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Short communication

Quantitative evaluation of carbon isotopic fractionation during reversed-phase high-performance liquid chromatography

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Abstract

The fractionation of ^{13}C during low-performance preparative LC and high-performance LC is reported quantitatively for methyl palmitate and using high-precision isotope ratio mass spectrometry (IR-MS). For both preparative and high-performance analytical columns, ^{13}C enrichment is about 7‰ greater than the parent starting material, drops sharply in the first section of the peak and then settles to a value about 1‰ below that of the starting material. Recycling over a single HPLC column did not induce greater fractionation. These results emphasize the importance of quantitative peak collection for high-precision IR-MS studies, particularly the first part of the peak where the isotope ratio changes rapidly.

Keywords: Isotope ratios; Carbon isotopic fractionation; Methyl palmitate; Fatty acid methyl esters

Isotope ratio mass spectrometry (IR-MS) is the method of choice for the high-precision determination of stable isotope ratios for C, H, N, O and S. High-performance liquid chromatography (HPLC) is commonly used for semi-preparative separation of organic compounds such as steranes, chlorophylls and porphyrins prior to isotopic analysis by IR-MS [1–5]. To ensure accuracy, it is necessary that any pretreatment steps, including separations, preserve the isotopic composition of the individual components. HPLC-induced isotopic fractionation followed by selective pooling of fractions is known to alter the original isotopic composition of the compound and produce erroneous results.

Carbon isotopic fractionation in gas chromatography (GC) is routinely observed in on-line GC–combustion–IR-MS (GC–C–IR-MS) [6]. LC-induced fractionation of hydrogen isotopes is well documented and has even been exploited for the separation of deuterated compounds from their corresponding unlabelled analogues [5,7,8]. It has generally been observed that the nature of the isotopic separation is dependent upon the polarity of the stationary phase. In normal-phase, polar columns, light isotopomers elute more rapidly while in reversed-phase chromatography heavy isotopomers elute more rapidly [9–13]. Although the mechanism for this separation is not completely understood, it has been argued that Van der Waals interactions with the non-polar component of the column are reduced for heavy isotopomers; that is, for the solvent in normal-phase chromatography and the stationary phase in reversed-phase chromatography.

It is clear that chromatography also induces fractionation of carbon isotopes. Unlike hydrogen, however, there appears to be some discrepancy con-

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cerning the effect of the stationary phase and the order of elution of the isotopes. For example, in reversed-phase chromatography of leucine, citric acid and tartaric acid ^{12}C isotopomers elute first, while in the reversed-phase separation of steroids and malic acid ^{13}C isotopomers elute first [13–15].

In this brief report we show a quantitative study of the fractionation of C isotopes of fatty acid methyl esters (FAMEs) during low-performance preparative-scale LC, high-performance analysis LC, and during recycling of analyte several times over a HPLC column to document the extent of fractionation across chromatography peaks.

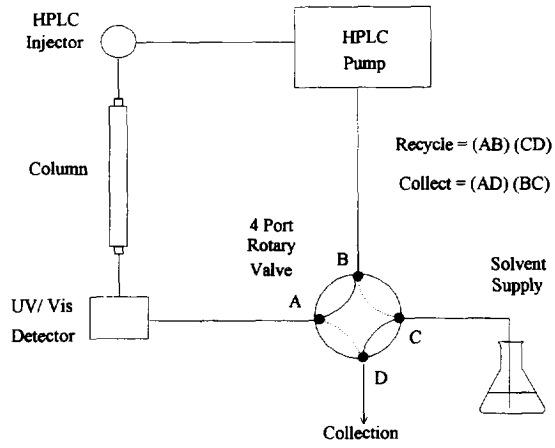

Fig. 1. Diagram of recycling chromatography system. Recycling is accomplished by connecting ports AB and CD. Sample is collected when the valve is rotated and AD and BC are connected.

1. Experimental

1.1. Low-performance analysis

Methyl palmitate ('Me16:0', >99% pure) was dissolved in acetonitrile and used as a test sample. This standard was injected onto a LiChro-Prep RP-8 preparative-scale column (40–63 μm particle size) and eluted isocratically with acetonitrile at 0.7 ml/min. Chromatography was monitored using an SSI (Scientific Systems, State College, PA, USA) variable-wavelength UV–Vis detector (210 nm) and HPLC fractions were collected at 2-min intervals across the peak yielding a total of 51 fractions eluting over 104 min. Carbon isotope ratios and analyte concentration in individual fractions were determined by IR-MS.

1.2. Recycling LC

Recycling LC was introduced in the 1960s by Porath and Bennich as a means of increasing the number of theoretical plates attainable for a given separation without increasing the effective bed height, and ultimately the backpressure, in the column [16]. In short this is accomplished by pumping the column eluent through the column instead of mobile phase from the reservoir, allowing a single analyte peak to be re-chromatographed several times. We used this approach to increase the degree of isotope segregation. Fig. 1 is a diagram of the recycling apparatus. It consisted of an SSI HPLC pump, an SSI injector with a 10-μl injection loop, a 4-port rotary valve (Rheodyne), a 25 mm ODS 3 (5 mm particles) column (Whatman), and an SSI UV–Vis detector (210 nm). Me16:0 was dissolved in methanol, injected onto the column, then eluted using methanol supplied at 1.5 ml min^{-1}. Prior to 16:0 elution, the rotary valve position was switched so that the column eluent was diverted back through the HPLC pump onto the column. The eluent was cycled from 1 to 5 times over the column before the rotary valve was reopened and the eluent was diverted to a collection port where multiple fractions were collected across each peak. Compound-specific isotope analyses (CSIAs) were performed on each fraction and the parent starting material as described below.

High-precision IR-MS analyses were performed on all fractions derived from the experiments described above using a Finnigan MAT 252 GC–C–IR-MS system. The chromatograph was equipped with a splitless injector and a J&W DB Wax column (50 m×0.32 mm I.D., 0.25 μm film thickness) with He carrier gas flowing at 1.5 ml min^{-1}. A typical chromatogram of m/z 44 versus time is presented in Fig. 2. Isotope values for the analytes in both sets of experiments were calculated using a working CO_2 standard calibrated versus NIST RM8541:USGS24 graphite reference standard. The standard CO_2 intensity was matched to within ±10% of the sample intensity for all of the analyses and isotope values

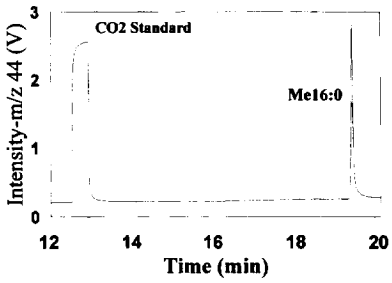

Fig. 2. Chromatogram of a GC–C–IR-MS analysis of Me16:0. The CO_2 standard is used as a reference for isotopic calibration.

are expressed in the $\delta^{13}C$ notation, standard in IR-MS and defined as follows:

$$\delta^{13}C_{PDB}(‰) = \left(\frac{R_{SPL} - R_{PDB}}{R_{PDB}}\right) \times 1000 \quad (1)$$

where 'PDB' is the internal standard PeeDee Belemnite with isotope ratio $R_{PDB} = 0.0112372$, the 'SPL' subscript refers to the sample, and the units are referred to as 'permil'.

2. Results and discussion

2.1. Low-performance preparative results

To quantitatively investigate the fractionation phenomena, Me16:0 was eluted from a reversed-phase column and 51 fractions were collected. These fractions contained between 100 and 6500 mg of carbon, as shown in Fig. 3. Isotopic analyses were performed in duplicate on all fractions as well as the starting material. The starting material had an isotope

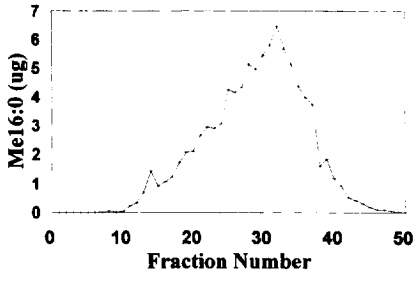

Fig. 3. Reconstructed chromatogram of the Me16:0 content of each HPLC fraction derived from the preparatory scale reversed-phase analysis. Quantification was accomplished using GC–C–IR-MS data.

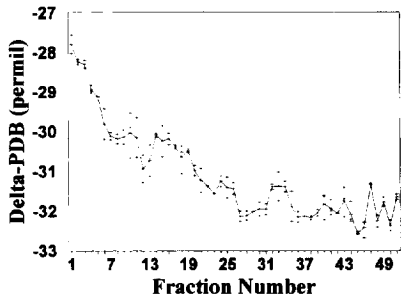

Fig. 4. Variation of isotope ratio across the preparatory scale, reversed-phase Me16:0 peak. '+' = individual CSIA analyses of the corresponding fraction; the solid black line corresponds to replicate means.

ratio of $\delta^{13}C_{PBD} = -31.39‰$; Fig. 4 shows that subsequent fractions exhibited isotope ratios of $-27.79‰$ at the beginning of the peak to $-32.4‰$ at the tail. The initial ^{13}C content dropped sharply and settled to a value slightly below that of the parent material. To establish the consistency of this result, the delta values and carbon content of each fraction were used to perform a mass balance. When total ^{12}C content and total ^{13}C content were determined, the isotope ratio of the cumulative fractions was within 0.2‰ of the isotope ratio of the starting material, which is well within the analytical precision.

Recycling chromatography was used to exaggerate isotopic segregation across chromatography peaks. Compound-specific isotope analyses were performed on all fractions as described above. Fig. 5 is a trace of the recycling chromatography at 210 nm. The first peak results from running the sample through the column once. Subsequent samples were recycled over the column; peak shapes show the expected

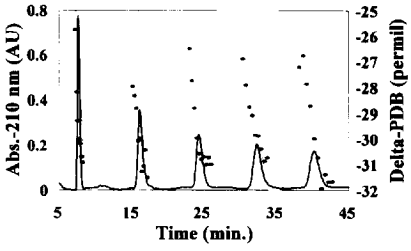

Fig. 5. The solid black line in this figure is the absorbance at 210 nm for the recycling chromatography of Me16:0. The dotted line depicts the carbon isotope ratio measured across the peak.

broadening with each subsequent recycling. Also plotted in Fig. 5 are the isotope ratios for each fraction collected across the peaks. This plot shows that the isotopes are distributed with ^{13}C enriched in the beginning of the peak and ^{12}C enriched in the tail with the overall isotope ratio varying up to 7‰. Once again, the isotope ratio drops sharply at the peak beginning and settles to values just below the isotope ratio of the parent material ($\delta^{13}C = -29.53$‰). While this distribution was maintained in recycled peaks, isotopic segregation was not further increased. This indicates that the band broadening associated with recycling occurs at a greater rate than the rate of isotopic separation under these conditions.

3. Conclusion

Our results show that the beginning of a peak has an isotope ratio sharply enriched relative to the parent material, while the end of a peak is mildly depleted. This observation suggests that quantitative collection of the beginning of LC peaks is particularly important for C isotope ratio analysis.

The recent development of on-line LC–combustion–IR-MS in our laboratory [17,18] and the related LC–chemical reaction interface system elsewhere [19] has made quantitative evaluation of the extent of LC-induced isotopic fractionation in reversed-phase separation necessary. The immediate practical consequence of these findings is that HPLC techniques must be applied with caution when used for sample preparation of complex mixtures for isotopic analyses. For instance, porphyrins and sterols are compounds that are almost exclusively purified through HPLC chromatography. Boreham et al. report significant carbon isotope variations of 4‰ within different groups of porphyrins isolated from the Julia Creek Shale [20]. Differences of this magnitude clearly require quantitative collection for accurate isotope ratio determination.

Acknowledgments

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