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^{13}C Isotopic analysis of an acetaminophen and diacetylmorphine mixture

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Abstract

When using gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) to measure the ^{13}C isotopic enrichment of acetaminophen, the nitrogen atom of the molecule can potentially distort the isotopic enrichment as NO_2 and N_2O can be formed during combustion and interfere with some of the CO_2 isotopomers. Nevertheless, the comparison of isotopic enrichments obtained either by elemental analyser–isotope ratio mass spectrometry (EA–IRMS) or by GC–C–IRMS leads to the conclusion that nitrogen atoms do not interfere and that it is possible to accurately measure the ^{13}C enrichment of acetaminophen by GC–C–IRMS even if the combustion interface does not include a reduction furnace. Moreover it is possible to measure isotopic enrichments from samples in which acetaminophen is mixed with diacetylmorphine (heroin) even if transacetylation occurs during the gas chromatographic process. It is then necessary to acetylate acetaminophen and to take into account the constant and reproducible isotopic fractionation induced by acetylation.

Keywords: Isotopic fractionation; Acetaminophen; Diacetylmorphine; Heroin; Paracetamol

1. Introduction

Samples of the totally unlawful diacetylmorphine (heroin) do not contain only pure diacetylmorphine: only a few percent of the drug are mixed with other components as cutting products. There are two kinds of cutting products: dilutants whose aim is to increase the volume of the sample and adulterants with pharmacological properties whose aim is to excite, relieve pain occurring during the first injection or stimulate during sniffing [1–4]. Acetaminophen is used as an adulterant. The measurement of the ^{13}C enrichment of drugs of abuse is one of the useful elements helping to trace the origin of a drug [5]. When GC–C–IRMS is used to measure the ^{13}C enrichment of acetaminophen from a mixture containing diacetylmorphine, a by-product corresponding to the transacetylation of acetaminophen by diacetylmorphine appears during the chromatographic process. Consequently, an isotopic fractionation can occur [6–8]. The aim of this study was to determine if it is possible to avoid this transacetylation in order to measure accurately the ^{13}C isotopic enrichment of acetaminophen, without any isotopic fractionation. Moreover, the interference of N_2O with measurement of ^{13}C isotope enrichment has

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been reported [9,10]. Consequently, we tried to demonstrate that the presence of nitrogen atoms in the molecule of acetaminophen does not cause any interference with GC–C–IRMS ^{13}C measurements, even if the combustion interface of the used GC–C–IRMS does not comprise a reduction furnace to reduce the various nitrogen oxides formed during the combustion process.

2. Materials and methods

2.1. Chemical reagents

Pure diacetylmorphine was kindly provided by the Laboratoire de Police Scientifique de Lyon. Various pure acetaminophen samples were obtained from Sigma and Aldrich (Saint Quentin Fallavier, France). Acetic anhydride was extra pure and obtained from Merck (Darmstadt, Germany). Pyridine was of analytical grade and obtained from Interchim (Montluçon, France).

2.2. Acetylation procedure

A sample of 5 mg of acetaminophen was dissolved into 300 μl of acetic anhydride and 200 μl of pyridine and slightly shaken. The resulting solution was then heated for 30 min at 50°C and shaken twice during that time. After heating, the solution was stirred and light protected at room temperature for 24 h. The solution was then evaporated to dryness at 60°C under a stream of nitrogen and the nitrogen flow was maintained for 5 min after the disappearance of pyridine and acetic anhydride odours; the residue was frozen until analysis. Before analysis, it was diluted into 500 μl of a mixture of ethanol–chloroform (1:1, v/v).

2.3. Gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS)

The chromatographic separation was achieved on an HP 5890 gas chromatograph (Hewlett-Packard) and the ^{13}C measurements on an ISOCHROM SIRA 10 spectrometer (VG Isotech). An on-line combustion furnace allows the transformation of the sample eluted from the capillary column into CO_2. Water formed during the combustion is removed by a cryogenic trap (−100±1°C). The results are expressed as $\delta^{13}C‰$:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000$$

The usual standard for carbon isotopes is PDB (Pee Dee Belemnite – a Cretaceous Belemnite, *Belemnitella Americana*). The ^{13}C enrichment of the CO_2 obtained from this calcium carbonate is taken as the zero value of the $\delta^{13}C$ scale.

Chromatographic separations were performed using a DB1 column (J&W Scientific, 30 m×0.25 mm I.D., film thickness 0.25 μm). The carrier gas was helium 5.5 and the column head pressure was maintained at 10 p.s.i. (constant pressure: at 100°C, the flow-rate was 1.1 ml/min and the linear velocity 43.7 cm/s). The oven temperature was maintained at 110°C for 1 min and raised up to 180°C at a rate of 40°C/min. After 1 min at 180°C, the oven was programmed from 180°C to 290°C at a rate of 4°C/min and held at 290°C for 1 min. The combustion furnace temperature was 820°C and the interface was maintained at 260°C. Injector and chromatographic detector (FID) temperatures were set at 260°C and 300°C, respectively. The water trap was set at −100±1°C. The solutions were injected in the splitless mode and the injected volume was 1 μl.

2.4. Elemental analysis–isotope ratio mass spectrometry (EA–IRMS)

Isotopic measurements of pure compounds were carried out using an elemental analyser (NA 1500, Carlo Erba) coupled with a VG ISOCHROM isotope ratio mass spectrometer. Results were also expressed as $\delta^{13}C‰$. Since there is no on-line chromatographic separation before the ^{13}C measurement, the compounds of interest to be analysed have to be pure or purified prior to analysis. Here too, the carbon atoms are transformed into CO_2 in an oxidation tube maintained at 1020°C. A reduction furnace at 650°C allows the reduction of nitrogen oxides into molecular N_2, thus avoiding the analytical interferences with CO_2 isotopomers. Amounts of 300 μg of

samples, equivalent to 200 μg of carbon, were used for analysis. The oven temperature of the chromatograph needed to separate the combustion gases as well as that of the thermal conductivity detector (TCD) were set at 60°C.

2.5. Gas chromatography–mass spectrometry

In order to clearly identify acetaminophen acetate, a GC–MS analysis was carried out using an HP 5890 gas chromatograph coupled with an HP 5970 mass spectrometer. The mass spectra were obtained under electron impact (70 eV). The interface was held at 300°C.

The same column and chromatographic conditions were used for both GC–C–IRMS and GC–MS analyses. The injected solution to observed transacetylation was 1 mg of acetaminophen and 1 mg of diacetylmorphine diluted in 1 ml of a mixture of ethanol–chloroform (1:1, v/v). The solutions were injected in the splitless mode and the injected volume was 1 μl.

3. Results and discussion

3.1. Influence of nitrogen on isotopic measurement

The acetaminophen molecule contains one nitrogen atom that can be oxidised by the combustion during GC–C–IRMS analysis and transformed into N_2O (M_r 44) and NO_2 (M_r 46). The molecular masses of these oxides are identical to those of some CO_2 isotopomers. Consequently, according to the structure of the molecule, they can interfere with ^{13}C isotopic measurement. Thus, it was of interest to compare acetaminophen isotopic enrichments obtained by GC–C–IRMS and EA–IRMS, which due to the reduction oven does not induce the formation of N_2O or NO_2 but only the formation of molecular nitrogen (N_2).

Table 1 gathers the results from two different samples of pure acetaminophen obtained from Sigma and Aldrich, respectively. The ^{13}C enrichment values obtained with both techniques were compared using a Student t-test. There is no significant difference between the results obtained either by GC–C–IRMS or EA–IRMS ($p<0.025$). The overall intrinsic reproducibility of both GC–C–IRMS and EA–IRMS systems, taking into account all the experimental random variations that can occur during isotopic measurement is given as 0.3 ‰ by the instruments manufacturer. Consequently, it is possible to conclude that the presence of nitrogen atoms does not induce any bias on the ^{13}C enrichment of acetaminophen measured by GC–C–IRMS. Since no significant difference between δ-value from EA–IRMS and GC–C–IRMS measurements could be observed we can state that from the compounds under study neither N_2O or NO_2 has been formed in the combustion furnace of the GC–C–IRMS or the ion sources of the isotope ratio mass spectrometer. These results can be explained by the fact that the Dumas combustion process is unlikely to produce nitrogenous products other than N_2 or NO at 820°C. N_2O is a very unlikely combustion product. Thus, isobaric interferences on m/z 44 and 45 are not a problem. Concerning interference on m/z 46, NO_2 ($2NO + O_2 \rightarrow 2N_2O$) is formed as the combustion products cool and is condensed in the −100°C cold trap, that would negate the possibility of coelution of NO_2 with CO_2. Moreover, NO_2 cracks to NO during electron impact ionisation (m/z 46→m/z 30).

Table 1
Comparison of δ‰ of pure acetaminophen obtained by EA–IRMS and GC–C–IRMS

	EA–IRMS			GC–C–IRMS			Δ
	δ‰	σ	n	δ‰	σ	n	
Aldrich	−27.92	0.05	3	−28.08	0.11	3	0.06
Sigma	−29.45	0.09	3	−29.22	0.14	3	0.23

Δ = difference between δ‰.

3.2. Transacetylation

When samples only containing diacetylmorphine and acetaminophen acetate are injected into the GC column, the amounts of acetaminophen and diacetylmorphine recovered are not equal to those injected and four compounds are eluted. Fig. 1 shows the reconstructed chromatogram obtained by GC–MS (total ion current). The difference in retention times for acetaminophen and acetaminophen acetate is 44 s (apex to apex). Nevertheless even if the GC separation between acetaminophen and acetamino-

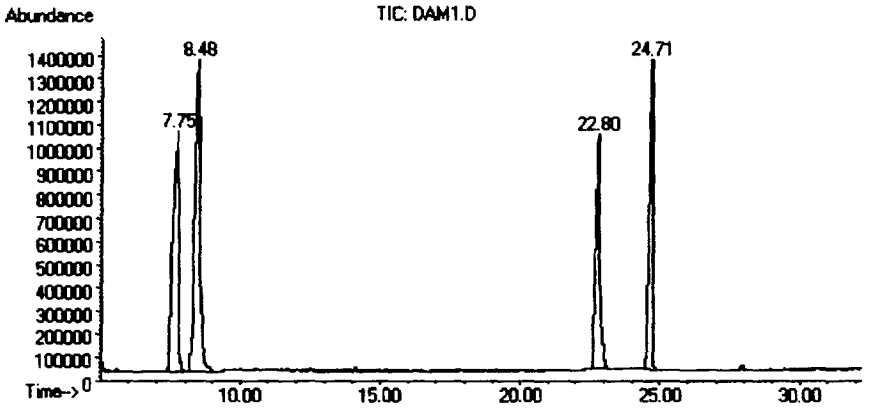

Fig. 1. Reconstructed chromatogram of an acetaminophen and diacetylmorphine mixture.

phen acetate is not maintained after the passage through the combustion interface, no isotopic fractionation due to the coelution of both compounds will occur as a total acetylation of acetaminophen will be realised prior to GC–IRMS analysis. Fig. 2 presents the mass spectra of these four compounds that can be identified as acetaminophen ($T_r = 7.75$), acetaminophen acetate ($T_r = 8.48$), monoacetylmorphine ($T_r = 22.80$) and diacetylmorphine ($T_r = 24.71$). These results show that in the gas chromatograph injector, acetaminophen undergoes a transacetylation with diacetylmorphine according to the reaction shown on Fig. 3. Moreover mixture of ethanol–chloroform (1:1, v/v) was used as it is used to analysed seized samples. Nevertheless, it has been verified that no transacetylation between ethanol and diacetylmorphine occurred, injecting a mixture of ethanol and diacetylmorphine. As isotopic effects may occur during the acetylation, the measurement of ^{13}C enrichment of both acetaminophen and diacetylmorphine can give wrong results. So in order to prevent transacetylation, an acetylation has to be performed prior to the GC–C–IRMS analysis. Therefore, it was important to verify if any isotopic fractionation could occur during the acetylation step and whether this fractionation was reproducible.

To calculate the acetaminophen ^{13}C enrichment from acetaminophen acetate values, the following equation has to be applied, if no isotopic fractionation occurs during acetylation:

$$n_A \times \delta_A = n_B \times \delta_B + n_C \times \delta_C \quad (1)$$

where A designates acetaminophen acetate, B acetaminophen and C the carbon in the acetate group added during derivatization, n_X = number of carbon atoms of compound X, $\delta_X = \delta\,^{13}C‰$ of compound X.

This equation is only valid if compounds are within ^{13}C natural abundance levels [−50 (natural gas) $< \delta\,^{13}C‰ < 0$ (PDB)]. Considering the reaction of acetaminophen acetylation according to Fig. 4:

$$10 \times \delta_{acetate} = 8 \times \delta_{acetaminophen} + 2 \times \delta_{anhydride}$$
$$(\delta_{anhydride} = -29.33\delta‰ \quad (2)$$

Acetylations were realised on pure acetaminophen in order to determine a potential isotopic fractionation and on a mixture of acetaminophen and diacetylmorphine (influence of diacetylmorphine and of acetylation on diacetylmorphine enrichment). Each reaction of acetylation was performed three times and each resulting samples analysed in triplicate. Table 2 gathered the values of ^{13}C measurements by both GC–C–IRMS and EA–IRMS, the $\delta\,^{13}C$ theoretical values for acetaminophen acetate and the difference between observed and theoretical values. An acetaminophen acetate sample has been injected in triplicate on three consecutive days. It appears that there is no variation of the ^{13}C isotopic enrichment whatever the day is (Student test, $p < 0.025$). Table 3 gathers the $\delta\,^{13}C‰$ values obtained from acetaminophen acetate and diacetylmorphine (DAM) after acetylation with comparison of theoretical and observed ^{13}C enrichments. As previously reported by Rieley [11], the uncertainty associated

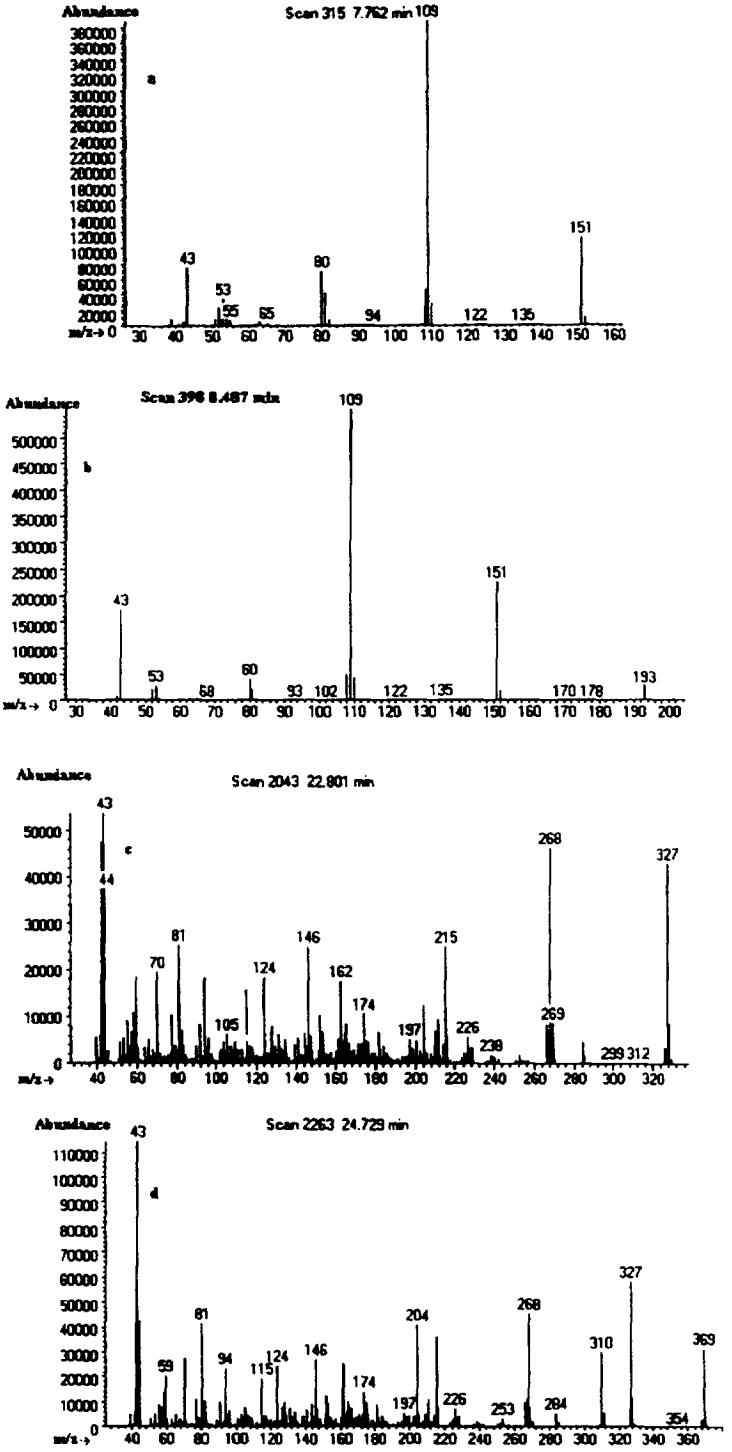

Fig. 2. Mass spectra of (a) acetaminophen; (b) acetaminophen acetate; (c) monoacetylmorphine; (d) diacetylmorphine.

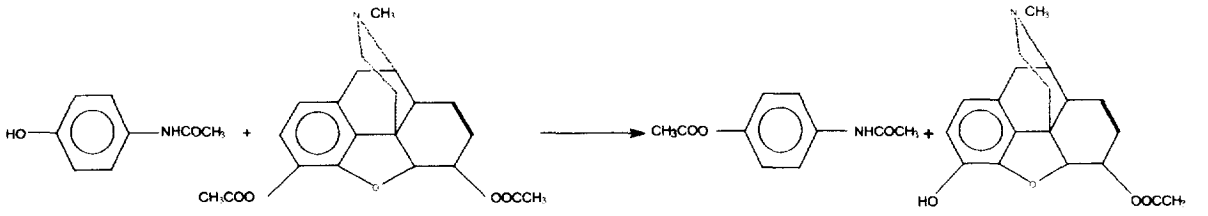

Fig. 3. Transacetylation of acetaminophen.

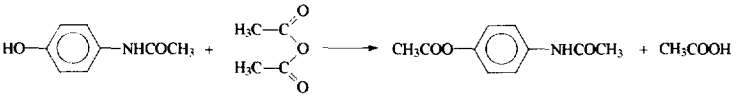

Fig. 4. Acetylation of acetaminophen.

Table 2
δ‰ of acetaminophen acetate – acetylation performed with pure acetaminophen

	EA–IRMS			GC–C–IRMS				Theoretical		Δ		
	δ‰	σ	n	δ‰	σ	n	Δ	δ‰	σ	EA-theo	GC-theo	EA-GC
Aldrich												
Acetylation No. 1	−32.04	0.03	3	−32.10	0.11	3		−28.20	0.04	3.84	3.90	0.06
Acetylation No. 2	−32.16	0.14	3	−31.94	0.01	3	0.16(2−1)	−28.20	0.04	3.96	3.74	0.22
Acetylation No. 3	−32.03	0.19	3	−31.95	0.20	3	0.15(3−1)	−28.20	0.04	3.63	3.75	0.08
Sigma												
Acetylation No. 1	−33.11	0.03	3	−33.11	0.16	3		−29.43	0.04	3.70	3.68	0.02
Acetylation No. 2	−33.10	0.06	3	−33.26	0.19	3	0.15(2−1)	−29.43	0.04	3.67	3.83	0.16
Acetylation No. 3	−33.18	0.03	3	−33.16	0.22	3	0.05(3−1)	−29.43	0.04	3.75	3.73	0.02

Δ = difference of δ‰; σ = standard deviation. Theoretical δ‰ = (8 × δ‰ of acetaminophen obtained by EA–IRMS + 2 × δ‰ of acetic anhydride)/10.

Table 3
δ‰ of acetaminophen acetate – acetylation performed with a mixture of acetaminophen and diacetylmorphine

	EA–IRMS			GC–C–IRMS				Theoretical		Δ		
	δ‰	σ	n	δ‰	σ	n	Δ	δ‰	σ	EA-theo	GC-theo	EA-GC
Acetaminophen acetate (Sigma)												
Acetylation No. 1	−33.13	0.03	3	−32.96	0.16	3		−29.43	0.04	3.70	3.53	0.17
Acetylation No. 2	−33.10	0.06	3	−32.91	0.05	3	0.05 (2−1)	−29.43	0.04	3.67	3.48	0.19
Acetylation No. 3	−33.18	0.03	3	−32.95	0.12	3	0.01 (3−1)	−29.43	0.04	3.75	3.52	0.23
Diacetylmorphine												
Acetylation No. 1	-	-	-	−32.75	0.10	3	-	−32.81	0.12	-	0.06	-
Acetylation No. 2	-	-	-	−32.79	0.14	3	-	−32.81	0.12	-	0.02	-
Acetylation No. 3	-	-	-	−32.72	0.12	3	-	−32.81	0.12	-	0.09	-

Δ = difference of δ‰. Theoretical δ‰ = (8 × δ‰ of acetaminophen obtained by EA–IRMS + 2 × δ‰ of acetic anhydride)/10. δ‰ theo DAM = δ‰ without acetylation.

with the calculation of the theoretical value was calculated following the equation:

$$\varepsilon_A^2 = \varepsilon_C^2[(n_A + n_B)/n_A]^2 + \varepsilon_B^2(n_B/n_A)^2$$

that gives:

$$\varepsilon^2_{\text{acetaminophen}} = \varepsilon^2_{\text{acetate}}[(n_{\text{acetaminophen}} + n_{\text{anhydride}})/n_{\text{acetaminophen}}]^2 + \varepsilon^2_{\text{anhydride}}(n_{\text{anhydride}}/n_{\text{acetaminophen}})^2$$

with: $\varepsilon_{\text{anhydride}} = 0.05$ ‰ and $\varepsilon_{\text{acetaminophen}} = 0.05$ ‰ (EA–IRMS determination [11]).

Consequently, uncertainty associated with theoretical $\delta^{13}C$‰ values of acetaminophen acetate is: $\varepsilon_{\text{acetate}} = 0.04$ ‰ [Theoretical δ‰ $= \delta_{\text{theoretical}} = (8 \times \delta$‰ of acetaminophen obtained by EA–IRMS $+ 2 \times \delta$‰ of acetic anhydride)/10].

Whatever the analytical technique used (EA–IRMS or GC–C–IRMS) and the type of sample acetylated (pure acetaminophen and mixture of diacetylmorphine and acetaminophen), there is a significant difference (Student test, $p < 0.025$) between theoretical and observed ^{13}C enrichments. These results point out an isotopic fractionation occurring during the acetylation process. The difference $\Delta = \delta_{\text{practical}} - \delta_{\text{theoretical}}$ is constant and quite reproducible whatever the technique and sample. The mean value is $\Delta = 3.71 \pm 0.13$, $n = 16$ ($s < 0.05$ $\delta^{13}C$‰ EA–IRMS reproducibility and $s < 0.3$ $\delta^{13}C$‰ GC–C–IRMS reproducibility). This correction factor 3.7 ‰ is in relatively good agreement with correction factors previously reported by Silfer et al. [12] for trifluoroacetylation of amino acids. A potential explanation of the constancy of the difference and its relative insensitivity to the substrate is that there is a possibility of an intramolecular isotope effect associated with the cleavage of acetic anhydride. Indeed, the 3.7 ‰ offset could occur because the ^{13}C-enriched half of the anhydride is preferentially lost as acetic acid and the ^{13}C-depleted half is preferentially transferred to the derivative. Consequently, it is possible to conclude that the isotopic fractionation induced by the acetylation is reproducible, thus:

$$10 \times \delta_{\text{acetate}} = 8 \times \delta_{\text{acetaminophen}} + 2 \times \delta_{\text{anhydride}} - 3.71 \quad (3)$$

Eq. (3) could be recast as follows:

$$(n + 2) \times \delta_{\text{derivative}} = n \times \delta_{\text{substrate}} + 2 \times (\delta_{\text{anhydride}} - 19) \quad (4)$$

where n is the number of carbon atoms in the substrate.

Indeed, considering that the δ-value of the acetylated Aldrich standard is about -32.00 ‰ (Table 2), since the δ value of the underivatized molecule is about -28.00 ‰ (Table 1), it appears that the average value of the carbon atoms in the acetate group must be -48.00 ‰, using Eq. (1). This result indicates an isotopic fractionation of 19 ‰ relative to the parent anhydride. Using the results of Silfer et al. [12], the same equation is recovered. Thus, all the substrate-specific effects disappear recognising that the constant factor is the isotopic difference between the transferred and the released acetate groups.

From these relations, it is possible to calculate the true ^{13}C enrichment of acetaminophen when measured as its acetate. Moreover, the acetylation of acetaminophen mixed with diacetylmorphine does not induce an alteration of the diacetylmorphine ^{13}C content as there is no difference (Student test, $p <$ 0.025) between theoretical values and practical values for that molecule.

4. Conclusion

This study wanted to bring to the attention again that, if a molecule which is analysed for its isotope content by gas chromatography–combustion–isotope ratio mass spectrometry without a reduction furnace contains nitrogen atoms, it is required to verify whether or not these atoms form nitrogen oxides capable of interfering with CO_2 isotopomers and distorting the isotopic measurement. In that specific case, it appears that it does not induce any interference.

Moreover these results demonstrate that isotopic effects can occur during sample handling and preparation which may lead to wrong results of the isotopic enrichment. Here the transacetylation of acetaminophen by diacetylmorphine during the chromatographic step of the analysis can lead to inaccu-

rate results in the determination of the ^{13}C content of both molecules. An acetylation carried out before the GC analysis, even if it induces an isotopic effect on acetaminophen acetate, allows the researcher to obtain accurate values of the ^{13}C enrichment measured by gas chromatography–combustion–isotope ratio mass spectrometry as the isotopic effect is quite constant and reproducible. Moreover, considering that the isotopic difference between the transferred and the released acetate groups is the constant factor, it appears that the substrate specific effects disappear. To conclude, in order to carry out accurate isotopic analysis, it is always necessary to validate the sample preparation, extraction, chromatographic purification and derivatization in order to avoid isotopic discrimination.

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