylalanine (M+H)+, 15: PTH carboxymethyl cysteine (M+H2O+H)+, 16: PTH histidine (M+H)+, 17: PTH tyrosine (M+H)+, 18: PTH arginine (M+H)+, 19: PTH tryptophan (M+H)+, and 20: PTC lysine (M+H)+. Notice that the mobility spectrum alone yields PTC/PTH amino acids that have similar mobilities. In fact from PTH proline to PTH tryptophan there are 16 PTH amino acids that have similar drift times. With the current resolving power of the APIMS (about 85 for singly charged ions) it would be difficult to see significant resolution of any two adjacent ions in this crowded region of the spectrum. For example, PTH glutamic acid and PTH glutamine would require a resolving power of 170 to achieve a 0.5 resolution separation of these ions. Resolving powers this high have been previously reported for singly charged ions using IMS [17, 18]. Further examination of Fig. 1 also established that the mass spectrum alone could not resolve all of the PTH amino acids. This was mainly due to the isomeric forms of PTH leucine and PTH isoleucine having the same mass-to-charge ratio. However, the combination of these two modes with IMMS provided a powerful 2-D mode of identification for the rapid identification and quantification of all PTC/PTH amino acids at once, making it possible to directly determine each of the product ions, mobility drift, and mass flight times produced from within a single experimental run.

Product ions, Ko, and limits of detection

Tabulated values for each of the products that were experimentally analyzed are shown in Table 1. Here the masses (M), product ions (M+N)n, drift times (td), reduced mobility (Ko), and limits of detection (LOD) are presented for each compound. The limits of detection determined by the amounts required to produce a signal that is three times that of the noise. The correlation coefficient calculated over two orders of magnitude using at least four data points for each compound is also listed.

### Table 1: Product ions, drift times, Ko, and limits of detection for the common PTH/PTC amino acid derivatives using IMMS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass (M)</th>
<th>Product ions (M+N)n</th>
<th>Drift time (ms)</th>
<th>Ko</th>
<th>LOD (ng)</th>
<th>CC (r²)</th>
<th>LOD (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH-alanine, A</td>
<td>207 (M+H)+</td>
<td>(M+H)+</td>
<td>12.38</td>
<td>1.50</td>
<td>2.25 (0.9975)</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>PTH-cysteine, Cc</td>
<td>297 (M+H2O+H)+</td>
<td>(M+H)+</td>
<td>14.73</td>
<td>1.26</td>
<td>2.44 (0.9865)</td>
<td>8.21</td>
<td></td>
</tr>
<tr>
<td>PTH-aspartic acid, D</td>
<td>251 (M+H)+</td>
<td>(M+H)+</td>
<td>13.46</td>
<td>1.38</td>
<td>3.52 (0.9958)</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>PTH-glutamic acid, E</td>
<td>265 (M+H)+</td>
<td>(M+H)+</td>
<td>13.96</td>
<td>1.33</td>
<td>3.49 (0.9904)</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>PTH-phenylalanine, F</td>
<td>283 (M+H)+</td>
<td>(M+H)+</td>
<td>14.64</td>
<td>1.27</td>
<td>2.02 (0.9948)</td>
<td>7.14</td>
<td></td>
</tr>
<tr>
<td>PTH-glycine, G</td>
<td>193 (M+H)+</td>
<td>(M+H)+</td>
<td>12.00</td>
<td>1.55</td>
<td>3.24 (0.9876)</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>PTH-histidine, H</td>
<td>273 (M+H)+</td>
<td>(M+H)+</td>
<td>14.92</td>
<td>1.25</td>
<td>1.16 (0.9982)</td>
<td>4.25</td>
<td></td>
</tr>
<tr>
<td>PTH-isoleucine, I</td>
<td>249 (M+H)+</td>
<td>(M+H)+</td>
<td>13.90</td>
<td>1.34</td>
<td>2.79 (0.9871)</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>PTC-lysine, K</td>
<td>399 (M+H)+</td>
<td>(M+H)+</td>
<td>18.20</td>
<td>1.02</td>
<td>3.31 (0.9978)</td>
<td>8.30</td>
<td></td>
</tr>
<tr>
<td>PTH-leucine, L</td>
<td>249 (M+H)+</td>
<td>(M+H)+</td>
<td>14.18</td>
<td>1.31</td>
<td>2.40 (0.9985)</td>
<td>9.64</td>
<td></td>
</tr>
<tr>
<td>PTH-methionine, M</td>
<td>267 (M+H)+</td>
<td>(M+H)+</td>
<td>14.20</td>
<td>1.31</td>
<td>2.64 (0.9762)</td>
<td>9.89</td>
<td></td>
</tr>
<tr>
<td>PTH-asparagine, N</td>
<td>250 (M+H)+</td>
<td>(M+H)+</td>
<td>13.58</td>
<td>1.37</td>
<td>1.86 (0.9936)</td>
<td>7.44</td>
<td></td>
</tr>
<tr>
<td>PTH-proline, P</td>
<td>233 (M+H)+</td>
<td>(M+H)+</td>
<td>13.02</td>
<td>1.43</td>
<td>1.38 (0.9994)</td>
<td>5.93</td>
<td></td>
</tr>
<tr>
<td>PTH-glutamine, Q</td>
<td>264 (M+H)+</td>
<td>(M+H)+</td>
<td>14.00</td>
<td>1.33</td>
<td>3.02 (0.9881)</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>PTH-arginine, R</td>
<td>292 (M+H)+</td>
<td>(M+H)+</td>
<td>15.46</td>
<td>1.21</td>
<td>1.04 (0.9957)</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>PTH-serine, S</td>
<td>223 (M+H)+</td>
<td>(M+H)+</td>
<td>12.80</td>
<td>1.46</td>
<td>3.37 (0.9869)</td>
<td>15.1</td>
<td></td>
</tr>
<tr>
<td>PTH-threonine, T</td>
<td>237 (M+H)+</td>
<td>(M+H)+</td>
<td>13.16</td>
<td>1.42</td>
<td>2.70 (0.9738)</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>PTH-valine, V</td>
<td>235 (M+H)+</td>
<td>(M+H)+</td>
<td>13.34</td>
<td>1.40</td>
<td>1.42 (0.9995)</td>
<td>6.04</td>
<td></td>
</tr>
<tr>
<td>PTH-tryptophan, W</td>
<td>322 (M+H)+</td>
<td>(M+H)+</td>
<td>15.66</td>
<td>1.19</td>
<td>2.31 (0.9835)</td>
<td>7.17</td>
<td></td>
</tr>
<tr>
<td>PTH-tyrosine, Y</td>
<td>299 (M+H)+</td>
<td>(M+H)+</td>
<td>15.18</td>
<td>1.23</td>
<td>2.85 (0.9987)</td>
<td>9.53</td>
<td></td>
</tr>
</tbody>
</table>

*a* limits of detection determined by the amounts required to produce a signal that is three times that of the noise

*Correlation coefficient calculated over two orders of magnitude using at least four data points*

*carboxymethyl*
constants ($K_a$), and limits of detection (LOD) for 1-min sample runs for both PTC/PTH amino acid reference materials are examined. While almost all compounds produced protonated product ions, $(M+H)^+$, one product formed an adduct that deviated from this trend. PTH carboxymethyl cysteine produced a protonated ion with the addition of a water molecule, $(M+H_2O+H)^+$. The extent of sodium adduct formation was negligible; only in few extreme cases of high PTC/PTH amino acid concentrations was this phenomena observed. Experimental $K_a$ values were calculated according to Eq. (2), thus providing, to our knowledge, the first ever published reduced mobility constants for these compounds. Finally the limits of detection for each compound were determined using the standard definition of the quantity required to give a signal-to-noise ratio of 3. For each compound a calibration curve was made using the five concentrations 25, 50, 100, 250, and 500 ng mL$^{-1}$. The lowest value, 25 ng mL$^{-1}$, was within one order of magnitude of the calculated detection limits for all of the PTC/PTH amino acids. The range of limits of detection was from 1.04 to 3.52 ng; which corresponded (depending upon respective molecular weights) to a range in terms of moles of ~3.5 to 17.0 pmol. As might be expected most PTH amino acids exhibited relatively similar LOD; mainly due to the common PTH group. However, it was still noted that both PTH arginine and PTH histidine showed the lowest LOD. These are the most basic amino acids besides lysine and were expected to be very sensitive using positive ESI. Lysine although most basic amino acids besides lysine and were expected might be expected most PTH amino acids exhibited relatively similar LOD; mainly due to the common PTH group. However, it was still noted that both PTH arginine and PTH histidine showed the lowest LOD. These are the most basic amino acids besides lysine and were expected to be very sensitive using positive ESI. Lysine although strongly basic was not found to be detected so sensitively to be very sensitive using positive ESI. Lysine although most basic amino acids besides lysine and were expected might be expected most PTH amino acids exhibited relatively similar LOD; mainly due to the common PTH group. However, it was still noted that both PTH arginine and PTH histidine showed the lowest LOD. These are the most basic amino acids besides lysine and were expected to be very sensitive using positive ESI. Lysine although strongly basic was not found to be detected so sensitively

Conclusions

IMMS can rapidly (<1 min) distinguish 20 PTC/PTH amino acids in a reference mixture from one another on the basis of their respective mobility/mass ratios; while still retaining a high degree of detection (<17.0 pmol). This is the first time that all 20 PTC/PTH amino acids have been separated in less than a minute. Although problems associated with ion suppression of reagents have not been investigated, the results from this initial evaluation of IMMS for rapid separation and identification of PTC/PTH amino acids appears promising. We envision that IMMS may be compatible with rapid LC methods in which many of the analytes are co-eluted from the column to improve sample throughput. Operating as a stand alone instrument or coupled with high speed LC system, IMMS technology offers added separation power and speed to the determination of complex mixtures such as PTC/PTH amino acids. When applied to PTC/PTH amino acids separation and detection, IMMS will improve sample throughput by eliminating or reducing the rate-limiting step of chromatography in protein microsequencing.

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