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Evidence of ^{13}C non-covalent isotope effects obtained by quantitative ^{13}C nuclear magnetic resonance spectroscopy at natural abundance during normal phase liquid chromatography

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ABSTRACT

Quantitative isotopic ^{13}C NMR at natural abundance has been used to determine the site-by-site $^{13}C/^{12}C$ ratios in vanillin and a number of related compounds eluted from silica gel chromatography columns under similar conditions. Head-to-tail isotope fractionation is observed in all compounds at the majority of carbon positions. Furthermore, the site-specific isotope deviations show signatures characteristic of the position and functionality of the substituents present. The observed effects are more complex than would be obtained by simply summing the individual effects. Such detail is hidden when only the global ^{13}C content is measured by mass spectrometry. In particular, carbon positions within the aromatic ring are found to show site-specific isotope fractionation between the solute and the stationary phase. These interactions, defined as non-covalent isotope effects, can be normal or inverse and vary with the substitution pattern present.

1. Introduction

Within the broad context of determining isotope ratios for measuring isotope effects, isotope fractionation or isotope incorporation, it is generally recognized that the procedure used to isolate the target molecule(s) must not *per se* modify the isotope content of the analyte(s). Although techniques such as liquid–liquid extraction [1] or crystallization [2] show negligible isotope fractionation, chromatographic procedures are well known to introduce substantial isotope fractionation [3].

Recently, we have introduced quantitative isotopic ^{13}C NMR spectroscopy at natural abundance as an efficient tool for determining the site-specific distribution of ^{13}C within a molecule [4]. Provided the acquisition conditions are carefully controlled, the internal long-term repeatability attained is about 1‰, concomitant with a signal-to-noise ratio (SNR) of about 500 [5]. Hence, in these experimental conditions, changes in values of isotope deviation ($\delta^{13}C$) <1‰ must be considered as insignificant, those of >1 but <2‰ to indicate a tendency, and those ≥2‰ as statistically significant at the 95% confidence limit, if the measurement is performed once. In the case on n replications, the standard deviation is further reduced by a factor of $(n)^{-0.5}$. In many processes where isotope deviation is exploited, such as isotope fractionation during reactions, authentication, and metabolism, $\delta^{13}C$ >2‰ is frequently found, enabling the routine measurement of $^{13}C/^{12}C$ ratios in the study of these applications. Smaller isotope fractionations could be exploited by improving the measurement precision, which is solely based on the signal-to-noise ratio (SNR) and therefore on the analysis time.

All these analytical objectives require the isolation of the target molecule(s) prior to quantitative ^{13}C NMR spectroscopy. Purification of the product under investigation has to be performed with great care in respect to possible isotope fractionation during the process: the molecule must show the same isotope profile before and after purification. As discriminatory behavior between heavy and light isotopes during chromatographic separation on solid phase is well documented [3], we have focused particular attention on this technique. Prior studies of both ^{2}H and ^{13}C have essentially been based on more or less enriched levels: results from experiments at natural abundance are less common [3,6,7]. Furthermore, there are even fewer studies in which site-specific variation has

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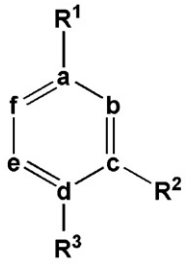

R^1 = CHO, R^2 = OCH_3, R^3 = OH: *vanillin*
R^1 = CHO, R^2 = OH, R^3 = OCH_3: *isovanillin*
R^1 = CHO, R^2 = H, R^3 = OH: *p-hydroxybenzaldehyde*
R^1 = CHO, R^2 = OCH_3, R^3 = H: *m-anisaldehyde*
R^1 = (t)CH=CHCOOH, R^2 = OCH_3, R^3 = OH: *ferulic acid*

Fig. 1. Molecular structure of the compounds studied with numbering of carbons.

been examined [2]: in the majority of reports fractionation has only been evaluated in terms of the global isotope deviation ($\delta_g{}^{13}C$ (‰)) determined using isotope ratio mass spectrometry (IRMS).

However, as we have reported in a preliminary study by ^{13}C NMR of isotope fractionation in vanillin (Fig. 1) during silica gel column chromatography, site-by-site fractionation in the $^{13}C/^{12}C$ ratios can be very much greater than $\delta_g{}^{13}C$ [8]. This study also demonstrates that quantitative ^{13}C NMR spectroscopy is a well-adapted tool to investigate site-by-site variation at natural abundance induced during column chromatography. In this paper we have shown that, with a specified solvent composition of the eluent, the elution order of mono-^{13}C isotopomers (molecules of the same compound with different isotope distribution) of vanillin with respect to ^{12}C molecules is site dependent during normal phase silica gel chromatography (see Fig. 2A). Critically, the induced fractionation at each site is not the same. Thus, the head of the eluted chromatographic peak contains slightly more of the isotopomers **d** and **e** (see Fig. 1 for carbon numbering and Section 2.5 for isotopomer definitions), while isotopomers **a1**, **a** and **f** tend to be slightly more present in the tail. This result was unexpected, as the trend in $\delta_g{}^{13}C$ observed by IRMS shows enrichment from the front to the tail (Fig. 2A). Vanillin was chosen as target analyte for a number of reasons. First, it displays a wide range of chemical shifts in NMR; secondly, it contains three substituents of differing polarities on the aromatic ring; thirdly, a family of closely related aromatic molecules is readily available.

The isotope effects observed during this work cannot be considered as a pure thermodynamic equilibrium isotope effect (EIE), because chromatography involves a flowing system, so that the compounds are not perfectly at equilibrium during partitioning between mobile and stationary phases. Rather, there is a continuous exchange between the free and ad(ab)sorbed populations of the molecules, which, provided the elution rate is not too rapid, will result in conditions close to equilibrium. Nonetheless, for a given molecule each population of isotopomers will exhibit an individual partitioning that will depend on a characteristic set of non-covalent primary and secondary isotope effects (NCIEs), which are themselves dependent on the precise chromatographic conditions used. Growing interest in this phenomenon has led to a number of studies for molecules enriched in ^{2}H [9–11] but, to our knowledge, no data are available for isotopomer selectivity for ^{13}C at natural abundance. As a first approximation, it can be suggested that NCIEs during the elution of vanillin originate from the interaction between the polar functional groups (hydroxyl, methoxyl, aldehyde) in vanillin and those of the stationary phase (mainly silanol groups [12]), but other forces involved in intermolecular interactions (e.g. hydrogen bond, van der Waals forces, permanent dipole, induced dipole, etc.) may also be contributing. Indeed as it

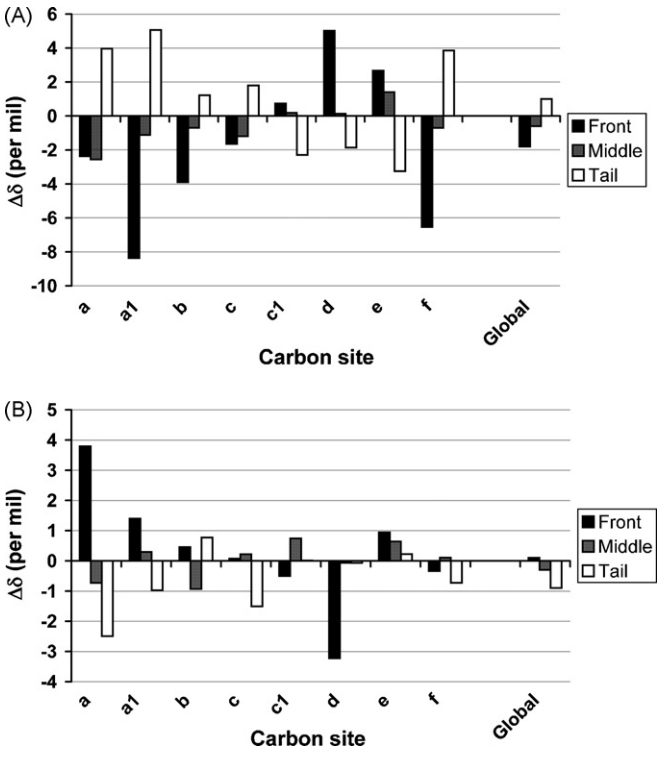

Fig. 2. Site-specific fractionation of ^{13}C of vanillin during silica gel column chromatography. (A) Normal phase silica [8]. (B) Reverse phase silica. The relative variation in $^{13}C/^{12}C$ is expressed as $\Delta\delta_i = (\delta_p - \delta_s)_i$ for each carbon position i of the eluted vanillin $(\delta_p)_i$ with respect to the initial value obtained for site i $(\delta_s)_i$ by NMR. See Fig. 1 for carbon numbering. In A (NP), the data presented is from the first portion (front = from 0 to 11% eluted), middle portion (middle = from 28 to 33% eluted) and the last portion (tail = from 76 to 100% eluted). In B (RP), the data presented is from the first portion (front = from 0 to 25% eluted), middle portion (middle = from 36 to 54% eluted) and the last portion (tail = from 81 to 100% eluted). Global is the total mean value for each of these portions determined by IRMS.

is observed that almost all ^{13}C isotopomers are affected, it can be inferred that either all atoms participate in the overall NCIE, or that both primary and secondary effects occur. However, it is beyond the scope of the current publication to consider in detail the potential participation of these diverse forces, which requires a theoretical treatment of the interactions. Rather, in order to try to rationalize the observed contrary behaviors within ^{13}C isotopomers of vanillin, we have studied the influence of a number of chromatographic and structural parameters on isotopomer selectivity. These include for a specified eluent, the type, position and presence of substituents, and their polarity combined with the aromaticity of the phenyl ring.

To date, the majority of studies of isotope fractionation use the global isotope deviation $\delta_g{}^{13}C$ measured by IRMS. Herein, we present methodology that allows the measurement of the individual isotope fractionation for each isotopomer. This has significant practical implications: if no isotope effect from the global ^{13}C value is observed, that does not mean that each ^{13}C isotopomer has a similar behavior. Rather, as we show, opposite (normal and inverse) effects can cancel out, giving an apparent global effect that reflects poorly the chromatographic behavior of individual isotopomers.

2. Experimental

2.1. Chemicals

4-Hydroxy-3-methoxybenzaldehyde (vanillin) and 4-hydroxybenzaldehyde (pHB), were purchased from MERCK (www.merck.com), 3-hydroxy-4-methoxybenzaldehyde (isovanillin) and 3-

methoxybenzaldehyde (*m*-anisaldehyde) from Sigma–Aldrich (www.sigmaaldrich.com), 4-hydroxy-6-methoxycinnamic acid (ferulic acid) (99%) from Wuhan Yuancheng Technology Development Co. Ltd., China (www.yuanchengtech.com). Cyclohexane, acetone and chloroform (RECTAPUR grade) and methanol (CHROMANORM grade) were purchased from VWR/PROLABO (fr.vwr.com). Cyclohexane was distilled prior use. Normal phase (NP) silica gel NORMASIL (40–63 μm) was obtained from VWR/PROLABO and reverse phase (RP) silica gel 60 RP-18 (40–63 μm) from MERCK. TLC aluminum sheets 20 cm × 20 cm Silica gel 60 F_{254} were purchased from Merck KgaA. Acetone-d_6 and hexadeuterated dimethylsulfoxide (DMSO-d_6) were purchased from Eurisotop (www.eurisotop.fr). Tris(2,4-pentadionato)chromium-III (CrAcac) (97%) was purchased from Acros Organics (www.acros.com).

2.2. Chromatographic procedures

2.2.1. Normal phase chromatography

About 80 g of silica gel NORMASIL (40–63 μm) in 200–240 mL cyclohexane was introduced into a glass column (i.d., 3 cm). To this was loaded 3 g of product dissolved in the minimum volume of appropriate solvent (pure ethanol, acetone, methanol, or as combinations) or as pure liquid for *m*-anisaldehyde. Elution was as follows and monitored by TLC (chloroform/ethyl acetate, 1:1 (v/v)): (i) *m*-anisaldehyde:acetone/cyclohexane 1:99 (v/v), (ii) vanillin, pHB, isovanillin:acetone/cyclohexane 6:94 (v/v), (iii) ferulic acid:acetone/cyclohexane 4:6 (v/v). The elutate was collected in fractions of 200 mL (4–6, depending on the experiment). Each experiment was replicated at least twice.

Flasks containing eluent were grouped into 3–5 pools in order to obtain at least 200–250 mg of the product under investigation in each pool. Solvent was removed by evaporation *in vacuo* at 40 °C max and the residue dried *in vacuo* over P_2O_5. When residual solvent was present, as detected by ^{1}H NMR spectroscopy, this was eliminated by recrystallization (notably for vanillin and ferulic acid) from a mixture of 5–10% (v/v) ethanol in water.

2.2.2. Reverse phase chromatography

Silica gel 60 RP-18 (40–63 μm) was introduced into a glass column (i.d., 3 cm) up to 15.5 cm height. The experiment on the reverse phase matrix was only performed for vanillin: the eluent was acetone/distilled water 13:87 (v/v). 100 mL fractions were collected until exhaustion.

2.3. NMR procedures

2.3.1. NMR acquisition parameters

Samples for ^{13}C NMR were prepared as follows: vanillin, 250 mg + 400 μL acetone-d_6 + 100 μL of 0.1 M CrAcac solution in acetone; ferulic acid, 250 mg + 200 μL DMSO-d_6 + 400 μL methanol + 100 μL of 0.1 M CrAcac solution in methanol; pHB, 250 mg + 500 μL acetone-d_6 + 100 μL of 0.1 M CrAcac solution in acetone; isovanillin, 250 mg + 500 μL DMSO-d_6 + 100 μL of 0.1 M CrAcac solution in DMSO; *m*-anisaldehyde, 250 mg + 500 μL acetone-d_6 + 100 μL of 0.1 M CrAcac solution in acetone.

Quantitative ^{13}C NMR spectra were recorded using a Bruker DRX 500 spectrometer fitted with a 5-mm-i.d. dual probe ^{13}C/^{1}H 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 10,000 Hz, sampling period 1 s. Inverse-gated decoupling was applied in order to avoid NOE. The decoupling sequence employed an adiabatic pulse with appropriate phase cycles, as described in Ref. [13]. The offset of the decoupler was placed at the middle of the proton frequency range. Five spectra per measurement were recorded. The number of scans was adjusted to ensure a SNR ≥500.

2.3.2. Data processing and calculation of the site-specific δ^{13}C (‰) values

Free induction decay was submitted to an exponential multiplication inducing a line broadening of 2 Hz. The curve fitting was carried out in accordance with a Lorentzian mathematical model using Perch™ Software (University of Kuopio, Finland). From the NMR spectral areas, the molar ratio (f_i/F_i) of ^{13}C/^{12}C under each peak can be calculated as described previously [14].

2.4. IRMS procedures

2.4.1. Global isotopic deviation in carbon

The global isotope deviation in carbon, δ^{13}C (‰), for purified compounds was determined by encapsulation and combustion to CO_2 using an elemental analyser (NA2100 Fisons Instruments, www.thermo.com) operating at 1050 °C in an oxygen-rich atmosphere coupled to a Finnigan MAT Delta-V Advantage isotope ratio mass spectrometer (www.thermo.com). Between 1 and 2.5 mg of compound was sealed in a tin capsule and the δ^{13}C determined by reference to a working standard of glutamic acid standardized against calibrated international reference material (IAEA-N1 or IAEA-N2; IAEA, Vienna). The results are expressed relative to the international reference PeeDee Belemnite (PDB), now designated V-PDB: $\delta^{13}C = 1000 \times (R_{sample} - R_{ref})/R_{ref}$ (‰), where $R = {}^{13}C/{}^{12}C$.

2.4.2. Global isotopic deviations in hydrogen and oxygen

The methodology used in this work followed the recommendations of Ref. [15]. The global isotope deviation in oxygen δ^{18}O and δ^2H values were measured by continuous flow thermal conversion isotope ratio mass spectrometry (CF-TC-IRMS) using a glassy-carbon thermal conversion column reactor operating at 1370 or 1450 °C for oxygen and hydrogen measurements, respectively, coupled to a Delta PlusXP mass spectrometer (www.thermo.com). Signals for reference peaks were, respectively, 5000 mV for δ^{18}O and 7000 mV for δ^2H. Complete elimination of water from sample was achieved by rigorous lyophilisation prior to analysis. Dried samples were finely ground to reduce crystal size and homogenized, placed in a dry-chamber and immediately analyzed. The following international standards were used: IAEA-601-Benzoic acid (δ^{18}O = 23.3‰) and V-SMOW (δ^{18}O = 0‰) for CO gas cylinder calibration (used for δ^{18}O measurements), IAEA-CH7-Polyethylene (δ^2H = −100.3‰) and V-SMOW (δ^2H = 0‰) for H_2 gas cylinder calibration (used for δ^2H measurements).

2.5. Numbering of the carbon sites and definitions

In order to compare the data for each carbon positioned at the same site in the molecules under investigation, the carbon sites have been designed by a letter, as shown in Fig. 1. Then, the numbering of the carbon within each substituent (R1, R2, R3) is achieved by incrementing the corresponding letter of the aromatic ring by a number: e.g. “a1” designs the carbon of the aldehyde in vanillin, etc. Bold lettering is used to indicate a positional isotopomer: thus, this example is isotopomer **a1**.

The definitions of normal or inverse isotope effects during chromatographic experiments are: a normal effect occurs when the interaction between the light isotopomer and the stationary phase is favored; an inverse effect occurs when the heavy molecule has a stronger interaction with the solid phase. Hence, a normal isotope effect means that heavy isotopomers are encountered in the front, and an inverse isotope effect means that they are encountered in the tail.

3. Results and discussion

3.1. Isotope fractionation in vanillin

It has previously been demonstrated [2,8,16] that vanillin shows isotope enrichment in the $\delta_g{}^{13}C$ (‰) during elution from NP silica gel (Fig. 2A). However, as can also be seen from Fig. 2A, the site-specific fractionation $\delta_i{}^{13}C$ in vanillin displays a much greater dynamic range than the $\delta_g{}^{13}C$ value. Four noteworthy features are illustrated by this data. First, all the carbon atoms of the molecule (Fig. 1) are subjected to isotope fractionation. Secondly, the extent of disparity is considerably variable depending on the isotopomer, with **a1** and **f** having the largest front-to-tail ranges. Thirdly, both enrichment and depletion during elution are seen, indicative of isotopomer-dependent normal and inverse isotope effects (see Section 2.5 for definitions). Fourthly, isotope fractionation is highly dependent on the stationary phase. When comparison is made with the same profile obtained by elution of vanillin from an RP silica column (Fig. 2B), not only is a different pattern found for the site-specific fractionation, but also the magnitude of the effect is diminished both for the global and the site-specific ^{13}C values. Indeed, with the exception of isotopomers **a** and **d**, isotope fractionation is insignificant. Moreover, these two isotopomers, as well as the $\delta_g{}^{13}C$ value, show an isotope effect on RP silica in the opposing direction (depletion) to that seen on NP silica. This contrary behavior of isotopomers between NP and RP has been observed on $\delta_g{}^{13}C$ for other compounds [17].

Isotope fractionation is not restricted to the ^{13}C isotopomers. Thus, the $^{18}O/^{16}O$ and $^{2}H/^{1}H$ ratios obtained on the same vanillin fractions as used for $\delta_g{}^{13}C$ measurements both show a similar effect on NP silica, also in the sense of isotope enrichment with retention (Fig. 3). Studies of ^{18}O fractionation are rare in the literature at natural abundance level and we have found no previous report of this phenomenon during chromatography. Similarly, for ^{2}H the tendency is for all heavy isotopomers to show retention (Fig. 3). This phenomenon has been observed during a study of the site-specific fractionation in ^{2}H of vanillin and pHB [2]. A particularly strong fractionation was observed for the ^{2}H isotopomer of the carbonyl group, implying that this group is indubitably a site of interaction with the stationary phase. Similarly, **a1** displays a particularly strong ^{13}C fractionation, supporting a major role for this group.

Furthermore, ^{13}C isotopomers at other positions, including those of the aromatic ring, show marked isotope fractionation. This observation is compatible with the hydroxyl at the *para* position

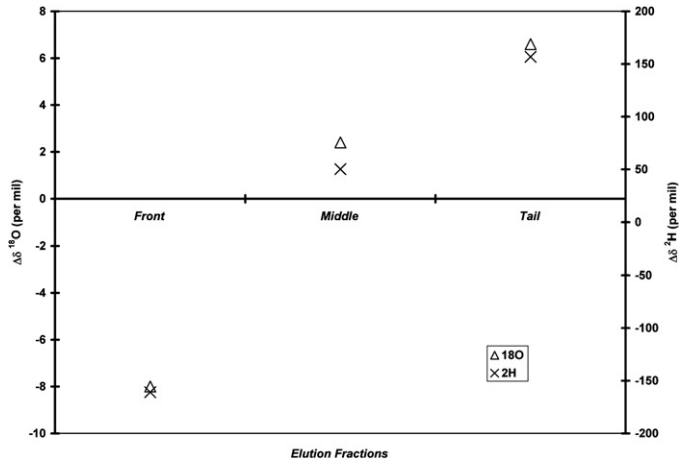

Fig. 3. Global ^{2}H and ^{18}O content measured by IRMS on vanillin during silica gel column chromatography. The fractions analyzed are those described in Fig. 2.

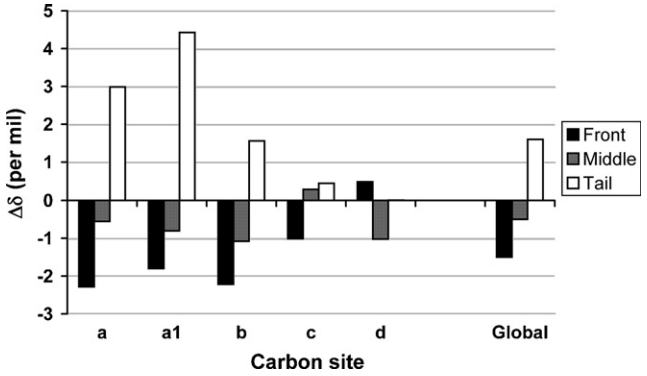

Fig. 4. Site-specific fractionation of ^{13}C of pHB during normal phase silica gel column chromatography. The relative variation in $^{13}C/^{12}C$ is expressed as $\Delta\delta_i = (\delta_p - \delta_s)_i$ for each carbon position i of the eluted pHB $(\delta_p)_i$ with respect to the initial value obtained for site i $(\delta_s)_i$ by NMR. See Fig. 1 for carbon numbering. The data presented is from the first portion (front = from 0 to 14% eluted), middle portion (middle = from 37 to 53% eluted) and the last portion (tail = from 88 to 100% eluted). Global is the total mean value for each of these portions determined by IRMS. Note that 'b' is b + f and 'c' is c + e (see text and Fig. 1).

also playing an important role. Delocalization of the lone pair electrons of the oxygen should reinforce the negative charge placed on the oxygen atom of the aldehyde, thus helping sustain a favorable interaction with the silanol hydrogen of the silica gel. That such charge–charge interactions play an important role in isotopomer selectivity is supported by the behavior of vanillin on RP silica (Fig. 2B), in which charge interaction can be expected to play a minor role compared with hydrophobic association. From the evolution of the $\delta_g{}^{13}C$ values for the different eluted fractions of vanillin from the NP silica phase it can be deduced that there is an overall inverse isotope effect, as has been previously described for other compounds. The site-specific data indicate that **a**, **f** and, to a lesser extent **a1**, are the main sources of this inverse effect. Interestingly, **d** (bearing the hydroxyl group) and **e** (adjacent), show normal effects. Focusing therefore on a closely related group of phenolic compounds that can be eluted from NP silica in similar stationary phases, we have asked the question as to what extent general criteria can be identified that are involved in the isotope fractionation process?

3.2. Site-specific ^{13}C data on p-hydroxybenzaldehyde

In the first instance, we have examined the influence of the *para*-hydroxyl group, since this is considered to play an important role in charge stabilization. pHB resembles vanillin in retaining the *p*-OH but is lacking the *m*-OCH_3 (Fig. 1). Thus, if the *p*-OH is important, but the *m*-OCH_3 much less so, then we can expect the isotope fractionation profiles for vanillin and pHB to be similar. Superficially, this would appear to be the case when only the $\Delta\delta_g{}^{13}C$ values are considered: vanillin and pHB both show the same range and sense of front-to-tail fractionation (Fig. 4).

The symmetry of the molecule means that the ^{13}C site-specific variations determined by NMR are the mean values for the pairs of isotopomers **b** + **f** and **c** + **e**. For **b** + **f**, the same profile showing enrichment is seen for pHB as for vanillin, although the magnitude is smaller. In contrast, for **c** + **e** there is almost no effect. However, in vanillin **c** is inverse and **e** is normal, which, if retained in pHB, would lead to a low mean value, as is observed. Alternatively, the difference could be due to the absence of the –OCH_3 group that averages out the NCIEs between normal and inverse effects. Might this also explain the absence of any fractionation in **d** in pHB, in contrast to the normal NCIE found for vanillin?

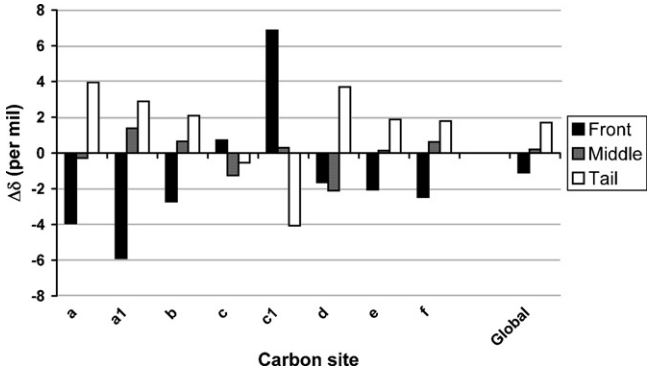

Fig. 5. Site-specific fractionation of ^{13}C of *m*-anisaldehyde during normal phase silica gel column chromatography. The relative variation in $^{13}C/^{12}C$ is expressed as $\Delta\delta_i = (\delta_p - \delta_s)_i$ for each carbon position *i* of the eluted *m*-anisaldehyde $(\delta_p)_i$ with respect to the initial value obtained for site *i* $(\delta_s)_i$ by NMR. See Fig. 1 for carbon numbering. The data presented is from the first portion (front = from 0 to 15% eluted), middle portion (middle = from 33 to 49% eluted) and the last portion (tail = from 81 to 100% eluted). Global is the total mean value for each of these portions determined by IRMS.

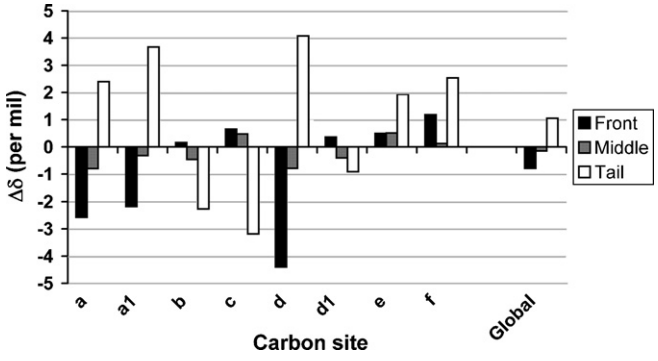

Fig. 6. Site-specific fractionation of ^{13}C of isovanillin during silica gel column chromatography. The relative variation in $^{13}C/^{12}C$ is expressed as $\Delta\delta_i = (\delta_p - \delta_s)_i$ for each carbon position *i* of the eluted isovanillin $(\delta_p)_i$ with respect to the initial value obtained for site *i* $(\delta_s)_i$ by NMR. See Fig. 1 for carbon numbering. The data presented is from the first portion (front = from 0 to 15% eluted), middle portion (middle = from 35 to 68% eluted) and the last portion (tail = from 87 to 100% eluted). Global is the total mean value for each of these portions determined by IRMS.

3.3. Site-specific ^{13}C fractionation in m-anisaldehyde

These options can be addressed by examining *m*-anisaldehyde, the alternative modification to vanillin lacking the *p*-OH but retaining the *m*-OCH_3 (Fig. 1). Again, it is found (Fig. 5) that this modification has negligible influence of the front-to-tail fractionation: the $\Delta\delta_g{}^{13}C$ values for *m*-anisaldehyde show both the same range and direction as those for vanillin and pHB.

Similarly the 'aldehyde' section of the molecule shows the same site-specific fractionation, **a**, **a1**, **b** and **f** all showing inverse NCIEs, the same as in vanillin and pHB. In contrast, **d**, **e** and **c** (a weak tendency in this case) all show a $\Delta\delta_i$ evolution opposed to that for vanillin: ^{13}C depletion in the front-to-tail direction, indicating normal NCIEs. Furthermore, the –OCH_3 in **c1**, which in vanillin only shows a small tendency for a normal NCIE, in *m*-anisaldehyde shows the largest $\Delta\delta$ evolution, indicating it now to be playing a major role in interaction. Does this indicate a different mechanism of interaction to that for those solutes possessing an –OH group? If this is the case, then the positioning of the –OCH_3 may be critical.

3.4. Site-specific ^{13}C fractionation in isovanillin

To probe this, the front-to-tail $\Delta\delta$ evolution in isovanillin has been examined. Will this molecule, in which the –OH is now at the *meta* position and the –OCH_3 at the *para* position with respect to the aldehyde, behave more like vanillin and pHB or more like *m*-anisaldehyde? Fig. 6 illustrates the evolution of the relative ^{13}C isotope deviation $\Delta\delta_i$ during the elution of the isovanillin peak.

Although the small $\Delta\delta_g{}^{13}C$ is in the same sense and magnitude as for the other compounds, the site-specific $\Delta\delta_i{}^{13}C$ values show a very different pattern. Indeed, it is only the isotopomers in the aldehyde group, **a** and **a1**, that resemble vanillin. The isotopomer **d**, carrying the –OH now behaves like **c** (–OCH_3) of vanillin, in showing an inverse effect, although of greater magnitude. Similarly, in **d1**, the –OCH_3 carbon, resembles the **c1** of vanillin in showing a tendency towards a normal NCIE, in marked contrast to the behavior of the **c1** in *m*-anisaldehyde in which a strong normal NCIE is found.

Which factors, then, are more important, the polarity of the group or its position? It is notable that ^{13}C isotopomers **b** and **c** of isovanillin elute first. These carbons are at the same positions with respect to the –OH function as in vanillin, **e** and **d**, respectively. This special architecture seems to be the origin of the inverse NCIE. In isovanillin, **d**, bearing the –OCH_3, shows a large normal effect, while in vanillin the similar isotopomer **c** undergoes a smaller effect.

3.5. Site-specific ^{13}C fractionation in ferulic acid

Ferulic acid, the natural precursor of vanillin [18,19], has a much more polar side group (prop-2-enoyl) in place of the aldehyde of vanillin (Fig. 1). To elute this organic acid from silica requires a much more polar eluent than the other molecules: it is constituted mainly of acetone, while cyclohexane is the main constituent for the others. With ferulic acid, no fractionation has been found either for the $\delta_g{}^{13}C$ value or for the site-by-site $\delta_i{}^{13}C$ values (data not shown). This observation clearly demonstrates that NCIEs are dependent also on the composition of the eluent. This means that the comparison of the behavior of various solutes with respect to one stationary phase is valid only if the composition of the mobile phase is similar, as is the case for the other molecules herein studied.

4. Conclusions

NCIEs associated with the physical processes of ad(ab)sorbtion during liquid/solid chromatography can be considered to characterize the non-covalent association/dissociation interactions between the solute and the solid phase. By exploiting quantitative ^{13}C NMR spectroscopy at natural abundance, which gives access to isotope fractionation at individual carbon position in the target molecules, it is shown that each ^{13}C isotopomer has a unique signature for this interaction and, as a result, elutes with its own retention profile. Furthermore, the behavior of different isotopomers is not predictable: in some the heavier molecules elute first while in others they are the more strongly retained, leading to a mixture of inverse and normal isotope effects. To an extent, a given set of observations can be rationalized within a restricted set of solute–solvent-phase interactions on the basis of the character of the substituent groups present. This has been shown by performing chromatography with solutes from the same family in a chromatography system in which the solid phase is identical and the solvent system (cyclohexane + X% acetone) is only minimally modified.

Intriguingly, however, high ^{13}C NCIEs (both inverse and normal) occur in the majority of isotopomers, even those in which ^{13}C is not in or adjacent to a substituent group, indicating that all the carbon positions could be subject to an isotope effect during column chromatography. Similar effects are documented for ^{2}H [20], which have been described as secondary NCIEs. Furthermore, it can be demonstrated that the intimate structure of the solute

may generate different sets of site-by-site NCIEs. It is clear that within the group of phenolic derivatives herein studied, both the polarity and the position of the functional groups are important for the elution order of ^{13}C isotopomers. Thus, the isomers vanillin and isovanillin, in which the –OH and $–OCH_3$ groups are inverted, show NCIEs that relate as much to the position of the substituents as to their structure. Moreover, combinations of functional groups give characteristic isotope effects. Thus, the addition of the patterns of pHB and *m*-anisaldehyde does not produce the pattern of vanillin (compare Figs. 2, 4 and 5). For NCIEs when the nature of the interaction is changed, the site-by-site isotope fractionation signatures also change, both in sense and in intensity. Hence, when the stationary phase is modified from NP silica to RP silica, or when the polarity of the eluent is increased, the NCIE signature for each isotopomer is redefined.

This phenomenon of NCIEs is completely veiled when the global ^{13}C content is measured by IRMS. It can therefore be proposed that the approach herein presented should allow models of solute–solvent-phase interactions – for example such as are used for the calculation for the sorption coefficients of organic contaminants in the environment [21] – to be greatly refined. Similarly, an intimate understanding of the behavior of each isotopomer may help in the adjustment of the parameters described in the LSER equation (Linear Solvation Energy Relationship) [22]. It is now pertinent to model the observed fractionation in relation to structural variation in the solutes and the solid phase by consideration of the ^{13}C isotope effects. The magnitude of this phenomenon is well within the capability of isotope ^{13}C NMR.

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