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HRMAS-NMR spectroscopy and multivariate analysis meat characterisation

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ABSTRACT

^{1}H-high resolution magic angle spinning-nuclear magnetic resonance spectroscopy was employed to gain the metabolic profile of *longissimus dorsi* and *semitendinosus* muscles of four different breeds: Chianina, Holstein Friesian, Maremmana and Buffalo.
Principal component analysis, partial least squares projection to latent structure – discriminant analysis and orthogonal partial least squares projection to latent structure – discriminant analysis were used to build models capable of discriminating the muscle type according to the breed. Data analysis led to an excellent classification for Buffalo and Chianina, while for Holstein Friesian the separation was lower. In the case of Maremmana the use of intelligent bucketing was necessary due to some resonances shifting allowed improvement of the discrimination ability. Finally, by using the Variable Importance in Projection values the metabolites relevant for the classification were identified.

1. Introduction

Meat is an essential food in the human diet since it is an excellent source of amino acids, fatty acids, vitamins, especially B group, and minerals such as iron, copper and zinc (Pearson & Gillett, 1999). In the western world its consumption has markedly increased in the last decades, and the quality demand increased accordingly. The term quality for meat encompasses several factors, above all hygiene, nutrition, technology and sensory (Verbeke, Van Oeckel, Warnants, Viaene, & Boucque, 1999). The most important aspects contributing to meat quality, thus determining its acceptability among consumers, are taste (Shahidi, Rubin, & Dsouza, 1986), texture (Maltin, Balcerzak, Tilley, & Delday, 2003), and juiciness (Cheng & Sun, 2008). The last two are determined by microscopic and macroscopic tissue organizations, while chemical composition defines the taste.

Several aspects of meat have been extensively investigated, including the use of metabolomics for evaluating some quality related properties. Metabolomics takes into account the pattern of the low molecular weight species, and is defined as the systematic study of the unique chemical fingerprints that specific cellular processes leave behind. Many analytical techniques have been used in metabolomics, including nuclear magnetic resonance spectroscopy. The latter was employed for determining the metabolic pattern of a large number of foods, predominantly fruits and vegetables. Tomato (Le Gall, Colquhoun, Davis, Collins, & Verhoeyen, 2003), lettuce (Sobolev, Brosio, Gianferri, & Segre, 2005), mango (Duarte, Goodfellow, Gil, & Delgadillo, 2005), juices (Spraul et al., 2009), and grape berries (Son et al., 2008) are examples. On the contrary, meat systems have been poorly investigated by NMR, most likely because of complicated and time demanding extraction and purification procedures. Bertram, characterized meat metabolome as a function of pre-slaughter exercise (Bertram, Oksbjerg, & Young, 2010) and heat stress (Straadt et al., 2010).

Recently, high resolution magic angle spinning-nuclear magnetic resonance (HRMAS-NMR) has been proposed as a reliable system based for assessing the metabolic profile. It offers the opportunity of measuring samples without any chemical and/or physical preparation, by producing highly resolved spectra. While HRMAS-NMR has been extensively used in medicine and related fields (Rocha et al., 2010; Wang et al., 2003), its application in food characterisation is still in its infancy, and few studies have been reported (Valentini et al., 2011), mainly referring to fresh vegetables (Perez, Iglesias, Ortiz, Perez, & Galera, 2010; Ritota, Marini, Sequi, & Valentini, 2010) and processed materials (Ciampa, Renzi, Taglienti, Sequi, & Valentini, 2010; Consonni, Cagliani, & Cogliati, 2011; Shintu & Caldarelli, 2005, 2006).

Brescia et al. (2002) reported a preliminary study on meat composition by HRMAS-NMR. Later, the characterisation of Apulian lamb meat according to geographical origin was determined, by combining HRMAS-NMR spectroscopy with other analytical techniques (Sacco, Brescia, Buccolieri, & Jambrenghi, 2005). Also Shintu used ^{1}H-HRMAS-NMR spectroscopy for the identification of molecular markers in the traceability of dried beef (Shintu, Caldarelli, & Franke, 2007). Finally, Renou characterized meat samples according to the production site and feed, by combining ^{18}O IRMS with NMR data (Renou et al., 2004).

Here is reported the comprehensive assignment of the metabolic pattern determined by ^{1}H-HRMAS-NMR. The multivariate NMR data

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analysis allowed the identification, to the best of our knowledge, for the first time, of the metabolites capable of discriminating *longissimus dorsi* and *semitendinosus* muscles according to breed.

2. Materials and methods

2.1. Meat samples

47 young bulls were used, 5 buffalo, 18 chianina, 15 maremmana and 9 Holstein. Longissimus dorsi (ld) and semitendinosus (st) muscles were sampled; 5 ld and 5 st for buffalo, 18 ld and 18 st for chianina, 15 ld and 12 st for maremmana and 9 ld and 9 st for Holstein Friesian. All animals were reared in the meat production and genetic improvement research center (CRA-PCM, Rome Italy) according to the national guide for animal care. Cattle were intensively reared in stalls and were fed hay and maize silage ad libitum and 800 g of concentrate/100 kg of live weight in the finishing period (3 months before slaughter). Animals were slaughtered at about 550 kg of liveweight according to EU legislation, Reg. CE n. 1099/2009. Samples were collected and frozen in liquid nitrogen prior to transportation to the laboratory, and then stored at −80 °C.

2.2. HRMAS-NMR measurements

Samples were prepared by inserting ca. 10 mg of meat in a 4 mm HRMAS rotor with a 50 μL spherical insert: approximately 40 μL of D_2O phosphate buffer, 0.01 M, pH 7.2 and containing 0.01% TSP, i.e. 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt, were then added. HRMAS-NMR spectra were recorded at 298 K with a Bruker AVANCE spectrometer operating at a ^{1}H frequency of 400.13 MHz, equipped with a 4 mm HRMAS dual channel probe head and spinning the rotor at 7 kHz. ^{1}H NMR spectra were referenced to the TSP methyl groups signal at $\delta = 0.00$ ppm. Similarly ^{13}C NMR spectra were referenced to the TSP signal $\delta = 0.00$ ppm.

^{1}H-HRMAS-NMR spectra were acquired by using a water suppression pulse sequence containing a NOE enhancement, with 32 K data points over a 4807 Hz spectral width and adding 256 transients. A recycle delay of 3 s, a NOE build up period of 150 ms and a 90° pulse length of 5.88 μs were used. NMR data were processed using ACD/Spec Manager 8.00 software (Advanced Chemistry Development Inc., Toronto, Canada). Each ^{1}H-HRMAS-NMR spectrum was FT transformed with 64 K data points, manually phased and base-lined, and a line broadening factor equal to 0.3 Hz was applied to the FID prior to FT.

Assignment of most resonances in the proton spectrum was made by connectivity information obtained from 2D spectra and the use, as guidelines, of chemical shift data reported in the literature (Brescia et al., 2002; Graham, et al., 2010; Shintu et al., 2007; Valentini et al., 2004).

^{13}C-HRMAS-NMR spectra were acquired with the power-gated decoupling sequence, using a 30° flip angle pulse of 5.0 μs. Experiments were carried out using 64 K data points over a 22,123 Hz (~220 ppm) spectral width by adding 64 K transients with a recycle delay of 3 s. Each spectrum was FT transformed with 128 K data points and manually phased and base-lined, and a line broadening factor of 0.5 Hz was applied to the FID.

^{1}H–^{1}H-TOCSY experiments were collected in the phase-sensitive mode using time proportional phase incrementation (TPPI) for quadrature detection in the direct dimension, with a 4807 Hz spectral width in both dimensions, 100 ms of spin-lock time, 2 K data points in f2, and 1 K increments in f1, each with 32 scans. The water signal was suppressed.

^{1}H–^{13}C-HMQC spectra were acquired in TPPI phase-sensitive mode, with a 4807 Hz spectral width in f2 dimension and a 15,083 Hz spectral width in f1. 1 K data points in f2 and 256 increments in f1, each with 32 scans, were used. HMQC was preferred to the HSQC since the latter is more sensible to the optimization of the acquisition parameters.

2.3. Multivariate data analysis

Each NMR spectrum was divided into intervals equal to 0.04 ppm by using the ACD bucketing or, when necessary, the intelligent bucketing. The spectral region from 4.63 to 5.20 ppm was neglected in order to remove any spurious effects of variability occurring during the suppression of HDO resonance. Also the regions containing only noise, usually addressed as dark regions, were ignored. Finally, to take into account variations in sample concentration, thus making meaningful comparisons between samples, whole spectrum intensity was normalized to 100. Each ^{1}H NMR bucketed spectrum was considered as a row vector and placed into a matrix of n rows (spectra) corresponding to the number of samples for a number of columns (variables) equal to the number of buckets used. Each variable was the integral of the spectrum area at the specific chemical shift. Multivariate analysis was performed using MATLAB (version 7.1, The Mathworks, Natick, MA, USA), PLS_Toolbox (version 502, Eigenvector Research Inc.) and home-made algorithms. Data were auto-scaled, that is variance scaled to unity and mean sets to zero, in order to avoid assigning large loadings to the highest intensity signals. The whole data set was used as training set and cross validation, by means of the leave-one-out method, was used to estimate the predictive ability of the model. The paired samples *t*-test was used to compare group means in the scores matrix, which is generally used when comparing two means that are repeated measurements for the same samples, scores might be repeated across different measures or across time, or comparing paired samples, as in a two treatment randomized block design. Furthermore, one-way ANOVA was used to test differences in means of the scores matrix for statistical significance.

3. Results and discussion

3.1. ^{1}H-HRMAS-NMR spectrum assignment

The ^{1}H-HRMAS-NMR spectra of the meat samples are dominated by the intense resonances of lactic acid and creatine and/or phosphocreatine. Several minor signals, such as fatty acids, amino acids, organic acids and nucleosides, are detectable, and are summarized in Table 1.

The high field region, top left panel of Fig. 1, contains signals belonging to the aliphatic groups of amino, organic and fatty acids. Signals in the range from 0.90 to 1.10 ppm belong to methyl or methylene groups of valine, leucine, and isoleucine, and the correct assignment was possible based on cross peaks in the TOCSY spectrum (not shown). In this region one finds also a series of broad peaks. The signal at 0.91 ppm has in the TOCSY spectrum cross peaks with proton atoms at δ equal to 1.33, 1.61, and 2.26 ppm, these were assigned to saturated fatty acids, i.e. palmitic and stearic acids which are the most abundant in meat. The HMQC spectrum, bottom right part of Fig. 1, confirmed this assignment, since ^{1}H at $\delta = 1.33$ and 0.91 ppm correlated with ^{13}C at 30.38 and 14.79 ppm, respectively.

Between 1.15 and 1.21 ppm different doublets with coupling constants varying from 6.16 to 6.95 Hz are present in several samples. Even though the corresponding correlations are not always clearly visible in the TOCSY spectrum, they were assigned to different hydroxybutyric acids, which are known to be involved in amino acid synthesis and degradation.

Cross peaks between the signal at $\delta = 1.32$ ppm and those at 2.04, 2.79 and 5.36 ppm indicates the presence of unsaturated lipid chains, with oleic and linoleic being predominant (Wood et al., 2008). The broad peak at $\delta = 0.74$ ppm was tentatively assigned to terminal CH_3 of sterols. One of the most intense peak in this region belongs to the β-CH_3 of lactic acid, $\delta = 1.33$ ppm; it shows correlations with