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NMR spectrometry isotopic fingerprinting: A tool for the manufacturer for tracking Active Pharmaceutical Ingredients from starting materials to final medicines

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ABSTRACT

In the frame of increasingly stringent quality assessment required by the regulators, the pharmaceutical industry has to face increasingly sophisticated counterfeiting practices. Counterfeits based on deliberate copying of processes or on the infringement of current patents for generic medicines are not straightforward to detect, unless the molecular probe is the active molecule itself. In this context, impurity profiling is limited. A tool based on the determination of intramolecular isotopic profiles has been developed to provide manufacturers of APIs (Active Pharmaceutical Ingredients) with a new solution to meet this double requirement. Stable isotope analyses by NMR gives direct access to site-specific isotope content at natural abundance. In this report, it is shown how both ^{2}H and ^{13}C NMR spectrometry can provide complementary and valuable information that could be applied to link APIs to their manufacturing source. Isotopic ^{13}C NMR offers additional benefits over ^{2}H NMR in using robust adiabatic polarization transfer methods, leading to a tremendous reduction in experimental time. Two approaches are illustrated. Firstly, the use of ^{2}H and single pulse ^{13}C NMR spectra obtained on 20 commercial ibuprofen samples from different origins show that this combined strategy leads to (i) a unique intramolecular isotope identification and (ii) a preliminary classification of the samples according to the synthetic pathways of the main industrial processes. An approach employing polarization transfer methods applied to 11 commercial naproxen samples, for which ^{2}H and single pulse ^{13}C NMR spectra are not exploitable and/or are not accessible in reasonable time. The relative and partial intramolecular ^{13}C distribution obtained on naproxen by applying this methodology is sufficiently informative to allow the same conclusions as for ibuprofen. The additional benefits that these approaches should bring to API manufacturers are discussed.

1. Introduction

The pharmaceutical industry today faces two major problems: counterfeiting practices and increasingly stringent quality assessment required by the regulators. However, for a pharmaceutical industry operating in a global market economy with extensive cross-border trade and internet purchasing, the task of finding and preventing counterfeit products is an ever greater challenge (Davison, 2011). On the one hand, counterfeit pharmaceuticals originate from a wide range of fraudulent practices, the most common being the re-use of salvaged packaging to sell a medicine with little or no API (Active Pharmaceutical Ingredient) involved. More sophisticated practices are also prevalent, including: (i) deliberate copying of existing patents for processes or formulations; (ii) stolen and relabeled drugs; and (iii) trans-shipment of goods, i.e. relabeling of a country of origin to circumvent anti-dumping measures or to benefit from the 'clear status' of a producing country. On the other hand, the increasing requirement from the regulatory instances can be illustrated by the sentence found in a recent concept paper proposed by the European Commission (EC): "an obligation could be placed on the manufacturer of the active substance to make ensure that the starting material is sourced from the premises claimed by the manufacturer of the starting material". The idea behind this measure is to extend GMP (good manufacturing practice) requirements to cover APIs as part of the falsified medicine directive (Taylor, 2012). At the present stage of this proposition it is still unclear how the EC will proceed, but

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pressure on the API manufacturers to verify the sources of their starting materials is increasing. The International Conference on Harmonization (ICH) (ICH, 2008), the Food and Drug Administration (FDA) (US Food and Drug Administration, 2006), the European Medicinal Agency (EMA) (European Commission, 2005), and the Japanese Ministry of Health, Labor, and Welfare (MHLW) rules (Ministry of Health, Labor, and Welfare, 2005) are all looking into this problem. A focus is put on the distinction between the API starting material (API-SM) and the API starting material for synthesis. The former (API-SM) is the point from which GMP applies, while the latter is the point in the whole supply chain from which detailed manufacturing information should be provided. Fig. 1 summarises the level of quality assessment applied during the manufacture of the API. The choice of API-SM is still a troublesome issue in the pharmaceutical industry (Scott, 2012). ICH recommends the risk-based approach (ICH, 2005). It is clear that the quality assessment of the raw materials used must extend beyond the material specifications to include the supplier's manufacturing process, quality systems and sourcing strategy. API-SM is included within this context.

Regulators require that impurities should be detected because it is accepted that the impurity profile is a marker of the different synthetic routes to a starting material (Gavin et al., 2006). This approach is also used to assess the genuineness of the API, in terms of counterfeiting. Most of the published data dealing with detection of counterfeiting and/or impurity profiles are based on the detection of residual organic or inorganic material via chromatographic methods (Fernandez et al., 2008). However these techniques only operate on the minor constituents: the active component – the key ingredient – is ignored! Techniques employing stable isotope analyses, in contrast, deal with the API itself (pure from the production batch or after extraction from medicinal products), and are able to strip down the atomic composition of a given molecule. Such an intimate constitution is unique, and therefore can be used as a means to characterize the drug substance at the different stages of its life. Of the available means of measuring stable isotope ratios at natural abundance levels, the two most commonly used are IRMS (Isotope Ratio Mass Spectrometry) and SNIF-NMR (Site-specific Natural Isotope Fractionation studied by Nuclear Magnetic Resonance spectrometry). Whereas IRMS provides a mean isotope ratio for the target element content, SNIF-NMR is able to measure the isotope ratios at individual sites of the molecule: hence, isotopic NMR spectrometry quantifies each isotopomer constituting a given molecule for a given element. ^{2}H SNIF-NMR spectrometry, which is used to measure ^{2}H/^{1}H isotope ratios, is a well-established technique for food authentication and is used for the official control of wine, spirits, fruit juices and flavors (for a full review of SNIF-NMR, its principles and applications, see Jamin and Martin, 2006; Martin et al., 2006a,b,c and references therein). Recently ^{2}H SNIF-NMR has been used to elucidate the synthetic pathway of commonly-used generic pharmaceuticals, demonstrating the potential of this technique to provide evidence in cases of patent infringement (Acetti et al., 2008). With a sufficiently large sample set, sophisticated chemometric tools can be applied to extract information useful for linking samples to their production process (Deconinck et al., 2008; Stanimirova et al., 2005), to discriminate between two or more possible reaction pathways, and/or to trace back to the company (or production plant) the origin of the raw materials used.

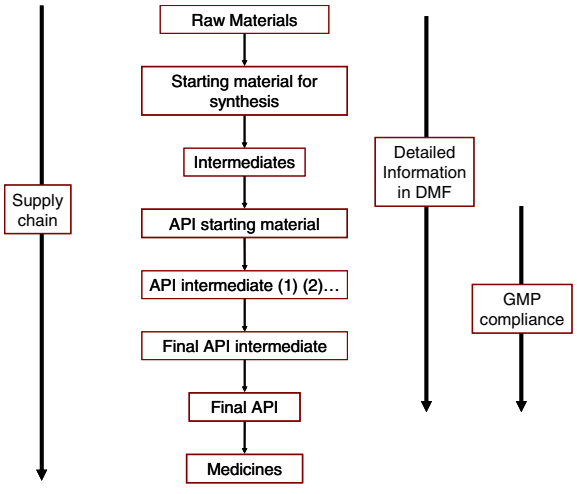

Fig. 1. Choosing the API starting material (API-SM) with respect of the quality assessment and regulatory requirements. DMF: drug master file. GMP: good manufacture practice.

However ^{2}H SNIF-NMR has a number of limitations, notably the low sensitivity of the deuterium probe, the risk of H-exchange during the manufacturing process, and the lack of resolution due to overlapping signals. ^{2}H NMR spectrometry require relatively large amounts of pure sample, which tends not to be a problem for food authentication, and should not be a major stumbling block either when studying APIs. Difficulties can arise, however due to the active ingredient only being available in limited quantities, i.e. extraction from a medicine only available in small amounts. The technique also suffers from the long measurement time that is required to obtain a ^{2}H spectrum with a sufficient signal-to-noise ratio (S/N) to carry out quantitative measurements. Furthermore, ^{2}H SNIFNMR is limited to molecules of low molecular weight (<250 g mol^{-1}).

In an attempt to overcome these difficulties, the possibility of measuring site specific ^{13}C/^{12}C ratios directly using quantitative ^{13}C NMR has been investigated (Tenailleau et al., 2004; Caytan et al., 2007a; Caytan et al., 2007b). The main difficulty of single-pulse isotopic ^{13}C NMR spectrometry is, however, meeting the requirement for a high level of precision: better than 1‰. Despite this, the approach has been successfully applied to several fields (Botosoa et al., 2009a; Botosoa et al., 2009b; Gilbert et al., 2012a; Gilbert et al., 2012b; Höhener et al. 2012), including isotope profiling on APIs such as paracetamol and aspirin (Silvestre et al., 2009). Recent technological developments have made further improvements, specifically on the exploitation of polarization transfer techniques, in which the abundance of the ^{1}H atom is exploited to enhance sensitivity (Thibaudeau et al., 2010). This latest approach has proved capable of providing a ^{13}C isotopic profile of ibuprofen with the required precision and within a reasonable measurement time (Bussy et al., 2011).

The aim of the investigation presented in this article is to take this approach a step further and to evaluate the potential of isotopic NMR spectrometry combining the information available from (i) both ^{2}H and ^{13}C, when possible and (ii) the polarization transfer technique, when appropriate, to characterize APIs by defining the most complete isotope profile possible. We present a re-evaluation within this context of results obtained from previous studies (Deconinck et al., 2008; Bussy et al., 2011) on ibuprofen and an extension of the method to another common non-steroidal anti-inflammatory drug (NSAID), naproxen. These examples illustrate the effectiveness of the strategy, which could be a means available to the manufacturer of defining the full history of an API from the starting materials to the final product. The strategy is generic and so has the potential for application to a large range of targets.

2. Materials and methods

2.1. Chemicals and samples

Acetone-d_6 was purchased from Eurisotop (Paris, France). Methanol, pentane, diethyl ether, P_2O_5 and silica gel were purchased

from Sigma Aldrich (Saint Quentin-Fallavier, France). 20 samples of ibuprofen (commercial packaged form) were purchased from pharmacies in five countries (Belgium, France, Portugal, UK, USA). 11 samples of naproxen (commercial packaged form and pure API) were purchased from pharmacies or from manufacturers in four countries (China, France, Israel, Russia).

2.2. Extraction and purification protocol

Ibuprofen and naproxen tablets (equivalent to 1 g of API) were powdered by manual grinding. Commercial naproxen pills are available in a pure form but also as the sodium salt. For these samples, a first step of acidification is needed: the sample is dissolved in a minimum quantity of hydrochloric acid solution at pH 1. The free acid form, which has low solubility in water, precipitates, and is recovered by simple filtration, then rinsed with water, and dried overnight at 40 °C. Ibuprofen or naproxen powders are then dissolved in 20 mL of methanol and filtered under vacuum through filter paper to remove the major excipients. Following solvent reduction to a maximum of 5 mL by rotary evaporation under reduced pressure, the sample was loaded onto the head of a silica gel column (3 cm o.d., 35–70 μm mesh, 120 g of silica per 1 g of API) and eluted with a 20/80 v/v mixture of pentane/ether. 50 mL fractions were collected and checked by TLC (Thin Layer Chromatography). All the fractions containing the product of interest were pooled in a round-bottomed flask and the solvent was removed by rotary evaporation under reduced pressure. The API (ibuprofen or naproxen) was dried overnight at 40 °C and stored under reduced pressure over P_2O_5 in a desiccator. The purity of the isolated compound was checked by qualitative ^{1}H NMR.

2.3. NMR spectrometry experiments

For quantitative ^{13}C NMR, ibuprofen samples were prepared by dissolving 300 mg of ibuprofen in 600 μL acetone-d_6. Naproxen samples were prepared by dissolving 200 mg of naproxen in 1000 μL acetone-d_6/DMSO 70/30 v/v). The solution was then carefully filtered into a 5 mm o.d. tube. INEPT NMR experiments on ibuprofen and naproxen were performed on Bruker spectrometers (Avance I 400 and DPX 400) fitted with a 5 mm i.d. ^{13}C/^{1}H probe carefully tuned to the recording frequency of 100.61 MHz. The temperature of the probe was set at 303 K. All ^{13}C NMR spectra were recorded with inverse gated adiabatic proton decoupling (Tenailleau and Akoka, 2007). Typical parameters for INEPT acquisitions were as follows: ^{13}C and ^{1}H offsets were set at 100 ppm and 4 ppm, respectively, 90° ^{1}H and ^{13}C high power pulse width 10 μs. The pulse lengths were calibrated at the beginning of each 'measurement session' and the probe tuning and matching were adjusted for each sample. For ibuprofen, 64 scans were accumulated with a repetition time of 17.8 s, τ_1 was adjusted to 2.3 ms and τ_2 to 1.3 ms. For naproxen, 700 scans were accumulated with a repetition time of 21 s, τ_1 was adjusted to 1.7 ms and τ_2 to 1.1 ms. The assignment of ^{1}H and ^{13}C NMR resonances of ibuprofen and naproxen was performed using HSQC and DEPT experiments.

Adiabatic full passage pulses were generated using Mathcad 8 (MathSoft, Surrey, UK). They were designed with a cosine amplitude modulation of the RF field (ω_2^{max} = 157.1 kHz or 93.89 kHz for ^{13}C or ^{1}H, respectively) and an offset independent adiabaticity (OIA) (Tannus and Garwood, 1996; Tenailleau and Akoka, 2007) by optimizing the frequency sweep ΔF (ΔF = 39 kHz or 17 kHz for ^{13}C or ^{1}H, respectively) according to the published procedure (Tenailleau and Akoka, 2007). For inversion pulses, adiabatic full passage pulses were used. For refocusing pulses, composite adiabatic pulses were used (Hwang et al., 1997). Five spectra were recorded for each measurement. The T_1 values were determined by using an inversion recovery sequence and the T_1 processing software of the spectrometer, with 35 inversion-time values ranging from 50 ms to 150 s for ^{13}C and with 40 inversion-time values ranging from 50 ms to 30 s for ^{1}H.

The ^{2}H NMR spectra of ibuprofen and naproxen were recorded at 76.7 MHz on a 10 mm ^{2}H probe fitted with a ^{19}F locking device.

2.4. NMR data processing and analysis

For each ^{13}C NMR spectrum, an exponential window function inducing a line broadening of 2 Hz was applied to the Free Induction Decay prior to Fourier Transform. An automatic polynomial baseline correction was subsequently applied to the resulting spectra. Peak areas of ^{13}C peaks were determined by the total lineshape fitting tool implemented within Perch (Perch NMR Software, University of Kuopio, Finland) or within Eurospec (Eurofins, Nantes, France): we have tested that both software gave the same results.

The peak areas measured in ^{13}C NMR spectra were corrected to take into account the presence of ^{13}C–^{13}C isotopomers in the molecule, which give rise to satellite lines.

Partial reduced molar fractions were calculated for each visible site according to the following equation:

$$f_{iR} = S_i/(F_i \cdot S_T) \quad (1)$$

where S_i defines the corrected peak area of the peak of site i in a specific spectrum and S_T represents the sum of the peak areas of all the measured sites. F_i corresponds to the statistical molar fraction of site i: F_i = n/N, where n corresponds to the number of nuclei contributing to the peak area of the peak of site i in the NMR spectrum, and N is the total number of carbons observed in the spectrum.

Deviation (in ‰) from the average value of the partial reduced molar fractions was calculated according to $\Delta_i = 1000 \times (f_{iR} - (f_{iR})_{av})$ where f_{iR} is the partial reduced molar fraction of site i and $(f_{iR})_{av}$ is its average value calculated over the total number of samples for each molecule. In the Results and Discussion (Section 3) these two parameters are used interchangeably and are also associated with the term f_i/F_i.

2.5. Multivariate data analysis

PCA (principal component analysis) was performed using SIMCA-P$^+$ software, version 12.0 (Umetrics, Umeå, Sweden) through its routine applications with no prediction used.

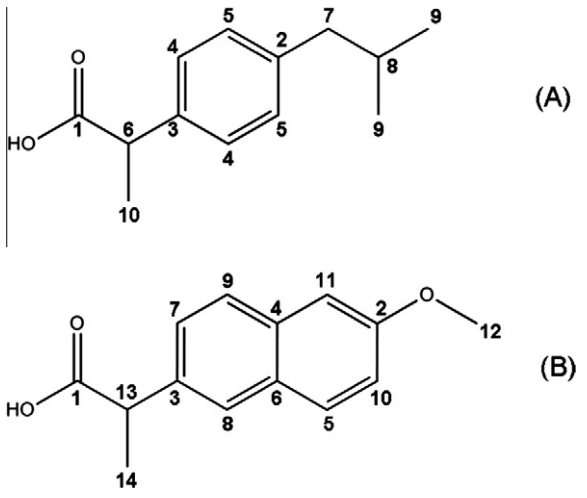

Fig. 2. Ibuprofen (A) and naproxen (B) molecules with carbon sites numbered in decreasing chemical shift. The same site numbering is used to name the $(D/H)_i$ ratios.

Table 1
Type and quality of information obtained from isotopic NMR, using similar equipment.

	^{2}H NMR		^{13}C NMR (single-pulse)		^{13}C INEPT	
	Resolution[a]	Duration[b]	Resolution[a]	Duration[b]	Resolution[a]	Duration[b]
Ibuprofen	Medium	Long	Good	Long	Good	Short
Naproxen	Bad	–	Good	Very long	Good	Long

[a] Quality of the spectrum in term of peak separation: bad = unusable spectrum, medium = isotope ratios are obtained with low precision, good = useful spectrum.
[b] Duration of the NMR analysis, including the repetition of 3 or 5 spectra according to the method (see experimental): short < 10 h, 10 h < long < 20 h, very long > 20 h.

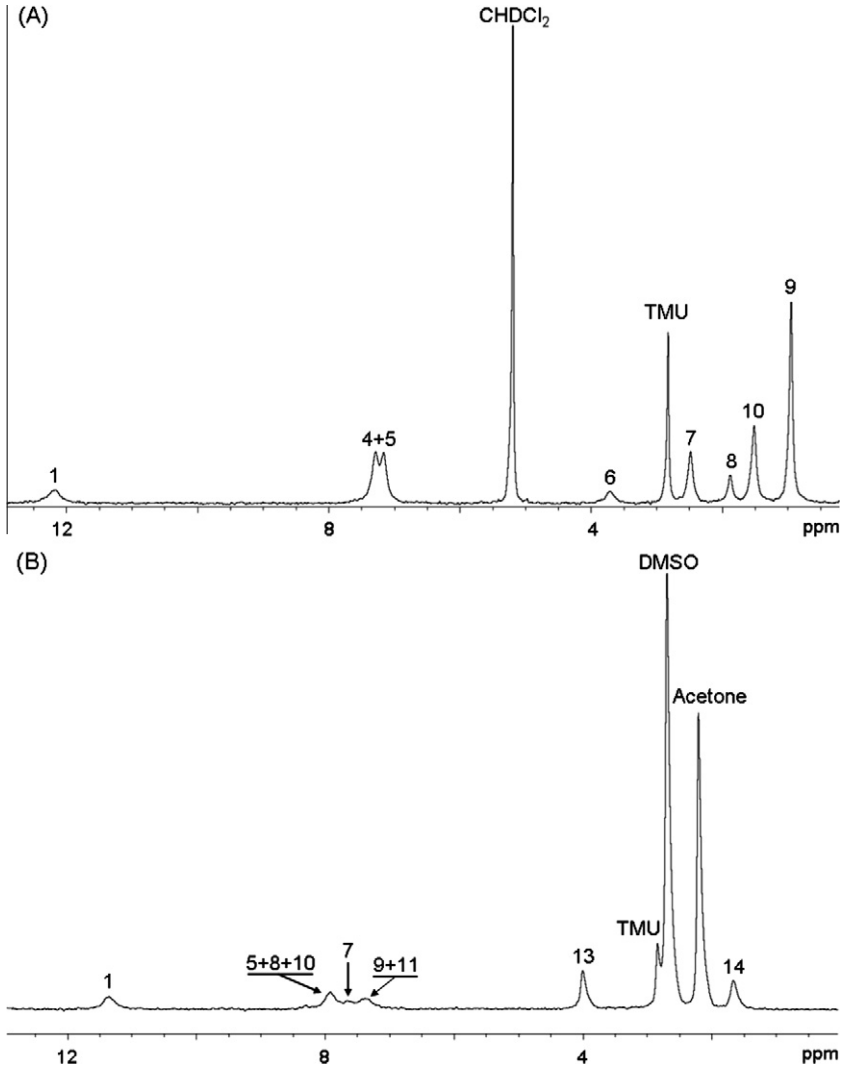

Fig. 3. ^{2}H NMR spectra of ibuprofen (A) (1 g in 2 mL of CH_2Cl_2 + CCl_4) and naproxen (B) (1 g in 3 mL of acetone + DMSO).

3. Results and discussion

3.1. Observable parameters

^{13}C and ^{2}H isotope contents are determined mainly by IRMS and/or NMR. The information provided by each technique is respectively a single value (global value) and site-specific values. It is clear that a more efficient origin labeling of the active molecule is achieved with a greater number of parameters. Ibuprofen and naproxen have potentially 7 and 9 $(D/H)_i$ (exchangeable sites excluded), respectively, and 10 and 14 different carbon sites, respectively (see Fig. 2, for numbering). But, these maximal numbers can be reduced in both ^{2}H and ^{13}C NMR spectra depending on the peak resolution and the duration of the analysis. Table 1 summarizes the information obtained and the difficulties encountered from isotopic NMR experiments for ibuprofen and naproxen. The classification has been established using equivalent NMR devices. It is

Table 2
Isotopic parameters from NMR experiments used in the present work.

	^{2}H	^{13}C (INEPT)
Ibuprofen	$(D/H)_6$, $(D/H)_7$, $(D/H)_8$, $(D/H)_9$ and $(D/H)_{10}$	f_4/F_4, f_5/F_5, f_6/F_6, f_7/F_7, f_8/F_8, f_9/F_9 and f_{10}/F_{10}
Naproxen	–	f_5/F_5, f_7/F_7, f_8/F_8, f_9/F_9, f_{10}/F_{10}, f_{11}/F_{11}, f_{12}/F_{12}, f_{13}/F_{13} and f_{14}/F_{14},

Table 3
Comparison between isotopic profiles obtained on the same ibuprofen sample using the single-pulse and INEPT experiments.

Carbon number[a]	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
b	0.9971	1.0096	1.0057	1.0040	1.0059	0.9998	1.0038	0.9994	0.9922	0.9804
c				1.0226	0.9744	1.1139	0.8913	0.8247	1.0808	1.0146

b: f_i/F_i calculated from a single-pulse experiment.
c: f_i/F_i calculated from an INEPT experiment, carbons 1–3 are not observed since they are quaternary carbons.
[a] According to Fig. 2.

Table 4
Identification of the different ibuprofen samples by geographical origin and company/distributor.

Sample number	Geographical origin	Company/distributor
1	Portugal	Abbott
2	Portugal	Ratiopharm
3	USA	Equate
4	USA	Wyeth
5	USA	Wal-Mart-Stores
6	USA	Wal-Mart-Stores
7	USA	Wal-Mart-Stores
8	USA	Wal-Mart-Stores
9	USA	McNeil
10	USA	McNeil
11	USA	McNeil
12	USA	Walgreens
13	USA	Walgreens
14	Belgium	Abbott
15	United Kingdom	Tesco
16	France	Upsa
17	United Kingdom	Wallis
18	United Kingdom	Tesco
19	United Kingdom	The Boots Company
20	United Kingdom	Wallis

clear that it can be modulated according to the magnitude of the magnetic field and the performance of the probe of the NMR spectrometer. As an illustration of this classification, Fig. 3 compares the ^{2}H NMR spectra of ibuprofen and naproxen. The actual parameters used in the present work are shown in Table 2. The $(D/H)_i$ data for ibuprofen have been collected from previous studies (Acetti et al., 2008; Deconinck et al., 2008). As indicated in Table 1, the single pulse ^{13}C NMR spectrum (90° pulse followed by a repetition delay of $10 \times T_{1max}$) has not been recorded for naproxen; for ibuprofen one sample only has been measured in order to assess the f_i/F_i profile obtained using INEPT experiments on an absolute basis (Table 3). The origin of the discrepancy between both experiments has already been discussed in previous work (Bussy et al., 2011; Thibaudeau et al., 2010). With such a sequence, ^{13}C intensities strongly depend on J_{CH} (^{13}C–^{1}H scalar coupling values) and on the number of attached protons. As a consequence, the observed relative intensities are no longer governed only by the intrinsic intramolecular ^{13}C composition. This 'calibration' (INEPT *vs* single pulse experiments) could be useful to interpret isotope effects and affiliation between molecules.

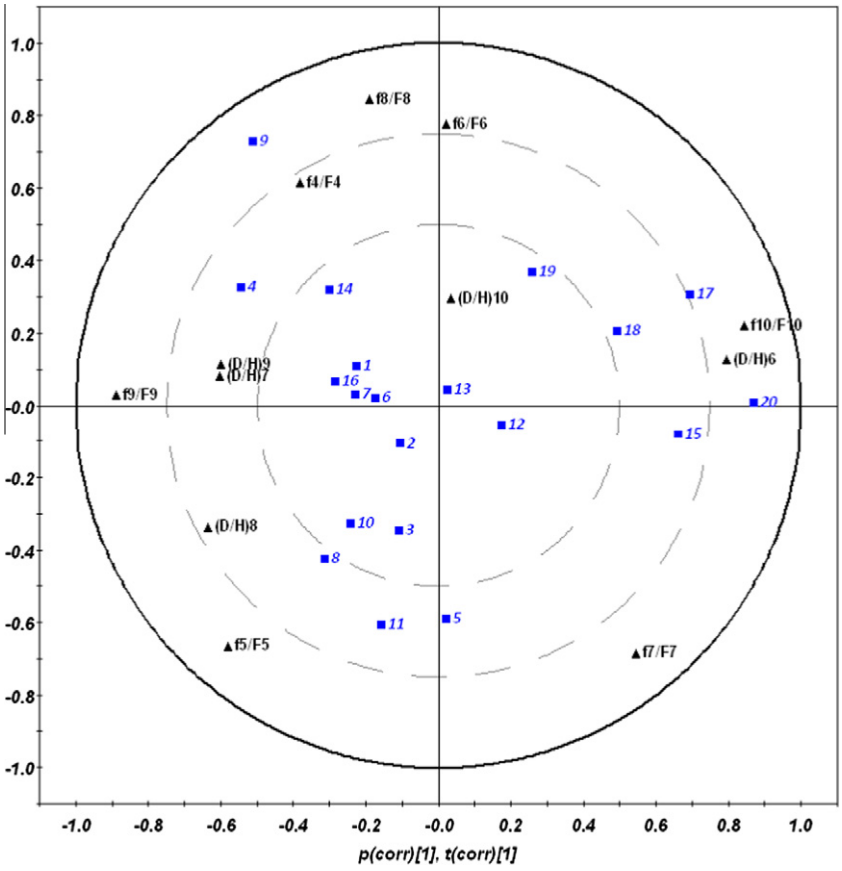

Fig. 4. Principal component analysis on ibuprofen: PC1 *vs* PC2 bi-plot (score + loading plots) using the whole experimental variables set from ^{2}H and ^{13}C NMR experiments (see Table 2). Triangle stands for the parameters (variables) used and square for the sample numbering.

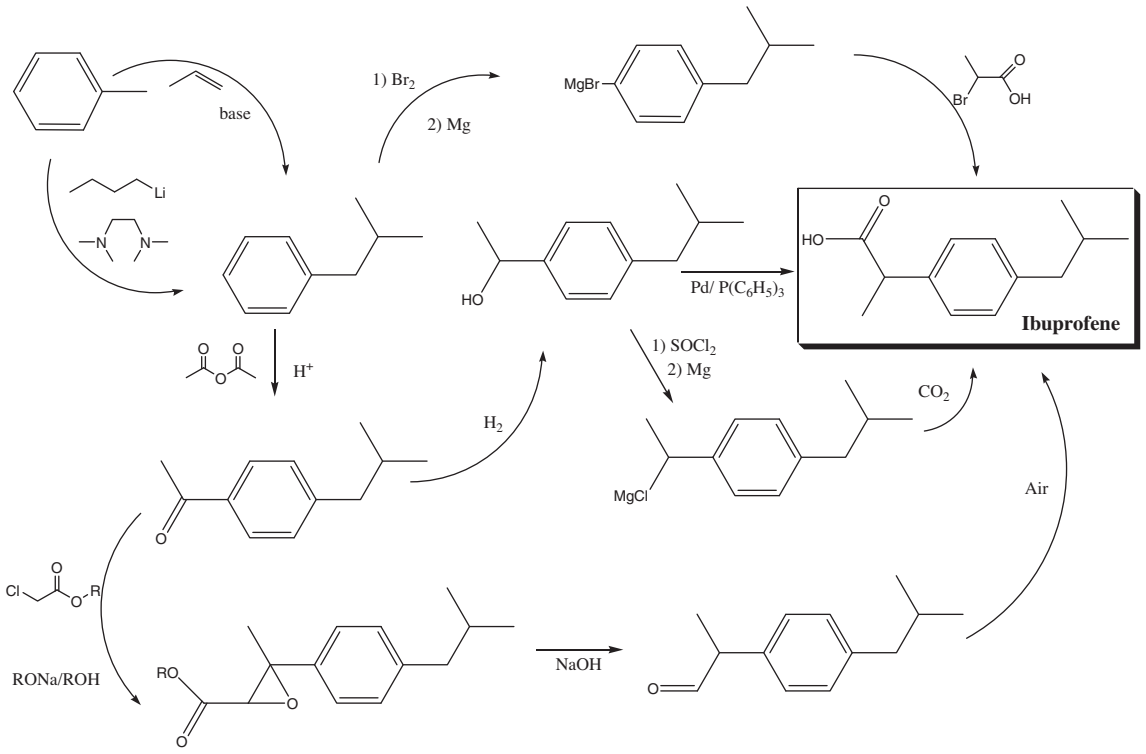

Fig. 5. Major synthetic pathways of ibuprofen. The Grignard pathway corresponds to the use of Br_2 and then Mg as the key reactants. The carbonylation route is identified by the introduction of CO_2 to create the carboxylic function of ibuprofen.

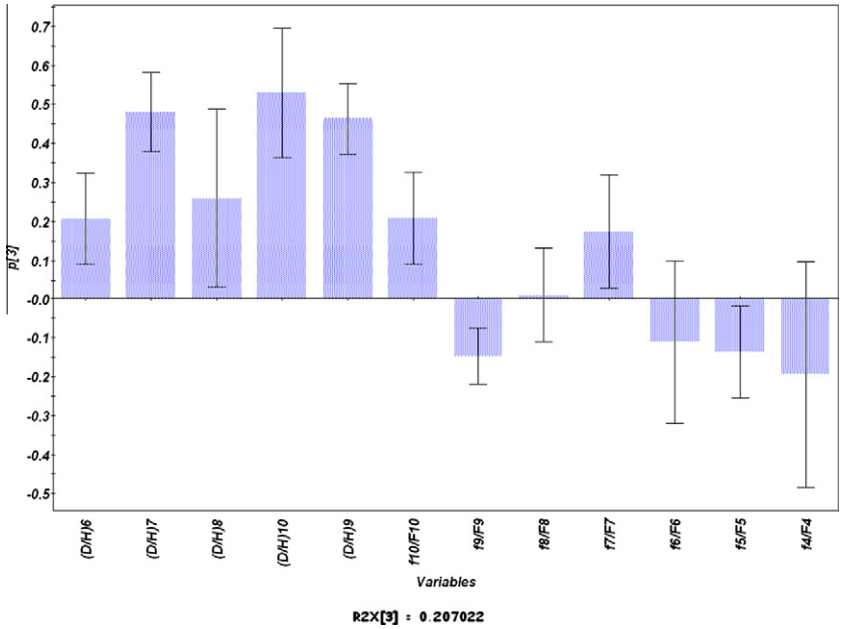

Fig. 6. Loading column plot associated to the component PC3 from a principal component analysis on ibuprofen samples using the same variables as in Fig. 4. The error bars give an estimate of the significance of each variable (experimental parameter).

3.2. Repeatability, reproducibility, data representation

Several analytical methods may be used for measuring isotope composition. If the focus is set on NMR, the group comprising ^2H NMR and single pulse ^{13}C NMR could be considered as able to produce 'true isotope ratios'. Their repeatability, even their reproducibility have been discussed (Martin et al., 2006a; Caytan et al., 2007a). As mentioned above, the same does not hold for INEPT experiments. However, the relative values provided by the technique are sufficient for establishing an isotope fingerprint for a given active molecule: the precision of the INEPT experiments is sufficient to ensure that a decrease of relative f_i/F_i between samples would indicate a ^{13}C impoverishment and the reverse would also be true (Thibaudeau et al., 2010). Reproducibility is not an issue

provided that repeatability is kept high enough: the profile obtained would be directly linked to a given spectrometer. The representation of the data can then be considered analogous to that carried out in 'metabolomic' (or any 'omic' approaches): comparison of data built up from a multiplicity of variables and collected from several samples using the same protocol for which the precision has been established. Thus the isotope profile could be considered as an 'isotopomic' study.

Table 5
Identification of the different naproxen samples by geographical origin, company and form.

Sample number	Geographical origin	Company	Form
A	China	Ningbo Pangs Lanza	Pure API
B	China	Zhejiang Tianxin Pharmaceutical	Pure API
C	China	Zhejiang Tianxin Pharmaceutical	Pure API
D	Russia	KRKA	Pills
E	China	Sunwell Chemical	Pure API
F	Russia	Akrihin	Pills
G	France	TEVA	Pills
H	France	ROCHE	Pills
I	Israel	TEVA	Pills
J	China	Suzhou Enhao Trade Ltd. Jinfeng International	Pure API
K	China	Shanxi TianHeTong Chemical	Pure API

In the present work the data from IRMS, ^{2}H NMR, single pulse ^{13}C NMR and INEPT ^{13}C NMR have been combined whatever their nature (absolute or relative) to create a profile based on isotope composition. The graphical display of the samples using the whole set of parameters can be assessed by principal component analysis (PCA). As a non-predictive tool, PCA is well adapted for studying data obtained from a relatively low number of samples and relatively high number of variables (at least >3). Briefly, PCA allows a two dimensional representation of the sample using synthetic parameters obtained from linear combinations of the experimental parameters. The construction of the resulting principal components is based on the maximization of the data variance. The score plot provides information on the similarities of samples and the loadings plot describes the contribution of each experimental parameter (variable) along a given principal component. In the present work PCA is used as a way to show the similarity level of the samples, so the word discrimination serves to describe a distinction between samples based on their unique isotope profiles. Once the repeatability of the methods used is established, the differentiation of samples originates from the data, not from hypothesis. Then, a pertinent link may be found between the data and the history of the samples.

3.3. Data set for ibuprofen

Twenty samples obtained from pharmacies in five different countries were collected. Table 4 shows all samples with their country of origin and manufacturer. Fig. 4 shows the PCA bi-plot (score plot + loading plot) of the ibuprofen data set using the two main principal components (PC1 and PC2) which explain 54% only

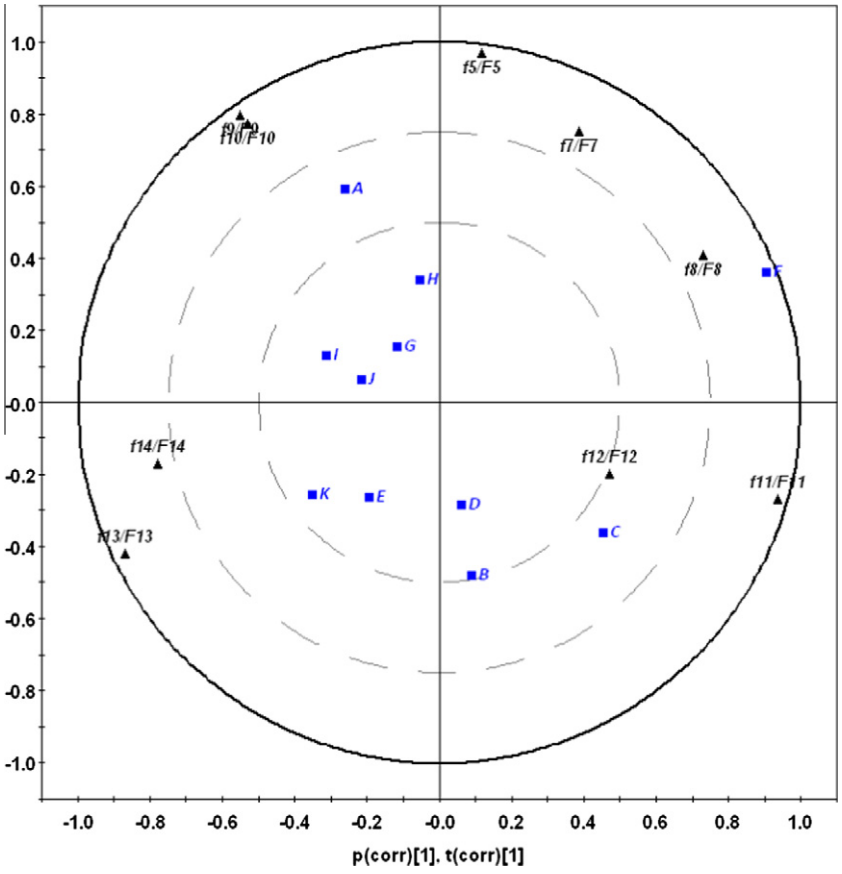

Fig. 7. Principal component analysis on naproxen, whole data set of f_i/F_i from ^{13}C INEPT NMR experiments (see Table 2): PC1 (41%) *vs* PC2 (36%) bi-plot (score + loading plots). Triangle stands for the parameters (variables) used and square for the sample identification.

of the variance (31% PC1 and 23% PC2). Nevertheless, there is no overlap of the sample position indicating that the isotope profile of each sample is different; enough to assume that it corresponds to an isotope fingerprint as a label for each manufacturing batch. Some samples (9, 15, 17, 18 and 20) are distant from the others. Examining the loading plot give some possible explanations on the origin of this discrimination. The parameters the closest to the correlated circle should contribute (for PC1 and PC2) the most to the differentiation of the samples, primarily f_5/F_5, f_6/F_6, f_7/F_7, f_8/F_8, f_9/F_9, f_{10}/F_{10} and $(D/H)_6$. The relative ^{13}C internal distribution is therefore the main contribution.

As shown in previous work (Acetti et al., 2008; Deconinck et al., 2008), the $(D/H)_i$ ratio of the CH group (No. 6 in Fig. 2) is the major probe for distinguishing between the main industrial synthetic pathways of ibuprofen (Fig. 5), through the (hydro-)carbonylation and Grignard intermediate. It has been shown that $(D/H)_6 \approx 95$ ppm for carbonylation while it is around 135 ppm for the Grignard pathway. Thus, as a first approximation, two groups among the ibuprofen samples can be distinguished. The use of INEPT for establishing the internal ^{13}C profile in ibuprofen has revealed that carbons 6, 7, 9 and 10 (Fig. 2) show the largest variation [23], in agreement with the observed PCA data (Fig. 4). This should be associated with the origin of the starting material (common synthon for each pathway, Fig. 5) for C-7 and C-9, while C-10 gives information on the origin of the methyl group of the 'acetate' moiety, and C-6 contains a significant part of the information for the isotopic discrimination of samples according to the remaining steps of the synthesis in which carbon 6 is involved. A clear separation is observed, based on the parameters $(D/H)_6$ and f_{10}/F_{10}, for samples 15, 17, 18 and 20 which come from the UK (Table 4). The samples with the USA as origin are more or less confined to the center of the PCA display. However there is not a full discrimination according to the geographical source of the product; samples are found outside their country of origin group: sample 9, for example, is very atypical. This heterogeneity within a geographical origin is likely to occur, since the name on the packaging is more often the distributor and not the manufacturer, and the same manufacturer may change raw material sources from one batch to another. This is seen to show up in the isotopic profiles. It is worthwhile noticing that for ibuprofen, the ^{13}C profile from isotopic INEPT experiments gave rise to larger discriminating parameters among the other isotopic parameters, still within a short analysis time. However a third component (PC3) can be calculated which contributes to 20.7% to the total variance. An inspection of the loading column plot (Fig. 6) indicates that the ^{2}H content of the sites 6, 7, 9 and 10 (see Fig. 2 for numbering) also bears valuable information for sample differentiation. Beside the case of $(D/H)_6$ already presented, the other will be a way to separate samples from the origin of the raw material rather than the process.

3.4. Data set for naproxen

Eleven samples obtained from four different countries were collected. Table 5 shows all samples with their country of origin, manufacturer and form. As discussed in sub-Section 3.1, ^{2}H NMR experiments on naproxen do not lead to useful data (see Fig. 3), even when the naproxen is esterified (Acetti et al., 2008). Other information has been obtained from ^{2}H, ^{13}C and ^{18}O IRMS measurements. Two subgroup origins from the naproxen samples studied could be identified from $\delta^{2}H$ values and three subgroups from the $\delta^{18}O$ data (Wokovich et al., 2005). It should be noted that the global ^{18}O content may be more influenced by the medium used

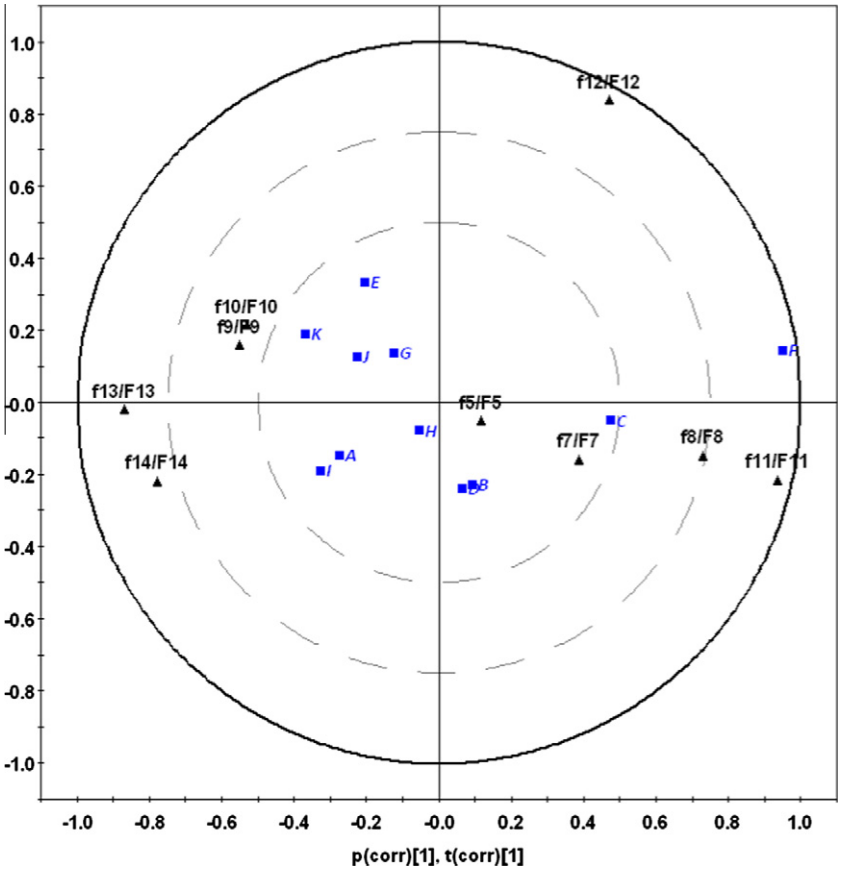

Fig. 8. Principal component analysis on naproxen, whole data set of f_i/F_i from ^{13}C INEPT NMR experiments (see Table 2): PC1 (41%) *vs* PC3 (10.7%) bi-plot (score + loading plots). Triangle stands for the parameters (variables) used and square for the sample identification.

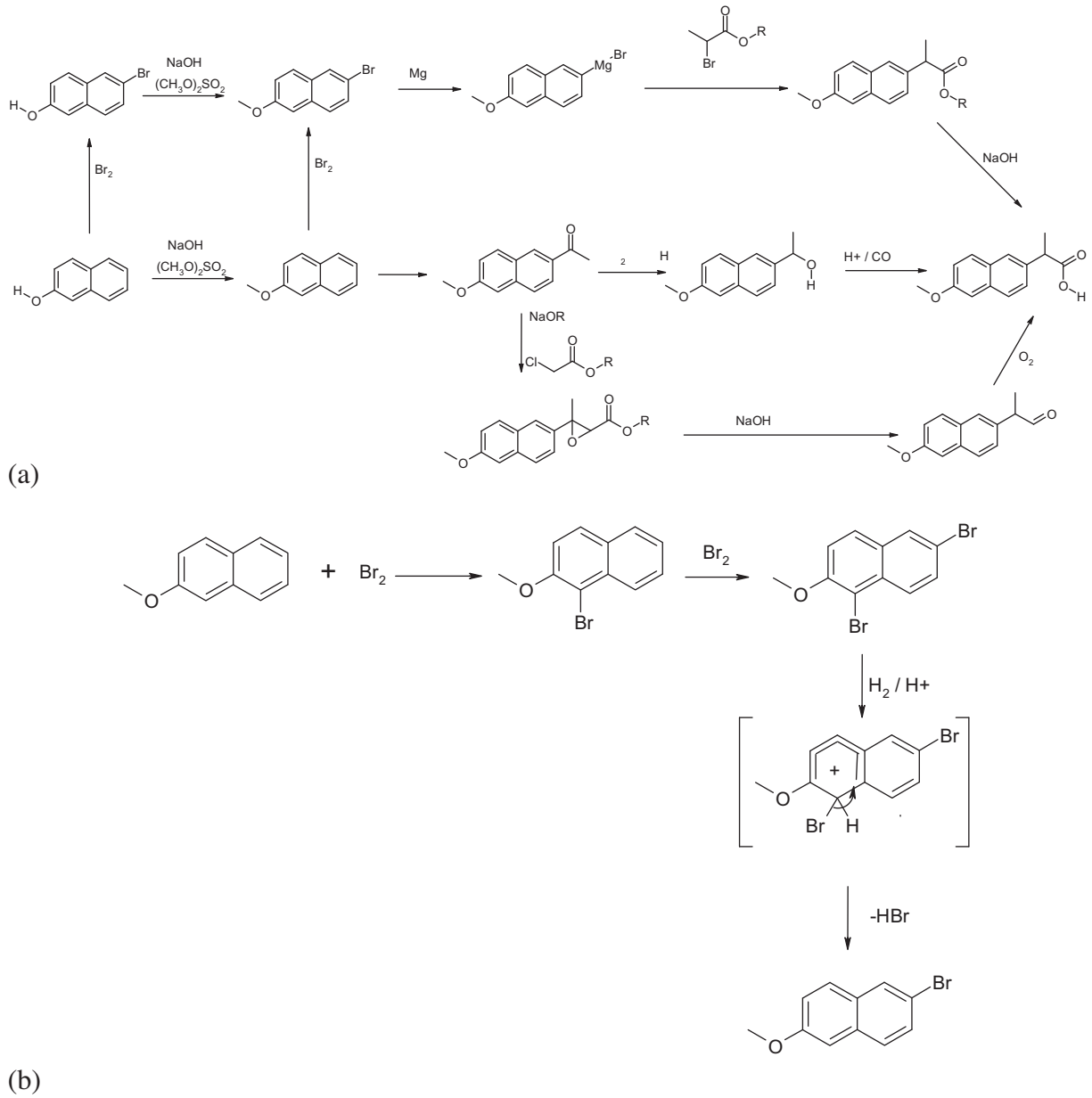

Fig. 9. Industrial manufacturing processes of naproxen: (a) major synthetic pathways and (b) zoom on the bromination step, involving carbon 11.

for crystallization rather than the process and the raw materials used for the synthesis, due to H-exchange.

In this work, the global $\delta^{13}C$ values span a range with no clear clusters. Fig. 7 shows the PCA carried out on the nine new parameters obtained by the isotopic INEPT experiment (Table 2). The explained variance of 77% (PC1 + PC2) is good, indicating that most of the parameters have a discriminating potential. A close inspection of the data shows that f_5/F_5, f_9/F_9, f_{10}/F_{10}, f_{11}/F_{11} and f_{13}/F_{13} account for the greatest discrimination. The component PC3 brings a non-negligible 10.7% of the total variance. A bi-plot, using PC1 and PC3, introduces f_{12}/F_{12} as a variable bearing further information (Fig. 8). Interestingly, these corresponding carbons are the main sites at which reaction occurs during the process used, as illustrated in Fig. 9. A contribution of each site to the discrimination could be established *a priori*: (i) C-12 is a marker for 2-methoxy naphthalene as raw material or during the methylation step from β-naphtol, (ii) C-11 should be affected through the bromination step either from β-naphtol or 2-methoxynaphthalene, (iii) C-13 comes from either the carbonyl group of the 'acetate' moiety (through EtCOCl as reagent) or from the carbon 2 of the 'propionate' moiety (through $CH_3CHBrCOOEt$ as reagent) and (iv) C-14 is the methyl group from the acetate or the propionate parts. As a result, the samples which lie closer to each parameter direction are indicative of the predominance of each synthetic pathway. Thus, a similar conclusion to that for ibuprofen can be drawn: the individual ^{13}C profile is a mark of the origin label for each batch (raw material and process).

4. Conclusions

Multinuclear NMR finger-printing, as demonstrated by these results, shows considerable promise as a means to establish an identity profile of a specific compound or substance that can be linked to its origin and/or the process involved in its manufacture. The additional parameters that are accessed specifically as a result of

using ^{13}C NMR either improve the statistical discrimination or, in some cases, provide the only means of discriminating between samples.

The tool has potential for being applied at several points in API production. In the first instance, the information will highlight whether the raw materials used in the manufacture of a pharmaceutical have been modified between different production batches. Second, on condition that the unique isotopic fingerprint of the compound has been established at the outset, the information could be used to track the product along the supply chain: each step depicted in Fig. 1 has a specific isotopic signature for the corresponding molecule, in a similar way to the unique barcode on its external packaging. Third, it can be used as a means of verifying a claimed or suspected manufacturing process by comparing the isotopic profile of the product with that of a sample made according to a known synthetic pathway. The former two applications require a proactive approach by the manufacturer, who will need to build up prior knowledge of any raw materials used, the reaction intermediates and final products involved, and to establish a data base of isotopic profiles. If this is done, an 'isotopomic' approach can be taken with classification of the samples based on predictive models: (i) approval of suppliers at each delivery level, (ii) improvement of product integrity and traceability, (iii) increasing transparency with respect to regulators, (iv) sourcing raw materials and, (v) tracking of the full supply chain. Thus, isotopic ^{13}C NMR can be considered as an ideal complement to chromatographic impurity profiles, being based on an independent set of criteria. The methodology can easily be implemented on current NMR spectrometers of medium magnetic field strength (400–500 MHz), likely to be available in facilities of the Pharmaceutical Industry. Likewise, regulatory bodies could use the same methodology to point out non-conformities, as is current practice in the food industry (Jamin and Martin, 2006; Martin et al., 2006c).

Within the wider context, these recent improvements in NMR methodology have a number of applications, not only for medicines, but also for establishing illicit drug affiliation, foodstuff and cosmetics counterfeits.

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References

Acetti, D., Brenna, E., Fronza, G., Fuganti, C., 2008. Monitoring the synthetic procedures of commercial drugs by ^{2}H NMR spectroscopy: the case of ibuprofen and naproxen. Talanta 76, 651–655.

Botosoa, E.P., Blumenstein, C., MacKenzie, D.A., Silvestre, V., Remaud, G.S., Renata A. Kwiecień, R.A., Robins, R.J., 2009a. Quantitative isotopic ^{13}C nuclear magnetic resonance at natural abundance to probe enzyme reaction mechanisms via site-specific isotope fractionation: the case of the chain-shortening reaction for the bioconversion of ferulic acid to vanillin. Anal. Biochem. 393, 182–188.

Botosoa, E.P., Silvestre, V., Robins, R.J., Moreno Rojas, J.M., Guillou, C., Remaud, G.S., 2009b. 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. J. Chromatogr. A 1216, 7043–7048.

Bussy, U., Thibaudeau, C., Thomas, F., Desmurs, J.R., Jamin, E., Remaud, G.S., Silvestre, V., Akoka, S., 2011. Isotopic finger-printing of active pharmaceutical ingredients by ^{13}C NMR and polarization transfer techniques as a tool to fight against counterfeiting. Talanta 85, 1909–1914.

Caytan, E., Botosoa, E.P., Silvestre, V., Robins, R.J., Akoka, S., Remaud, G.S., 2007a. Accurate quantitative ^{13}C NMR spectroscopy: repeatability over time of site-specific ^{13}C isotope ratio determination. Anal. Chem. 79, 8266–8269.

Caytan, E., Remaud, G.S., Tenailleau, E., Akoka, S., 2007b. Precise and accurate quantitative ^{13}C-NMR with reduced experimental time. Talanta 71, 1016–1021.

Davison, M. (Ed.), 2011. Pharmaceutical Anti-Counterfeiting: Combating the Real Danger from Fake Drugs. John Wiley & Sons Inc., Hoboken, New jersey, USA.

Deconinck, E., van Nederkassel, A.M., Stanimirova, I., Daszykowski, M., Bensaid, F., Lees, M., Martin, G.J., Desmurs, J.R., Smeyers-Verbeke, J., Vander Heyden, Y., 2008. Isotopic ratios to detect infringements of patents or proprietary processes of pharmaceuticals: two case studies. J. Pharm. Biomed. Anal. 48, 27–41.

European Commission, 2005 (Enterprise Directorate General). EMA Guidelines to Good Manufacturing Practice, Medicinal Products for Human and Veterinary Use, Part II, Basic Requirements for Active Substances used as Starting Materials. Brussels, Belgium, October 2005.

Fernandez, F.M., Green, M.D., Newton, P.N., 2008. Prevalence and detection of counterfeit pharmaceuticals: a mini review. Ind. Eng. Chem. Res. 47, 585–590.

Gavin, P.F., Olsen, B.A., Wirth, D.D., Lorenz, K.T., 2006. A quality evaluation strategy for multi-sourced active pharmaceutical ingredient (API) starting materials. J. Pharm. Biomed. Anal. 41, 1251–1259.

Gilbert, A., Silvestre, V., Robins, R.J., Remaud, G.S., Tcherkez, G., 2012a. Biochemical and physiological determinants of intramolecular isotope patterns in sucrose from C_3, C_4 and CAM plants accessed by isotopic ^{13}C NMR spectrometry: a viewpoint. Nat. Prod. Rep. 29, 476–486.

Gilbert, A., Robins, R.J., Remaud, G.S., Tcherkez, G.B., 2012b. Intramolecular ^{13}C pattern in hexoses from autotrophic and heterotrophic C_3 plant tissues. P. Natl. Acad. Sci. USA 109, 18204–18209.

Höhener, P., Silvestre, V., Lefrançois, A., Loquet, D., Botosoa, E.P., Robins, R.J., Remaud, G.S., 2012. Analytical model for site-specific isotope fractionation in ^{13}C during sorption: determination by isotopic ^{13}C NMR spectrometry with vanillin as model compound. Chemosphere 87, 445–452.

Hwang, T.-L., Zijl, P.C.M., Garwood, M., 1997. Broadband adiabatic refocusing without phase distortion. J. Magn. Res. 124, 250–254.

International Conference on Harmonization, 2005. Q9, Quality risk management. Geneva, Switzerland, November 2005.

International Conference on Harmonization, 2008. Q8(R1), Pharmaceutical development. Geneva, Switzerland, November 2008.

Jamin, E., Martin, G.J., 2006. SNIF-NMR-Part 4: applications in an economic context: the example of wines, spirits and juices. In: Webb, G.A. (Ed.), Modern Magnetic Resonance. Springer, pp. 1659–1665.

Martin, G.J., Akoka, S., Martin, M.L., 2006a. SNIF-NMR-Part 1: principles. In: Webb, G.A. (Ed.), Modern Magnetic Resonance. Springer, pp. 1629–1636.

Martin, M.L., Zhang, B.-L., Martin, G.J., 2006b. SNIF-NMR-Part 2: isotope ratios as tracers of chemical and biochemical mechanistic pathways. In: Webb, G.A. (Ed.), Modern Magnetic Resonance. Springer, pp. 1637–1645.

Martin, G.J., Martin, M.L., Remaud, G., 2006c. SNIF-NMR-Part 3: from mechanistic affiliation to origin inference. In: Webb, G.A. (Ed.), Modern Magnetic Resonance. Springer, pp. 1647–1658.

Ministry of Health, Labor, and Welfare. Pharmaceutical and Food Safety Bureau, 2005. Guidelines on Mentions in Manufacturing/Marketing Approval Application Dossiers for Pharmaceuticals and Others Based on Revised Pharmaceutical Affairs Law, PFSB/ELD 020001. Tokyo, Japan, 2005 February 10.

Scott, B., 2012. Pharmatech.com, 2-Jan-2012 FDA Perspectives: designation of regulatory starting materials in the manufacturing of drug substances: impact on ANDA review time.

Silvestre, V., Maroga Mboula, V., Jouitteau, C., Akoka, S., Robins, R.J., Remaud, G.S., 2009. Isotopic ^{13}C NMR spectrometry to assess counterfeiting of active pharmaceutical ingredients: site-specific ^{13}C content of aspirin and paracetamol. J. Pharm. Biomed. Anal. 50, 336–341.

Stanimirova, I., Daszykowski, M., Van Gyseghem, E., Bensaid, F., Lees, M., Smeyers-Verbeke, J., Massart, D.L., Vander Heyden, Y., 2005. Chemometrical exploration of an isotopic ratio data set of acetylsalicylic acid. Anal. Chim. Acta 552, 1–12.

Tannus, A., Garwood, M., 1996. Improved performance of frequency-swept pulses using offset-independent adiabaticity. J. Magn. Res. Ser. A 120, 133–137.

Taylor, N., 2012. EU could make API suppliers verify starting material sources. In-Pharma Technologist.com, 26-Jan-2012.

Tenailleau, E., Lancelin, P., Robins, R.J., Akoka, S., 2004. NMR approach to the quantification of nonstatistical ^{13}C distribution in natural products: vanillin. Anal. Chem. 76, 3818–3825.

Tenailleau, E., Akoka, S., 2007. Adiabatic ^{1}H decoupling scheme for very accurate intensity measurements in ^{13}C NMR. J. Magn. Res. 185, 50–58.

Thibaudeau, C., Remaud, G., Silvestre, V., Akoka, S., 2010. Performance evaluation of quantitative adiabatic ^{13}C NMR pulse sequences for site-specific isotopic measurements. Anal. Chem. 82, 5582–5590.

US Food and Drug Administration, 2006. Guidance for industry: BACPACI: intermediates in drug substance synthesis: bulk actives post-approval changes: chemistry, manufacturing, and controls documentation. Rockville, MD; 2001 February Withdrawn Fed. Regist. Notice June 2006.

Wokovich, A.M., Spencer, J.A., Westenberger, B.J., Buhse, L.F., Jasper, J.P., 2005. Stable isotopic composition of active pharmaceutical ingredient (API) naproxen. J. Pharm. Biomed. Anal. 38, 781–784.