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SHORT COMMUNICATION

High resolution nuclear magnetic resonance spectroscopy (NMR) studies on meat components: potentialities and prospects

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ABSTRACT

In recent years, increasing application of nuclear magnetic resonance (NMR) spectroscopy in the study of agricultural food products has been remarked, thanks to the advantages of this technique over other conventional analytical techniques. This preliminary work presents, for the first time, the application of an innovative NMR technique, the proton high resolution magic angle spinning (¹H HR-MAS), for studying meat features. It stresses that this method makes it possible to acquire qualitative and quantitative information about chemical composition, both quickly and without any particular preparation of the sample to be analysed. Finally, the study highlights the potentiality of this method in defining the origin of meat and the possibility of identifying meat adulteration.

Key words: NMR, ¹H HR-MAS, Meat.

RIASSUNTO

LA SPETTROSCIA DI RISONANZA MAGNETICA NUCLEARE (NMR) AD ALTA RISOLUZIONE NELLO STUDIO DELLA COMPOSIZIONE DELLE CARNI: POTENZIALITÀ E PROSPETTIVE.

Negli ultimi anni si è riscontrato un sempre crescente impiego della spettroscopia di risonanza magnetica nucleare (NMR) come mezzo di indagine nel campo agro-alimentare. Ciò è dovuto ai numerosi vantaggi che questa tecnica presenta rispetto ad altre tradizionali tecniche di analisi. In questa indagine preliminare, uno stesso pezzo di carne bovina è stato sottoposto a due metodiche di preparazione e di analisi NMR ad alta risoluzione. Nel primo caso si è seguito un procedimento, riportato in letteratura, che ha permesso di ottenere un estratto del campione in HCl 1M, successivamente analizzato con uno strumento NMR a 500 MHz; nel secondo, il campione di carne, dopo liofilizzazione, è stato direttamente analizzato attraverso una innovativa tecnica NMR (¹H HR-MAS) per l’analisi di solidi. Gli spettri NMR così ottenuti permettono di mettere in evidenza numerose classi di composti chimici, ma da un confronto è emerso che la tecnica ¹H HR-MAS è da preferirsi in quanto non necessita di alcuna particolare preparazione del campione da sottoporre ad indagine. L’assegnazione di gran parte dei segnali presenti negli spettri è stata effettuata grazie all’ausilio di tecniche di spettroscopia NMR bidimensionale. Infine, vengono messe in evidenza la potenzialità del metodo nella caratterizzazione della provenienza delle carni e la possibilità di individuare eventuali sofisticazioni delle stesse.

Parole chiave: NMR, ¹H HR-MAS, Carni.
Introduction

Italian meat production satisfies internal demand only for the poultry and rabbit sectors. This shortage imposes the need to import large quantities of pork and beef and smaller quantities of sheep, goat and horse meat. The 2000 NOMISMA report indicated that the Italian meat output supplies 43% of demand for beef, 63% for pork, and 55% for sheep and goat meat with the shortfall made up by meat imported from the European partners. In the '93-'96 period the production of beef increased by 9% in the USA and by almost 10% in developing countries, while in the European Union it decreased by over 3%. In Italy, according to an ISMEA report (1999), the situation worsened in the years '97-'98 with a further output reduction of 3% and a consequent increase of the imports, which - sometimes exceeding demand - tend to balance the productive drop. Recently production has dropped further because of the vertical fall in beef consumption caused by the Bovine Spongiform Encephalopathy (BSE). In order to regain consumer confidence offering a secure product, and simultaneously protecting the typical productions of the different EU countries, the EC has promoted programmes to boost the recognition of typical primary products by endorsing the Protected Designation of Origin (PDO) and the Protected Geographical Indication (PGI) trade-marks. In addition to these initiatives, the EC also published a White Paper which sets out the requirements to meet in order to maintain safety standards for each productive stage of all typical products. The appreciation of qualified national products, together with a guaranteed reference for consumers, has become a must for all who are engaged in business concerning beef. Italy has a long-standing tradition of highly qualified products (Parmigiano Reggiano cheese, Buffalo Mozzarella, Parma Ham, olive oil etc.). These products have a clear-recorded history, which have helped consumers to trust them. A similar record for beef would certainly help the confused and worried consumer to have a new approach to this product. At the present time only "Vitellone bianco dell'Appennino Centrale" meat has obtained PGI in Italy. In this framework the traditional quality indicators, i.e. bromatology, organoleptic, hygiene and technological parameters, appear inadequate. This is the reason why the scientific community feels strongly that there is the need for new and innovative chemical markers, able to anchor the products with their territorial origin.

Large-scale livestock farming characterised by mass-production which only the big distribution chains are able to handle and on which there are several restrictions regarding the use of hormones, health and safety, and acceptable residues, necessitated by the harshness of the productive technologies is now becoming obsolete. A new productive scenario is gaining momentum, based on the nutritional and preventive health value of foods produced by quality livestock farming, and this productive growth needs well-defined biological indicators, which will characterise the nutritional value, the territorial origin and the healthiness of the goods, thus reassuring the consumer.

In the last few years the application of spectroscopic techniques in the analysis of food products has been developed. Among these, nuclear magnetic resonance spectroscopy (NMR) has grown to a relevant position. NMR is a versatile technique that allows the gathering of quantitative and qualitative information from a single spectrum, a task that, generally, requires twenty minutes at the most. Similar information could be gained via other conventional analytical techniques, but this would require several instruments and more time. A further advantage of NMR is the lack of sample pre-treatments, which is instead a general requirement for the other techniques.

For these reasons, the use of NMR in food research is expanding greatly. This is shown by the large number of scientific publications appearing in the last years (only a few of the most representative are reported: Rapp and Markowetz, 1993; Martin and Martin, 1995; Martin et al., 1995; Belton et al., 1996; Belloque and Ramos, 1999; Vlahov, 1999) and the regular International Conference on Application of Magnetic Resonance in Food Science (Belton et al., 1999).

Our research group is applying NMR to the characterisation of the variety and geographical origin of food products. NMR was used to investigate durum wheat flours (Sacco et al., 1998) and
olive oils (Sacco et al., 2000). Investigation on wines (Brescia et al., 2002a) and Italian wheat flours (Brescia et al., 2002b) have recently been published and have been presented to national (Brescia et al., 2000a; Caldarola et al., 2000) and international conferences (Brescia et al., 2000b; Brescia et al., 2001).

In this paper we present a preliminary NMR investigation on meat, trying to show the potentiality and the prospective of this innovative technique.

To our knowledge there is not a great number of scientific studies concerned with the application of NMR on meats. In fact, a rather detailed bibliographic search gave as results: (a) a review (Renou, 1995) in which low field NMR was used to measure the amount of protein and fat in meat, (b) an application for checking the nutritional value of pork (Brown et al., 2000) by measuring the relaxation time of the water in the tissues, and (c) a high field (500 MHz) application, followed by a statistical analysis of the collected data, in order to highlight adulteration coming from the combination of different meat cuts (Colquhoun et al., 2001).

Material and methods

In the present study, one cut of fresh meat was purchased from local retailers and investigated by NMR analysis using two different methodological approaches.

In the first one, the cut of beef was minced and frozen, following a procedure suggested by Colquhoun et al. (2001). Afterwards, 5 g of sample were thawed and homogenised in 5 ml of HCl 1M, using a Ultra-Turrax for 60 seconds. The obtained suspension was centrifuged (10,000 rpm), at room temperature, for 30 minutes. 0.5 ml of supernatant were removed, mixed with 0.1 ml of D$_2$O containing 0.5% TSP (sodium 3-trimethylsilyl-propionate-2,2,3,3-d$_4$), and the solution was analysed with a Bruker AVANCE 500 MHz NMR spectrometer.

The mono-dimensional NOESY sequence, with presaturation of the water signal contained in the sample, was used to record the NMR spectrum. The following experimental conditions were applied: 160 transients with 32,768 data points; 3 seconds of recovery delay and total experimental time 15 minutes. The resulting spectrum has been reported in figure 1.

In the second preparative approach, the meat sample was minced and freeze-dried. 30 mg of D$_2$O were added to 40 mg of this sample obtaining a semi-solid pulp in order to apply the $^1$H HR-MAS technique. This innovative technique allows to obtain high-resolution NMR spectra of the semi-solid samples by means of the rotation at high fre-
frequency of the sample rotor (up to 15,000 Hz). In this way, the molecular interactions that would produce the broadening of NMR signals are eliminated. A NMR spectrum containing broad lines could not be used for diagnostic purposes.

The NMR spectrum of the semi-solid meat sample was obtained with a Bruker AVANCE 400 MHz NMR spectrometer equipped with a HR-MAS probehead. The sample was brought to the magic angle (54°44’) with respect to the direction of the static magnetic field and spun at 4,500 Hz in order to minimise the chemical shift anisotropy effects. NOESY mono-dimensional pulse sequence, modified by adding a sequence for suppression of water protons contained in the deuterated solvent, was employed. The experimental conditions were: 32,768 time domain points; 1.5 seconds of recycle delay and 160 scans. The total experimental time was 15 minutes. The obtained spectrum is shown in figure 2.

In order to assign unknown peaks, 2D-NMR experiments were performed: a) TOCSY (Total Correlation Spectroscopy), that correlates protons in a molecule, and b) $^1$H-$^{13}$C heterocorrelated spectroscopy, that relates $^{13}$C nuclei with directly connected protons. TOCSY spectrum was obtained using 2,048 points, 256 increments and 16 transients per increments; $^1$H-$^{13}$C spectrum was recorded with 1,024 X 200 points and 16 transients per increment. Both spectra were acquired with a 400 MHz NMR spectrometer equipped with a HR-MAS probehead.
Results and discussion

The $^1$H-NMR spectra of meat show a large number of signals and a high degree of overlap (figures 1 and 2). The $^1$H HR-MAS spectrum shows very efficient water suppression, whose residual signal is visible at 4.8 ppm, and resolution comparable to that of the extract spectrum. Moreover, it seems to contain more detailed chemical information on the meat components.

This behaviour was expected since with the first sample preparation methodology used, only signals from the soluble meat components are obtained, while with the second methodology all the components give a signal. Moreover, the $^1$H HR-MAS spectrum, obtained using the same number of scans and with a lower field instrument, is characterised by a better signal to noise ratio.

NMR is a powerful tool for structure determination. In fact, the information on the structure of molecules constituting the analysed sample is readily obtained from the parameters measured in NMR spectra, as the chemical shift ($\delta$), spin multiplicity and coupling constants. Anyway, in some cases, due to the strong overlap of the resonances, the determination of the aforesaid parameters can be carried out only by means of the bi-dimensional NMR spectra (2D-NMR). This is true in the case of mono-dimensional $^1$H HR-MAS meat spectra. Consequently, TOCSY (figure 3) and $^1$H-$^1^3$C hetero-correlated (figure 4) 2D-NMR experiments have been carried out. Bi-dimensional analysis allowed to assign signals to particular metabolites through the examination of the existing correlations. The assignments are based on the comparison of chemical shifts and spin multiplicities with data reported in literature (Fan, 1996). The spectra contain resonances from amino acids, sugars and organic acids. A partial assignment of the signals present in the mono-dimensional $^1$H HR MAS spectrum (figure 2) is reported in table 1.

Figure 3. TOCSY HR-MAS NMR spectrum of a freeze-dried meat sample.
most intense peaks arise from creatine, but other compounds are detected in the aliphatic and aromatic regions. In the aliphatic region signals arise mainly from amino acids and creatine. In addition, peaks from carnosine are also observed. In the range 3-5.5 ppm the main contributions arise from creatine and lactate. Resonances from α, β-glucose and carnosine can also be seen. In the aromatic region signals arise from the amino acid tyrosine and from carnosine. From the comparison of the two spectra it is possible to notice that some signals, in particular lactic acid CH₂ and creatine N-CH₂ resonate at slightly different chemical shifts. This is due to the sensibility of these protons to the pH of the medium, that is almost neutral for the solid sample and acid for the extract.

Signals present in the spectra could be quantified using an integration procedure providing quantitative information on the studied meat sample.

Figure 4. ¹H-¹³C HR-MAS NMR heterocorrelated spectrum of a freeze-dried meat sample.
Conclusions

This preliminary approach has shown how it is possible to obtain information on a high number of metabolites by means of a simple extraction carried out on a cut of meat. In other words, the NMR spectra can be regarded as the fingerprint of the analysed sample. Moreover, it has been shown that high resolution NMR spectra can be obtained directly on the meat sample through the 1H HR-MAS NMR technique. The advantages of this innovative technique consist in easy preparation of the sample, in the possibility of carrying out more reliable quantitative measurements and in obtaining more information. Moreover, since ten minutes suffice to obtain a 1H HR-MAS spectrum of a meat sample, hundreds of samples could be analysed in an acceptable time. This is the necessary condition for the statistical applications that are essential for the aims we intend to reach through an already planned research. Different cuts of beef could give spectra characterised by compositional differences that would result in different intensities of some peaks. These differences could be used to distinguish beef cuts through the application of statistical analysis on most discriminant signals’ intensities.

In conclusion, we think that this preliminary study prepares the way for a wide range of specific applications in meat field:

1) protection of quality products through the discrimination of meats coming from different species or cuts of different commercial value;

2) determination of discriminating parameters to be processed with multivariate statistical analysis in order to identify the geographical area of origin of the meat samples analysed;

3) constitution of data banks with samples of different origins.

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Table 1. 1H and 13C chemical shift assignment of compounds detected in a meat sample.

| δ 1H (ppm) | Group | δ 13C (ppm) | Multiplicity | Compound       |
|------------|-------|-------------|--------------|----------------|
| 0.99       | CH3   | Doublet     | Valine       |
| 1.04       | CH3   | Doublet     | Valine       |
| 1.34       | CH3   | Doublet     | Lactate      |
| 1.48       | CH3   | Doublet     | Alanine      |
| 2.14       | CH3   | Doublet     | Lactate      |
| 2.45       | CH3   | Doublet     | Glutamine    |
| 2.70       | CH2   | Multiplet   | Carnosine    |
| 3.04       | CH3   | Multiplet   | Creatine     |
| 3.15       | CH3   | Multiplet   | Carnosine    |
| 3.25       | C2H   | Multiplet   | β-glucose    |
| 3.30       | CH    | Multiplet   | Carnosine    |
| 3.40       | C4H   | Multiplet   | β-glucose    |
| 3.42       | C4H   | Multiplet   | α-glucose    |
| 3.52       | C3H, C5H | Multiplet | β-glucose    |
| 3.55       | C2H   | Multiplet   | α-glucose    |
| 3.62       | C3H   | Multiplet   | α-glucose    |
| 3.79       | CH    | Multiplet   | Alanine      |
| 3.79       | CH    | Multiplet   | Glutamine    |
| 3.85       | C5H   | Multiplet   | α-glucose    |
| 3.94       | NCH3  | Multiplet   | Creatine     |
| 4.13       | CH    | Multiplet   | Lactate      |
| 4.51       | CH    | Multiplet   | Carnosine    |
| 4.65       | C1H   | Doublet     | β-glucose    |
| 5.25       | C1H   | Doublet     | α-glucose    |
| 6.88       | H3, H5| Doublet     | Tyrosine     |
| 7.19       | H2, H6| Doublet     | Tyrosine     |
| 7.26       | C4H   | Multiplet   | Carnosine    |
| 8.56       | C2H   | Multiplet   | Carnosine    |
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