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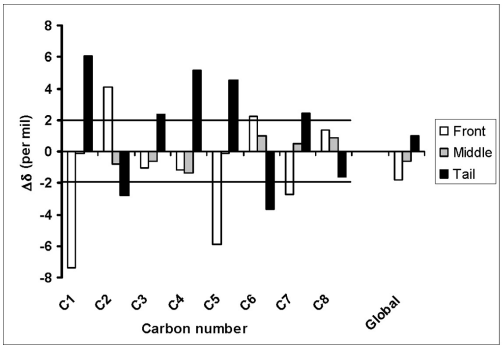

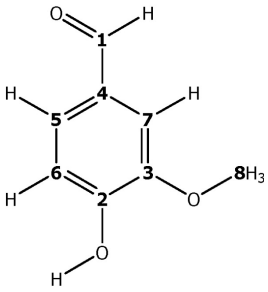

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Unexpected Fractionation in Site-Specific ^{13}C Isotopic Distribution Detected by Quantitative ^{13}C NMR at Natural Abundance

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Isotopic fractionation phenomena are associated with many physical and chemical processes. In all cases, this fractionation reflects a change in the concentration of each isotopomer between the starting material and the product when the chemical or physical transformation is not complete.[1] The method of choice for measuring each isotopomer concentration—hence the associated isotope effects—is quantitative NMR.[2] The present study exploits this positional resolution, made possible by the recently developed method to measure the site-specific natural ^{13}C isotopic fractionation by NMR (^{13}C SNIF-NMR),[3] to examine site-by-site variation at natural abundance induced by two common physical processes: distillation and column chromatography. The target molecules, ethanol and vanillin, respectively, both show fractionation that differs in size and/or in sense at each carbon atom. The observation of this disparity in behavior is novel and unexpected since previous studies using isotope ratio mass spectrometry (IRMS) could only access the global or partial site-specific content of the heavy element.

Recent improvements in quantitative ^{13}C NMR have established conditions of sufficiently high trueness and precision to make routine measurements at natural abundance possible.[4] Thus, the ^{13}C content of each carbon can be determined with a long-term repeatability of the order of 1‰ on the absolute isotopic deviation scale δ.[5] This is sufficiently accurate to observe natural variation from a statistical distribution in the internal $^{13}C/^{12}C$. Consequently, ^{13}C SNIF-NMR is becoming a tool of interest for discriminating the origin of natural molecules. However, the extraction and purification of molecules such as ethanol or vanillin require great care in order to avoid further fractionation being introduced by the procedures employed. It has been shown by IRMS that incomplete distillation of ethanol generates fractionation for the global $^{13}C/^{12}C$, $^{2}H/^{1}H$, and $^{18}O/^{16}O$ ratios.[6−9] Similarly, separation on silica gel chromatography disfavors the heavy isotope ^{13}C during the elution of vanillin or 4-hydroxybenzaldehyde (pHB),[10,11] an example of a general phenomenon observed for several stable and radioactive isotopes in organic substances subjected to different types of chromatography.[12,13] Data on site-specific fractionation at natural abundance are much less common for such phenomena. It has only been reported for ^{2}H during the distillation of some organic compounds[7] and during vanillin purification on fast column chromatography[11] but has not previously been described for ^{13}C.

To observe site-specific isotopic fractionation at individual carbon sites during distillation, we have measured the variation in $^{13}C/^{12}C$ ratios by quantitative ^{13}C NMR[14] at the two carbon positions of ethanol during distillation using a Cadiot column. This device enables distillation under steady-state conditions since only a very small quantity of distillate is collected. Thus, the isotopomer profile of the distillate represents that of the vapor phase. In the case of a small transformation of the substrate, such as the collection of a very small distillate volume (<3%), isotope effects (IE) are likely to occur in the distillate. In contrast, the liquid phase can be considered as having the isotopomer profile of the starting material since the change in the isotope composition in the bulk liquid phase during the distillation is negligible. The converse effect, which occurs in the residual substrate, can only readily be observed at an advanced state of the distillation procedure (>90%). The corresponding separation factor α is defined as

$$\alpha = (N'/N)_{vap}/(N'/N)_{liq} \quad (1)$$

where N' and N are the mole fractions of light and heavy isotopomers, respectively, in the system at equilibrium. The specific isotopic fractionation of site i of a ^{13}C isotopomer is then defined as

$$\alpha = (^{13}C/^{12}C)_{i,liq}/(^{13}C/^{12}C)_{i,vap} \quad (2)$$

Thus, a normal isotope effect leads to $\alpha > 1$, while an inverse effect favoring the heavy atom gives $\alpha < 1$. Unfortunately, there is a misuse in the literature of eqs 1 and 2 which are often inverted, as argued in ref 7. Table 1 presents the results obtained from the distillation of pure ethanol.

The α value calculated from IRMS data (global value) is in agreement with previous results.[6−8] The ^{13}C SNIF-NMR measurements reveal that it is the methylenic carbon that undergoes the largest inverse isotopic fractionation. This discrimination between the CH_2 and the CH_3 has already been observed for ^{2}H and was explained on the basis of the presence of hydrogen bonds involving the hydroxyl group: the presence of a ^{2}H on the CH_2 alters the energy required to rupture the hydrogen bond.[7] The results presented herein indicate that the presence of a ^{13}C on the CH_2 can also influence the thermodynamics of the system, similarly altering the energy required to rupture the noncovalent interactions.

Chromatographic separation is also generally recognized as introducing isotopic fractionation, the isotope ratio changing from the head to the tail of the eluted peak.[13] This phenomenon has been described using either ^{2}H- or ^{13}C-enriched molecules, but the discrimination of ^{13}C isotopomers at natural abundance has not previously been reported. Fractionation in the site-specific $^{13}C/^{12}C$ ratio has been measured using ^{13}C SNIF-NMR for vanillin (Figure 1) during silica gel 60 chromatography. Figure 2 shows the evolution of the relative ^{13}C isotopic deviation $\Delta\delta_i$ during the elution of the vanillin peak. Taking into account the standard deviation of 1 ‰ for long-term repeatability achieved for ^{13}C NMR on vanillin,[4] confidence limits can be set at −2 and +2 ‰ for delineating significant effects. It is evident that different carbon positions show disparate fractionation patterns: at carbons C2 and C6, retention of the light isotope is favored; at carbons C1, C4, and C5, retention of the heavy isotope is favored; at carbons C3, C7, and C8, only

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Table 1. Values of $\delta^{13}C$ Measured by ^{13}C NMR During Distillation of Ethanol

	Global (IRMS)		CH_2 (NMR)		CH_3 (NMR)	
product	$\delta^{13}C$ (‰)	α	$\delta^{13}C$ (‰)	α	$\delta^{13}C$ (‰)	α
ethanol to be distilled	−29.3		−32.7		−25.9	
vapor fraction (2.6%)[a]	−25.1	0.9955 (±0.0004)	−26.4	0.994 (±0.001)[b]	−23.8	0.998 (±0.001)[b]

[a] Percentage of the collected distillate, with respect to the starting material. [b] Confidence interval calculated from the long-term repeatability recently achieved by ^{13}C SNIF-NMR measurement (SD $\delta^{13}C$(‰) < 0.5‰).[4]

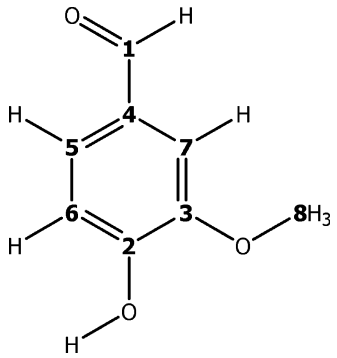

Figure 1. Molecular structure of vanillin with numbering of carbons in relation to decreasing ^{13}C chemical shift in the NMR spectrum.

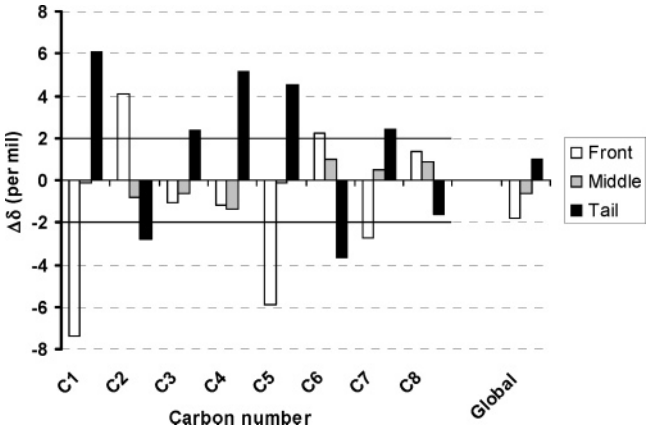

Figure 2. Site-specific fractionation of ^{13}C of vanillin during silica gel column chromatography. The relative variation in $^{13}C/^{12}C$ for each carbon position is expressed as $\Delta\delta_i = (\delta p - \delta s)_i$ for each carbon position i of the eluted vanillin (δp_i) with respect to the initial value obtained for site i (δs_i) by NMR. See Figure 1 for carbon numbering. The data presented are from the first portion (front = from 0 to 11% eluted), middle portion (middle = from 28 to 43% eluted), and the last portion (tail = from 76 to 100% eluted). Global is the total mean value for each of these portions determined by IRMS.

slight fractionation occurs. Furthermore, the intensity of $\Delta\delta_i$ is much larger for some carbons (C1, C2, C4, C5, and C6) than for others (C3, C7, and C8): in contrast, the relative global variation observed by IRMS is much smaller (Figure 2).

The explanation for such behavior is not straightforward since it cannot be explained simply as a thermodynamic isotope effect (TIE). Rather, during the successive equilibria between the stationary and eluent phases, each isotopomer is subjected to an individual noncovalent isotope effect (NCIE).[16,17] Although there is increasing interest in this phenomenon for ^2H isotopomers,[18,19] relatively little is yet known about NCIEs in relation to ^{13}C isotopomers.

While it is not currently possible to interpret these observations fully in theoretical terms, it is pertinent that the most significant isotope effects are related to the presence of the aldehyde and hydroxyl groups, suggesting that their polarities combined with the aromaticity of the phenyl ring are key factors promoting isotope-dependent interactions between the eluate and the stationary phase. Interestingly, the observed isotope effect is propagated to carbon atoms distal to the probable interaction sites between vanillin and the stationary phase. Although this secondary NCIE is well-documented for ^2H,[19] it has not previously been detected for ^{13}C. Experimental and theoretical studies that take into consideration the roles of steric, electrostatic, and polar parameters, including the possible alteration of polarity by adjacent isotopes, are being carried out in order to construct a model integrating both chemical and physical properties to interpret the underlying causes of the observed positional ^{13}C fractionation.

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(5) $\delta_i = (R_i/R_{VPDB} - 1) \times 1000$ (in ‰), where $R_i = {}^{13}C/{}^{12}C$ isotopic ratio of site i and $R_{VPDB} = {}^{13}C/{}^{12}C$ isotopic ratio (= 0.0112372) of the international standard reference product, Vienna−Pee Dee Belemnite (VPDB).
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(14) Quantitative ^{13}C NMR spectra were recorded using a Bruker DRX 500 spectrometer fitted with a 5 mm i.d. dual probe $^{13}C/^1H$ carefully tuned at the recording frequency of 125.76 MHz. The temperature of the probe was set at 303 K. The experimental parameters for ^{13}C NMR spectral acquisition were the following: pulse width 4.3 μs (90°), spectral width 10000 Hz (for ethanol) or 30030 Hz (for vanillin), sampling period 1 s. Inverse-gated decoupling was applied in order to avoid NOE. The decoupling sequence employed a cosine adiabatic pulse with appropriate phase cycles, as described in ref 15. Free induction decay was submitted to an exponential multiplication inducing a line broadening of 2 Hz. Curve fitting was carried out in accordance with a Lorentzian mathematical model using Perch Software (Perch NMR Software, University of Kuopio, Finland). Preparation of the samples: for ethanol at natural abundance (700 μL + 200 μL of distilled water + 100 μL of acetone-d_6), 32 scans using a repetition delay of 101 s; for vanillin at natural abundance (250 mg + 400 μL of acetone-d_6 + 100 μL of 0.1 M CrAcac (tris(2,4-pentadionato)chromium(III)) solution in acetone), 92 scans using a repetition delay of 21 s.
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