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^{13}C isotopomics of triacylglycerols using NMR with polarization transfer techniques†

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A rapid and very precise method is proposed to simultaneously determine the position-specific ^{13}C content and the metabolomic profile of triacylglycerols (isotopomics). ^{13}C-NMR spectra are obtained by using an adiabatic INEPT sequence; the experiment time is reduced by a factor of 7 with respect to a one-pulse acquisition.

Nuclear Magnetic Resonance (NMR) is highly recognized as a fast and accurate technique for identification and quantitation of tracers in food matrices.[1] In this respect, ^{1}H NMR spectroscopy allows precise determination of the compositional profile.[2] However, ^{13}C NMR offers an interesting alternative as it provides a much better discrimination of resonances,[3] but it suffers from lower sensitivity.

Isotopic ratios can also be used to characterize food matrices. They are routinely determined using mass spectrometry (irm-MS: isotope ratios monitoring by Mass Spectrometry).[4] However, this technique, based on the complete combustion of the molecules, only provides average values of the heavy isotope content of each element.[5] Valuable information on the isotope intramolecular distribution is therefore inevitably lost. NMR is the only analytical technique which allows a site-by-site isotopic ratio determination. The isotope ^{13}C profile determined by ^{13}C NMR should reflect the history of the molecule, including the raw materials used and the chemical and/or biochemical pathways.

^{13}C NMR permits therefore simultaneous determination of both metabolomic and isotopic profiles (isotopomics). But in the case of ^{13}C, a precision of about 1 per mil is necessary for isotopic analysis since the range of ^{13}C isotopic compositions (δ^{13}C) within a given molecule could be highly restricted, *e.g.*, $\Delta\delta^{13}$C between C1, *C*2, and C3 of glycerol is between 1.6 and 40.9 per mil.[6] The determination of the intramolecular isotopic profile with a high degree of accuracy is therefore challenging and is a lengthy experiment due to the low natural abundance of ^{13}C, its small gyromagnetic ratio and long longitudinal relaxation.

A substantial reduction in the acquisition time of ^{13}C NMR can be achieved by sensitivity enhancement using polarization transfer from ^{1}H to ^{13}C nuclei.[7,8] Besides, the repetition time of the experiment becomes ^{1}H-T_1 dependent, thus allowing further time reduction. DEPT (Distortionless Enhancement by Polarization Transfer)[8] and INEPT (Insensitive Nuclei Enhanced by Polarization Transfer)[7] pulse sequences are widely used to this end. However, the regular versions of these experiments do not satisfy the isotopic analysis requirement in terms of precision. A low repeatability, approximately 5%, is obtained due to off-resonance effects and radiofrequency inhomogeneity in the 180° pulses applied to ^{1}H and ^{13}C nuclei.[9] A dramatic improvement in precision (repeatability of per mil) was reached *via* the incorporation of adiabatic 180° ^{1}H and ^{13}C pulses into the sequences.[7,9] Nevertheless, it was proved in a previous study that the long time stability of DEPT is not efficient for isotopic measurements at natural abundance, while the use of the INEPT sequence for ^{13}C isotopic measurements affords a substantial reduction in the experiment time without deterioration of short and long time stability.[10] Such a high precision offers extended potentialities in the position-specific isotopic composition analysis at natural abundance, provided that the exact stoichiometry of the different carbon sites to be compared is available. An isotopic profile can therefore be obtained from the peaks for which the concentrations are precisely known and have a signal-to-noise ratio of $\approx$ 500, while the other peaks of the ^{13}C NMR spectrum can be used for metabolomic profiling of the samples.

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† Electronic supplementary information (ESI) available: Details of ^{13}C-INEPT spectra acquisition and processing, pulse sequence scheme for adiabatic INEPT, designation of carbon atoms in triacylglycerol molecules, and enlargements of different regions in the ^{1}H and ^{13}C NMR spectra of olive oil for a resolving power comparison. See DOI: 10.1039/c5ay01250c

In this paper, we report the optimization of the adiabatic INEPT sequence for the metabolomic and isotopic characterization of triacylglycerols, which are quasiuniversal components of vegetable and animal matrices. Thus, metabolomic and isotopic profiling of these molecules are very powerful tools for food authentication issues and for metabolic studies at natural isotopic abundance. The composition in fatty acids of an oil sample cannot be known with a precision as high as a few per mil; therefore, most of the peaks observed in the ^{13}C NMR spectrum can only be used as compositional profile elements and not for the measurement of the intramolecular isotopic distribution. However, the NMR signals related to the carbons of the glycerol moiety (C1,3 and *C*2) and the first part of the fatty acids (*C*2 and C3) are always present and are common to all triacylglycerol molecules which permits their use for isotopic purposes. The other signals will be used for the profiling.

To illustrate the high potential of the adiabatic INEPT experiment in the origin classification of triacylglycerols, olive oil samples (bought from ethical and fair-trade shops) from different geographic origins (Crete, Lebanon, Spain, and Tunisia) were analyzed. Here we have to mention that the global ^{13}C content measured by irm-MS does not allow any classification of the samples.

Description of experimental parameters used for sample preparation, NMR acquisition and data processing is given in the ESI.† However, we want to describe here the way by which the INEPT sequence was optimized for the characterization of triacylglycerols.

In the refocused INEPT sequence (see Fig. S1 in the ESI†), initial longitudinal ^{1}H magnetization is converted by the 90° ^{1}H pulse into in-phase ^{1}H coherence in the transverse plane. The first spin-echo aims to convert this coherence into antiphase ^{1}H coherence. $\tau_1 = 1/4J_{CHav}$ ensures efficient coherence transfer (where J_{CHav} is the median value of the ^{1}H–^{13}C coupling constant). Simultaneous ^{1}H and ^{13}C 90° pulses transform the antiphase ^{1}H coherence into antiphase ^{13}C coherence. The 180° ^{13}C pulse during this second echo serves to refocus ^{13}C chemical shift evolution during τ_2 delays. τ_2 needs to be adjusted to an optimal value that depends on the nature of the resonance observed (1/4J for CH, 1/8J for CH_2 and 1/10J for CH_3 spin systems, respectively). The optimized τ_2 (see ESI†, NMR spectrometry experiments) ensures efficient refocusing for CH, CH_2 and CH_3 and the glycerol signals (C1,3 and *C*2) are then in the same ratio as in the one pulse quantitative ^{13}C NMR experiment for a ^{13}C isotopic analysis. Biases remaining on the other signals are highly reproducible. For signals used in isotopic analysis, RSD values ≤1.2 per mil were observed. For other signals, only used for profiling, RSD values ≤3.1% were observed.

The recovery delay in the one pulse sequence must be greater than 10 times the longest ^{13}C T_1;[11] this condition is required to avoid the influence of the nuclear Overhauser effect (NOE). Here, it induces TR = 42 s (since the longest ^{13}C T_1 of the measured signals was 4.0 s). Furthermore, 72 scans are needed with this sequence to obtain a signal-to-noise ratio higher than 500 on the *C*2 of glycerol (Fig. 1), which is a prerequisite for a precision around 1 per mil. The measurement time was therefore 5 hours 45 min for six spectra. Since, in the INEPT sequence, the proton magnetization is transferred to the bounded carbons, the intensity of the detected signal is only dependent on the ^{1}H longitudinal magnetization before the first 90° pulse and no longer depends on the carbon longitudinal relaxation. This avoids any influence of the NOE on the detected signal. The recovery delay is therefore only governed by the ^{1}H proton relaxation and must be greater than 7 times the longest ^{1}H T_1 in order to obtain an accuracy of 1 per mil. Here, the longest ^{1}H T_1 was measured for the methyl group of the linolenic acid (2.9 s). We have chosen to use a recovery delay equal to 8 times this T_1, in order to overcome any variation in relaxation times from one sample to another; this condition induces TR = 24 s. The occurrence of the polarization transfer increases the sensitivity, thus only 16 scans were required to obtain a signal-to-noise ratio higher than 600 on the *C*2 of glycerol (Fig. 1). The measurement time was therefore 50 min 10 s for six spectra. The experiment time is therefore reduced by a factor of 7 with respect to the quantitative one pulse ^{13}C NMR.

For each olive oil sample, peak areas of the following signals were determined (see Fig. S2† in the ESI for signal designation): *C*2, C3, Cω1, Cω2 and Cω3 of fatty acids; *C*2 and C1,3 of glycerol; the carbons 9 and 10 (oleic acid), 9, 10, and 12 (linoleic acid) at sn-1,3 and sn-2 positions of glycerol; allylic and di-allylic carbons, 24 signals related to the aliphatic methylene groups, other than those aforementioned, carbons 10, 12, 13, and 15 (linolenic acid) and carbons related to squalene were determined as well.

From the physical description of polarization transfer, it follows that the sensitivity gain in INEPT is only obtained for hydrogen-bearing carbons. In other words, only CH, CH_2 and CH_3 groups will be observed, while quaternary carbons will be missing. Carbonyl carbon signals are therefore lost, while they allow the determination of saturated, oleic, linoleic, and linolenic acids at sn-1,3 and sn-2 positions of the glycerol backbone. However, this information can still be obtained from other regions of the spectra such as from the olefinic region ranging from 132 to 127 ppm and from the region related to the *C*2 of fatty acids ranging from 34.2 to 33.7 ppm.[12]

As an example of the pertinence of these variables, we show in Fig. 2 that the plot of the peak area ratio of carbon 9 of linoleic acid at the sn-1,3 position of glycerol to that at the sn-2

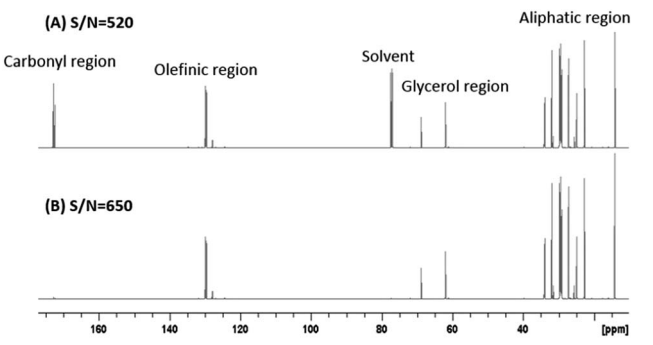

Fig. 1 ^{13}C NMR spectrum of an olive oil sample obtained by (A) one pulse ^{13}C acquisition in 54 min 30 s, and (B) adiabatic refocused INEPT in 8 min 21 s.

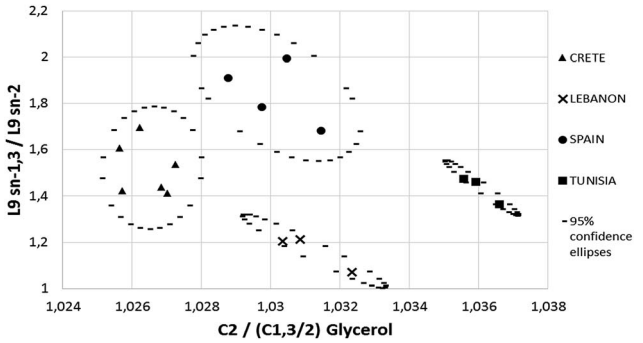

Fig. 2 Scatter plot of olive oil samples, from Crete, Lebanon, Spain, and Tunisia, based on the ratio C2/(C1,3/2) of the glycerol backbone and the ratio of C9 linoleic acid-glycerol sn-1,3/C9 linoleic acid-glycerol sn-2.

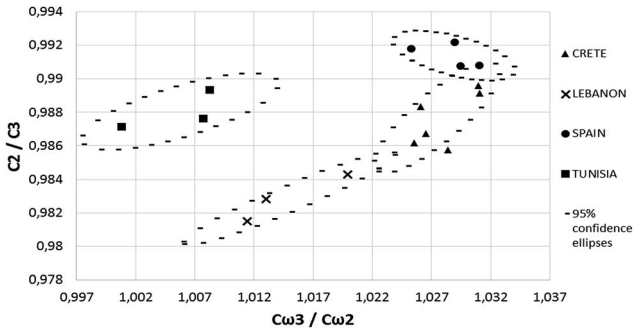

Fig. 3 Scatter plot of olive oil samples from 4 different countries (Crete, Lebanon, Spain, and Tunisia) based on the C2/C3 and Cω3/Cω2 of fatty acids.

position against the isotopic ratio of the glycerol carbons [C2/(C1,3/2)] permits an excellent separation between the different olive oil groups. It should be noted that the variables related to the position of fatty acids at sn-1,3 and sn-2 positions of glycerol could not be obtained by means of ^{1}H NMR spectroscopy due to its lower resolution owing to its narrower range of resonance frequencies relative to ^{13}C NMR (see Fig. S3† in the ESI for a comparison).

As a result, the olive oils could be classified according to their geographical origin using solely the information related to the position of linoleic acid on the glycerol skeleton and the isotopic content of carbons in this moiety.

In the second example (Fig. 3), we show that it is also possible to distinguish olive samples from different countries using the ratios C2/C3 and Cω3/Cω2 of fatty acids.

In conclusion, adiabatic INEPT is a rapid and very precise method that allows the relative site specific ^{13}C content of triacylglycerols to be determined at the same time as the metabolomic profiling with the information related to the position of fatty acids on the glycerol moiety being available.

Nevertheless, numerous elements are extracted from the corresponding ^{13}C INEPT spectra but only few of them were exploited in this paper. A global analysis of all the elements using a larger number of samples and statistical tools is currently underway in our laboratory, aiming to draw out their full characterization potential.

This method is a valuable tool for origin tracing and metabolic studies using triacyglycerols, extracted from vegetables, animal tissues or finished products, as probe molecules. Its characterization power is brought to a higher level since faint deviations between carbon signals in the spectra become rapidly quantifiable with high reproducibility and robustness, allowing the isotopomic information inherited by the triacylglycerol molecules to be revealed.

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