Introduction

Amino acids are the molecular units that make up peptides and proteins. There are 20 α-amino acids that are relevant in mammalian proteins. Several other amino acids can be found in the body in free forms and in non-protein associated states performing specialized functions. Most analytical protocols for amino acid analysis use gas or liquid chromatography (GC, LC), often coupled with mass spectrometers (GC/MS, LC/MS). Isotope ratio monitoring GC/MS (irm-GC/MS) is well-established for the determination of $^{13}$C/$^{12}$C and $^{15}$N/$^{14}$N isotope ratios of amino acids. However, amino acids cannot be analyzed directly by GC due to the presence of polar groups because polar groups (e.g., carboxyl, amino and hydroxyl functions) decrease the compound’s volatility, making them thermally labile at GC temperatures. Prior to the analysis of amino acids by GC, it is necessary to quantitatively convert the amino acids into volatile derivatives.

However, high-performance liquid chromatography (HPLC) is the preferred separation method for non-volatile components such as amino acids. Isotope ratio monitoring LC/MS (irm-LC/MS) with the Thermo Scientific LC IsoLink allows determination of $^{13}$C/$^{12}$C ratios of individual amino acids without derivatization, eliminating all of the possible errors, limitations, and time requirements of derivatization. For amino acids water is the best solvent; HPLC columns are available on which the separation of amino acids can be performed with an aqueous mobile phase.

The LC IsoLink offers new access to $^{13}$C-tracer experiments which investigate amino acid and protein metabolism.

irm-LC/MS Technology

The LC IsoLink is the first high sensitivity interface connecting High Performance Liquid Chromatography (HPLC) with Isotope Ratio Mass Spectrometry (IRMS) for reproducible and accurate on-line determination of $^{13}$C/$^{12}$C isotope ratios (Figure 1). Each of the organic compounds eluting from an HPLC column can be isotopically analyzed for $^{13}$C/$^{12}$C while maintaining the chromatographic resolution.

In the LC IsoLink, a sample is oxidized to CO$_2$ within the aqueous solvent eluting from the HPLC system. The oxidation is obtained by adding oxidation reagents to the mobile phase. These consist of sodium peroxodisulfate (0.8 M) and phosphoric acid (1.7 M), which are pumped separately at a flow rate of 30 mL/min and added to the mobile LC phase. Within this mixture, all organic compounds eluting from the HPLC column are oxidized quantitatively into CO$_2$ when passing through a heated reactor. The reaction is carried out in a reactor maintained at 99.9°C.

In a downstream separation unit, the CO$_2$ is removed from the liquid phase and entrained into a stream of helium. The individual CO$_2$ peaks in helium (which correspond one to one with the peaks of the individual compounds) are subsequently dried in an online unit (Nafion$^\text{®}$) and then admitted to the IRMS system via an open split interface. The helium flow rate is at 2 mL/min.

The LC IsoLink also enables a second operation mode for $^{13}$C-bulk analysis. The flow injection or μ-EA mode offers fast analysis of bulk samples, working standards and reference materials. Samples can be injected via a 6-port-valve of variable size which is located between the HPLC column and the interface.
Experimental Section
The on-line determination of the $^{13}\text{C}/^{12}\text{C}$ isotope ratios was performed using a Thermo Scientific Surveyor™ MS Pump coupled to a Thermo Scientific DELTAplus XP isotope ratio mass spectrometer via the LC IsoLink interface. The interface was used in both operational modes, HPLC (compound specific isotope analysis, CSIA) mode and bulk stable isotope analysis (flow injection or μ-EA) mode.

HPLC Setup 1
In a first approach to the separation of amino acids, the HPLC separation was effected on a C18 column 60 Å (4 μm, 3.9 mm x 300 mm, Nova-Pak®) using a 10 mM NaH$_2$PO$_4$ buffer (pH 4.7) as mobile phase and a flow rate of 0.3 mL/min.

HPLC Setup 2 (McCullagh et al.)
In the second approach a single column was used, utilizing a mixed-mode stationary phase which enabled separation using both hydrophobic and ion-exchange interactions. Amino acid separation was performed on a Primesep A column (4.6 mm x 250 mm, particle size 5 μM; SIELC Technologies, Prospect Heights, IL, USA) by applying a linear gradient programme. Two mobile phase compositions were used in this program. Pump A (100% water) was used for the first 10 min of the separation. From 10 to 30 min a linear gradient took place to 100% pump B (0.2% sulfuric acid by volume). After 30 min 100% pump B was maintained until the end of the run (total 110 min). Throughout the run, the flow rate of the mobile phase was maintained at 0.7 mL/min and the column at 25°C. A 10 mL sample loop was used for injection of the sample. Although the analysis described in reference 1 utilized a 4.6 mm x 250 mm Primesep A column at a mobile phase flow rate of 0.7 mL/min, a 3.2 x 250mm Primesep A column was also tested and found to produce very similar retention times using a flow rate of 0.5 mL/min.

Flow Injection (μ-EA Mode)
A 10 μl loop was installed for flow injection. The loop was rinsed and filled by loading of about 30 μl of sample volume. The measured δ$^{13}$C value is the bulk δ$^{13}$C of all of the water soluble components. Memory effects of the loop can be avoided by flushing the loop 5 times of the subsequent sample material.

Calculation
The δ$^{13}$C value in per mil (%) is the $^{13}$C/$^{12}$C ratio of the sample related to the $^{13}$C/$^{12}$C ratio of a reference material:

$\delta^{13}\text{C} = \left(\frac{^{13}\text{C}^{12}\text{C}}{^{12}\text{C}^{12}\text{C}}\right)\text{Sample} / \left(\frac{^{13}\text{C}^{12}\text{C}}{^{12}\text{C}^{12}\text{C}}\right)\text{Reference} - 1 \times 1000.$

$\delta^{13}$C can be approximated by dividing atom% by 1000.

Results and Discussion
Precision and linearity of the LC IsoLink were evaluated in the flow injection (μ-EA) mode. Leucine was injected with different concentrations, each sample 10 times, using a 10 μl loop (Figure 2).

![Figure 2: Irn-LC/MS traces (m/z 44, 45, 46) of leucine (130 ng/μl) analyzed by flow injection (μ-EA mode).](image)

The precision of the analyses are shown in Table 1.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>65 ng/μl</th>
<th>130 ng/μl</th>
<th>195 ng/μl</th>
<th>325 ng/μl</th>
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<tbody>
<tr>
<td>$\delta^{13}$C (%)</td>
<td>-25.30</td>
<td>-25.28</td>
<td>-25.43</td>
<td>-25.34</td>
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<tr>
<td>Std dev.</td>
<td>0.05</td>
<td>0.09</td>
<td>0.11</td>
<td>0.15</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 1: Precision of $\delta^{13}$C values of leucine analyzed by flow injection Irn-LC/MS (μ-EA mode).

The $\delta^{13}$C reproducibility is ± 0.15 ‰. The LC IsoLink allows reproducible $^{13}$C isotopic analysis of a wide range of concentrations. The mean $\delta^{13}$C value over range of sample size from 650 ng to 3250 ng was - 25.34 ‰ with an overall standard deviation of 0.07 ‰ (Figure 3). Figure 4 shows the correlation between the area (in Volt-seconds, Vs) and the amount injected (ng) of leucine.

![Figure 3: Linearity of leucine analyzed by flow injection Irn-LC/MS (μ-EA mode).](image)

![Figure 4: Flow injection Irn-LC/MS (μ-EA mode) of leucine, quantitative analysis by correlation of area [in Volt-seconds, Vs] and amount injected [ng].](image)
The excellent linearity ($R^2$ of 0.99999) proves that there is quantitative conversion of the C into CO$_2$ and that there is complete separation of CO$_2$ from the mobile phase by the membrane exchanger. Consequently, the LC IsoLink can be used for precise quantification of concentrations as well as precise determination of $^{13}$C/$^{12}$C isotope ratios.

The HPLC (CSIA) mode enables the analysis of complex mixtures which are injected into a loop injection valve in front of the HPLC column performing a separation of individual components. A mixture of alanine, leucine, phenylalanine and tryptophan is separated on a C18 column using 10 mM NaH$_2$PO$_4$ buffer as mobile phase. Figure 5 shows the m/z 44 trace (mV of $^{13}$C$^{16}$O$_2$ vs time) of this standard mixture.

Figure 5: *reLC/MS chromatogram (m/z 44 trace) of a standard mixture of Ala (113 ng/µl), Leu (123 ng/µl), Phe (79 ng/µl), Try (120 ng/µl).

All amino acids eluting from the HPLC column are quantitatively converted into CO$_2$ and the integrity of chromatographic resolution is maintained during this conversion. The separation of the amino acids is performed within 30 min. At the end of the chromatogram three flow injections of benzoic acid demonstrate the possibility for additional referencing with working standards of the same or different compounds.

The maximum concentration which can be injected is determined by the amplifiers which offer a linear response up to 50 V. Each collector has its individual amplifier. The different amplifications for m/z 44, 45 and 46 reflect the natural abundances of the carbon isotopes $^{13}$C and $^{12}$C. Figure 6 shows the HPLC chromatogram (m/z 44, 45 and 46 traces) of amino acids in higher concentrations.

Figure 6: *reLC/MS chromatogram (m/z 44, 45, 46 traces) of a standard mixture of Ala (1135 ng/µl), Leu (1470 ng/µl), Phe (949 ng/µl), Try (1108 ng/µl).

Table 2 shows the precision of the $\delta^{13}$C values of amino acid mixtures. The samples were injected with different concentrations using a 5 µl loop. The analyses of mixture 1 and 2 representing concentrations from 79 to 368 ng/µl resulted in an excellent reproducibility, between 0.35 ‰ and 0.11 ‰. The more highly concentrated mixtures (between 474 and 1354 ng/µl) show reproducibility of better than 0.16 ‰. These results are in agreement with precision calculated from counting statistics.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Alanine</th>
<th>Leucine</th>
<th>Phenylalanine</th>
<th>Tryptophan</th>
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</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>113 ng/µl</td>
<td>123 ng/µl</td>
<td>79 ng/µl</td>
<td>120 ng/µl</td>
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<tr>
<td>$\delta^{13}$C (‰)</td>
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<td>0.26</td>
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<tr>
<td>N</td>
<td>5</td>
<td>5</td>
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<td>5</td>
</tr>
</tbody>
</table>

Table 2: Precision of $\delta^{13}$C values of amino acids analyzed by compound specific isotope analysis *reLC/MS (HPLC mode).

Figure 7 shows the linearity of $\delta^{13}$C values of amino acids analyzed by HPLC mode. The measurements were performed on different days using reagents were prepared daily. Nevertheless, the $\delta^{13}$C values of different concentrations are both reproducible and linear, indicating the robustness of the method and the excellent long term reproducibility which is required for routine analysis.

Figure 7: Linearity of $^{13}$C/$^{12}$C values of amino acids analyzed by compound specific isotope analysis *reLC/MS (HPLC mode).
Recently, a new chromatographic method for the HPLC separation of underivatized amino acids using an acidic, aqueous mobile phase in conjunction with a mixed-mode stationary phase was presented (McCullagh et al.). The method utilizes a reversed-phase Primesep-A column with embedded, ionizable, functional groups providing the capability for ion-exchange and hydrophobic interactions.

Baseline separation of 15 amino acids and their carbon isotope values are reported. Standard deviations for all amino acids were between 0.06% and 0.38% with an average of 0.18% (n=6).

The LC IsoLink interface is shown here to enable the analysis of underivatized amino acids, reducing sample preparation and analysis time. Amino acids and other bio-molecules (e.g. carbohydrates, organic acids, peptides and nucleic acids) can also be isotopically analyzed by irm-LC/MS. This new technique can be applied to many applications in medical, biological, pharmaceutical and environmental studies.

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**References**
