ISOTOPE-RATIO-MONITORING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (IRM-LCMS): FIRST RESULTS FROM A MOVING WIRE INTERFACE SYSTEM

W. A. BRAND AND P. DOBBERSTEIN

Finnigan MAT, Bremen, Deutschland

(Received December 5, 1995; accepted April 22, 1996)

A Liquid Chromatography-Combustion (LC-C) Interface, based on a moving wire technique, has been built and tested. The LC effluent is deposited onto a transport wire, which carries the sample through solvent evaporation and combustion ovens. CO₂ from the combustion step is analysed in an isotope ratio mass spectrometer. Performance of the interface was tested by loop injections of sucrose and glucose into a liquid flow of methanol/water (80/20). Accuracy and precision of δ¹³C < 1% was achieved for sample concentrations > 500 ng/ul (5 µl loop), sufficient for studies at natural isotope ratios. In case of ¹³C tracer applications the detection limit was determined to be about 20 pg carbon tracer (on wire).

KEY WORDS Carbon 13, isotope ratios, liquid chromatography, mass spectrometry, on-line coupling

INTRODUCTION

Isotope-ratio-monitoring GCMS (irm-GCMS), first described in [1] and introduced as a commercial product in [2], has gained acceptance as a technique for the measurement of ¹³C and ¹⁵N of single organic compounds. Because most biological molecules are not volatile, it is desirable to examine a comparable methodology based on liquid chromatography, irm-LCMS.

The first steps in this direction have been taken by Abramson and co-workers [3–5] for tracer applications. Their system (named CRIMS, Chemical Reaction Interface for MS) combined a particle beam interface for solvent removal, a microwave reaction chamber and a quadrupole MS. This technique is restricted to tracer applications and can not be used for studying variations of isotope ratios in natural abundance. Abramson and Moni [6] combined a moving belt interface with CRIMS. This work appears to have been discontinued, presumably due to memory effects and other technical problems with the moving belt. Caimi and Brenna [7]...
explored using a moving wire interface to achieve LC-IRMS-coupling. They modified a Pye Unicam interface, originally built to couple an LC with a FID. Caimi et al. reported a precision of 0.5% for δ\textsuperscript{13}C\textsubscript{FDB} in the range of natural isotope ratios for multiple loop injections of linoleic acid standards. They improved the low yield of the original device by applying a spray technique for the solvent deposition [8].

In our laboratory, we also have investigated the moving wire because of its superiority with respect to quantitative solvent removal. The first results obtained with our breadboard design, reaching an overall yield of 20%, have already been reported [9,10]. Precision, however, was only 5% for δ\textsuperscript{13}C\textsubscript{FDB}, using 100% water for the liquid carrier.

**LC COMBUSTION SYSTEM**

A nickel wire runs continuously from a feed spool to a collect spool, carrying sample through the different sections of the interface, which include (Fig. 1):

- the **cleaning oven**, for removal of organic impurities and surface oxidation,
- the **coating assembly**, where a portion of the HPLC effluent, typically 1/10\textsuperscript{th} of the 1ml/min coming from the LC pump, is deposited onto the wire,
- the **drying stage**, where the solvent is completely evaporated, leaving the sample content of the eluent as a coating on the wire,

![Fig. 1 Schematics of LC Combustion Interface](image-url)
- the combustion reactor, where the sample is oxidized to CO₂ and H₂O; helium flowing through the combustion reactor carries the combustion products via an open split interface and a fused silica capillary into a Finnigan MAT IRMS delta C for detection of CO₂ (m/z = 44, 45 and 46).

For testing the system loop injections of the sample (Rheodyne injector, 5 µl loop) were made directly into the LC flow of water/methanol (80/20) in front of the effluent splitter (no LC column).

The effect of wire speed on the peak shape and the peak area was marginal, between 5 and 15 cm/s. A wire speed of about 7 cm/s was found to be a good compromise between a stable background signal and good reproducibility of the detected peak area. The cleaning oven and the combustion reactor were operated between 800 °C and 1000 °C. In the drying stage a temperature of about 150 °C was sufficient for quantitative solvent evaporation (water/methanol).

DISCUSSION

Two prerequisites for measurement of isotope ratios with high precision are quantitative yield and linearity.

Linearity was tested by injecting sucrose with different concentrations ranging from 100 ng/µl up to 2 µg/µl. In order to test the precision, a sequence of 5 injections with the same sample solution was performed, before switching to the next concentration level (time between injections typically 1 min). Data acquisition and evaluation were performed with the ISODAT® software package (Finnigan MAT) applying the peak detection algorithm developed for irm-GCMS measurements. The measured peak area increases linearly with the sample concentration (Fig. 2). The relatively large scatter (typically ±10%) is believed to be generated by the coating device and will be investigated in the future. The dependence of the δ values upon sample concentration could be due to a drift of the measured δ with time, because a drift of the same magnitude was also seen in a measurement, in which the same sample solution was injected over 1 h. The scatter of the δ values is lower on higher sample concentrations, reaching values of better than ±0.5‰ for concentrations > 500 ng/µl.

The yield of the moving wire interface is estimated as follows: From an injection of a 2 µg/µl solution 1 µg is deposited onto the wire. 1 µg sucrose contains 35 nmoles carbon, which yields 35 nmoles CO₂ gas upon combustion. The He flow carrying the CO₂ is split 10/1 at the open split interface. Thus, 3.5 nmoles CO₂ enter the source of the IRMS. Taking into account the ionization yield of the mass spectrometer (operated at a sensitivity of 1 ion/2000 molecules) and the shunt resistance of the Faraday cup (3 × 10⁶Ω), a peak area of 51 Vs should be detected under conditions of no sample loss. A yield of 55% is estimated from the detected peak area of 26.2 Vs (Fig. 2). Other experiments have shown yields of 78%. Because no significant loss of sample occurs in the interface, we believe that the missing signal is due to sample losses in the coating device.

For testing the precision of the irm-LCMS system beet sugar and cane sugar were used. Both are chemically identical molecules differing only by their carbon isotope
Fig. 2 Linearity test. Beet sugar injected into water/methanol (80/20). Each datapoint is the rms average for 5 subsequent injections with the same sample solution (same concentration level). The error bars represent the standard deviation for 5 injections. **Upper part:** Detected peak area for 44 trace. **Lower part:** Calculated δ values. The δ value for the first peak of the first 5 injections was set at −26.2‰, determined by sealed tube combustion.

ratios due to their different origin. In our test run beet sugar was used as the “standard” and cane sugar as the “unknown”. The use of an internal standard for calibration was necessary, because the breadboard design was not equipped with a reference inlet. The experimental conditions have been similar to the previous
Fig. 3 "Chromatogram" obtained for injections of beet- and cane-sugar (2 µg/µl, water/methanol 80/20). The injected sample changed after 5 injections. **Lower part**: 44 trace. **Upper part**: 45/44 ratio trace

experiment: A sequence of 5 injections of the "standard" solution was followed by a sequence of 5 injections of the "unknown", and this procedure was repeated 3 times (Fig. 3). In the evaluation of this test, the 2nd peak in the chromatogram was selected as the "standard" (−26.2%), because the first peak is often a statistical outlier. The three different δ values obtained for the "unknown" (Tab. 1) are within the error intervals for the value −10.6% determined independently by sealed tube combustion.

**Tab. 1** Summary of the evaluation of the measurement of Fig. 3. Standard: beet sugar, "unknown": cane sugar. The δ values given are the rms average for 5 subsequent injections with the same sample solution and their standard deviation

<table>
<thead>
<tr>
<th>sequence</th>
<th>sample</th>
<th>δ&lt;sup&gt;13&lt;/sup&gt;C&lt;sub&gt;PDB&lt;/sub&gt;[‰]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>standard</td>
<td>−26.1 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>unknown</td>
<td>−10.0 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>standard</td>
<td>−26.4 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>unknown</td>
<td>−10.7 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>standard</td>
<td>−26.2 ± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>unknown</td>
<td>−10.2 ± 0.4</td>
</tr>
</tbody>
</table>
In order to extend the range of $\delta$ values under test, we prepared solutions in the range of $\delta = -10$ to $+50\%$ in steps of $10\%$ (concentration 2 $\mu$g/µl). Again, sequences of 5 injections with the same solution were performed, before switching to the next solution. In addition, one injection of the first solution ($\delta = -10.0\%$) was performed also after the 4th and the 7th sequence for calibration purposes.

<table>
<thead>
<tr>
<th>sequence</th>
<th>calculated $\delta[%]$</th>
<th>measured $\delta[%]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-10.0</td>
<td>-10.1 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>+1.6 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>+10.0</td>
<td>+13.4 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>+20.0</td>
<td>+22.6 ± 0.7</td>
</tr>
<tr>
<td>5</td>
<td>+30.0</td>
<td>+36.2 ± 0.8</td>
</tr>
<tr>
<td>6</td>
<td>+40.0</td>
<td>+47.5 ± 0.9</td>
</tr>
<tr>
<td>7</td>
<td>+50.0</td>
<td>+57.5 ± 0.7</td>
</tr>
</tbody>
</table>

Although the results of the measurements (Tab. 2) differ markedly from the calculated $\delta$ values, the data can be fit by a straight line (Fig. 4). To confirm the irm-LCMS results, one of the sample solutions was measured by bulk combustion with the result of $\delta = +24.6 ± 0.8\%$ for a solution with the (calculated) $\delta$ value of $+20.0\%$, matching the straight line on Fig. 4. We interpret this to mean that the labelled glucose has a higher $^{13}$C concentration than was assumed, due to enrichment of all C atoms with $^{13}$C during synthesis. The NMR analysis, run by the manufacturer, probably had insufficient precision to detect this level of enrichment.

The use of $^{14}$C tracers has inherent drawbacks. There is a demand to replace this technique with a non radioactive tracer method. To evaluate the sensitivity of irm-LCMS in tracer studies, the following experiment was performed: Natural and $^{13}$C enriched glucose (tracer) of varying concentrations were injected into the LCMS, as shown on Fig. 5. Due to the 1000-fold lower concentration the tracer peaks have a peak height of 6 mV only on the 44 trace, thus, they cannot be distinguished from the background noise (ca. 60 mV peak to peak). On the

$$m_a/m_b = M_a/M_b \times (\delta_a - \delta_b)/(\delta_a - \delta_b)$$

(1)

$m$ is the amount of unlabelled (index a) and labelled (index b) glucose, $M$ is the corresponding molecular weight and $\delta$ is used in the common definition (2) with index $x$ representing the solution to be prepared.

$$\delta^{13}\text{C}(\%o) = 1000 \times (R_s - R_p)/R_p \text{ with } R = [^{13}\text{C}]/[^{12}\text{C}]$$

(2)

With indexes $s$ and $p$ indicating sample and PDB standard and $R_p = 0.0112372$. We used $\delta_p = -10\%o$, determined by bulk combustion of the unlabelled glucose and $\delta_p = 13.683.4\%o$ calculated under the assumption, that 1 of the 6 C atoms in the molecule is $99\%$ $^{13}$C labelled (information from Sigma literature).
Fig. 4  Plot of data from Table 2. Each datapoint is the rms average for 5 subsequent injections with the same sample solution (same nominal $\delta$ value). The error bars represent the standard deviation for 5 injections. The additional datapoint is a bulk combustion of the solution with the calculated $\delta$ value 20.0‰.

Fig. 5  "Chromatogram" obtained from flow injections of natural glucose (2$\mu$g/$\mu$L) and $^{13}$C labelled glucose (2ng/$\mu$L, 1ng/$\mu$L and 0.5ng/$\mu$L)
45/44 ratio trace, however, the situation is different: The $^{13}$C/$^{12}$C ratio is about 20 times that of the background, resulting in an enhancement of the tracer peak$^2$.

The measurement at a tracer concentration of 0.5 ng/$\mu$l (last 3 injections on Fig. 5) shows a signal to noise ratio of about 3 to 1. Taking this as the detection limit, the amount of $^{13}$C applied is 167 pg, or 17 pg on the wire after the eluent split.

CONCLUSIONS

The first measurements on a breadboard design of a moving wire interface for irm-LCMS are encouraging. A liquid flow up to 0.1 ml/min of methanol/water (80/20) can be applied to the wire. The peak area of the detected CO$_2$ signal (m/z = 44) is proportional to the sample concentration. Accuracy and precision of $\delta^{13}$C/DB for natural samples are better than 1‰. The detection limit for $^{13}$C tracers is around 200 pg carbon label. The next step will be to test the performance of the interface from life effluents of an LC.

References


$^2$For a plausibility assessment of the expected peak height the average height of the background signal has to be known. From the 44 signal of natural glucose (5 V on top of a 1 V background for a 10 µg injection) the background signal of 1 V is estimated to be equivalent to a continuous $^{12}$C flow of 40 ng/s. Thus the corresponding $^{13}$C flow is about 0.4 ng/s.

The situation for the tracer (concentration of 0.5 ng/$\mu$l) is as follows:

2.5 ng tracer are injected, corresponding to 0.02 ng/s $^{13}$C deposited on the wire at the apex of the peak. 1 out of 6 carbon atoms is labelled, therefore 0.004 ng/s 13C are deposited on the wire. The sample signals at 44 and 45 added to the background signals resulting in a 45/44 ratio of 0.404/40.02 = 1.01% at the apex of the peak, sufficient to be distinguished from the background (1.00%).
