Food Chemistry 240 (2018) 514–523

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

^{1}H HR-MAS NMR-based metabolomics analysis for dry-fermented sausage characterization

Ana Belén García-García[a], Santosh Lamichhane[c], David Castejón[b], Mª Isabel Cambero[a], Hanne Christine Bertram[c,*]

[a] Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain
[b] CAI de Resonancia Magnética Nuclear y Espín Electrónico, Universidad Complutense de Madrid, 28040 Madrid, Spain
[c] Department of Food Science, Aarhus University, Research Centre Aarslev, Kirstinebjergvej 10, DK-5792 Årslev, Denmark

ARTICLE INFO

Keywords:
Dry-fermented sausages
Fermentation
Ripening
^{1}H HR-MAS NMR spectroscopy
Meat metabolites
Culinary meat

ABSTRACT

Proton high-resolution magic angle spinning (^{1}H HR-MAS) nuclear magnetic resonance (NMR) spectroscopy in combination with principal component analysis (PCA) was employed to characterize dry-fermented sausages *salchichón type* throughout the manufacturing process. ^{1}H HR-MAS NMR metabolite profiling was achieved from a small sample of intact sausage after 0, 2, 4, 7, 11 and 14 days of drying. Intriguingly, the obtained results enabled the identification of the three main stages in the traditional production of *salchichón*. Formulation, fermentation and drying-ripening periods showed distinct and characteristic metabolomic profiles. Compositional changes related to microbial activity, as well as proteolytic and lipolytic phenomena, decisive steps in such a ripening process, could be monitored through the NMR spectra. This study shows the potential of ^{1}H HR-MAS as a rapid method for probing metabolomic profiles and compositional changes during sausages processing.

1. Introduction

Drying represents one of the oldest forms of meat preservation. Within the category of cured meat products, *salchichón* is one of the most popular dry sausage type produced and consumed in Spain. The main distinguishing feature of this group of meat products is that they rely on microbial fermentation to achieve their final properties. Production occurs by stimulating the growth of the desired fermentation microbiota, while the growth of spoilage bacteria is suppressed, mainly due to the pH decline in the product and the controlled decrease of moisture, related to water activity (a_w) reduction (Fernández, Ordóñez, Bruna, Herranz, & de la Hoz, 2000). In naturally produced dry-fermented sausages without starter cultures, the typical microbiota (lactic acid bacteria (LAB) and *Micrococcaceae*) highly contributes to obtain the desired taste, appearance and texture of fermented sausages (Ordóñez, Hierro, Bruna, & de la Hoz, 1999). However, the final properties of the product depend not only upon the products of carbohydrate fermentation but are also strongly influenced by biochemical and physical changes occurring during the drying process with the consequence of more concentrated flavour components and firmer sausage texture (Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Álvarez, 2008).

From a production point of view, the satisfaction of consumers must be ensured by offering high quality products, including both organoleptic and nutritional value. In this regard, an elucidation of the metabolite profile of the product is essential. Metabolomics seeks to identify and quantify the complete set of metabolites in a cell or tissue type, and to do so as quickly as possible and without bias (Weckwerth, 2003). To achieve this objective, NMR spectroscopy is a quantitative nondestructive, noninvasive, nonequilibrium perturbing technique that provides detailed information on molecular structures, based on atom-centered nuclear interactions and properties. It allows the detection of a wide range of structurally diverse metabolites simultaneously, providing a metabolic 'snapshot' at a particular time point (Beckonert et al., 2007). Generally, NMR spectroscopy has been used in the elucidation of the chemical compounds in solution, but in recent years its application in the field of food, together with *Metabolomics*, has opened new perspectives, allowing changing and increasing the targets in the study of food samples (Laghi, Picone, & Capozzi, 2014).

Newly, NMR analysis has been employed to obtain metabolic profiles of meat samples and to investigate different factors affecting meat properties including the geographical origin of beef (Jung et al., 2010), the muscle type according to bull breed (Ritota, Casciani, Failla, & Valentini, 2015), the effects of different irradiation doses on

* Corresponding author.
E-mail address: HanneC.Bertram@food.au.dk (H.C. Bertram).

http://dx.doi.org/10.1016/j.foodchem.2017.07.150
Received 5 April 2017; Received in revised form 24 July 2017; Accepted 27 July 2017
Available online 29 July 2017

the beef metabolome (Zanardi et al., 2015), the wooden breast abnormality in chicken (Sundekilde, Rasmussen, Young, & Bertram, 2017) and the evaluation of beef exudates as suitable matrix to study beef ageing (Castejón, García-Segura, Escudero, Herrera, & Cambero, 2015). However, few NMR spectroscopic studies focused on the examination of fermented sausages have been conducted. Siciliano et al., 2013 determined the variations in the fatty acid composition based on analyses of lipid extracts and Mati, Staruch, & Soral, 2015 studied the quantitative changes in carnosine and free amino acids in the aqueous fraction during ripening. Consequently, studies on the application of NMR-based metabolomics for the detection of compounds in dry-fermented meat are still limited and no detailed investigations on the complete profiling of intact sausages have been reported. Therefore, the aim of this study was to investigate the potential of a ^{1}H HR-MAS methodology for an explorative and untargeted elucidation of changes in the metabolites of dry fermented sausages *salchichón type* during a traditional manufacturing process and to explore the ability of this technique to monitor and predict the early days of ripening.

2. Materials and methods

2.1. Dry fermented sausages manufacture

Traditional dry fermented sausages *salchichón type* were produced using a basis of pork (*Longissimus lumborum*) and 20% of fat (w/w). The following non-meat ingredients were also added to this basis to get the final features of the product: sodium chloride (2.5% w/w), lactose (1.0% w/w), dextrose (0.8% w/w), dextrine (1.8% w/w), sodium glutamate (0.25% w/w), sodium ascorbate (0.046% w/w), NO_2 (0.0065% w/w), NO_3 (0.0085% w/w) and black pepper (0.14% w/w). In order to study the spontaneous fermentation process, no starter cultures were added to the meat batter. A fine chopping of meat and fat was carried out at cooled temperature, then the remaining ingredients were added and the final mixture was refrigerated for 24 h to facilitate interactions between its components. Fifty mm collagen casings were stuffed with 400–500 g emulsion, which resulted in sausages with a length of 20 cm. The units were fermented and dried for a period of 14 days, under controlled conditions of temperature, relative humidity and air flow. During the first 24 h sausages were kept at 22 °C and relative humidity of 95%. Within 48 h, storage conditions were reduced gradually to 89% and 17 °C. Hereafter, units were stored at 12 °C for the rest of the manufacturing time. Air flow was established in 1 m/s. Eighteen independent *salchichón* units were manufactured in total and sampling was carried out at different ripening times: 0 (24 h after batter meat preparation and just before stuffing into casings), 2, 4, 7, 11 and 14 days. Three different units of each ripening time were collected for the analysis.

2.2. Physicochemical analyses

In order to monitor the process and ensure the proper ripening development and behavior of the manufactured products, conventional physicochemical analyses were performed. Water activity (a_w) measurements were carried out at 25 °C using a Decagon CX1 hygrometer (Decagon Devices Inc., Pullman, WA, USA). The dry matter (DM) content of the samples was determined by drying the sample at 110 °C to constant weight and the results were expressed as a percentage (AOAC, 2006). The pH was determined in a homogenate of the sample with distilled water (1:10) (w/v), using a Crison Digit-501 pH meter (Crison Instruments LTD, Barcelona, Spain). Physicochemical parameters were determined in triplicate. The obtained results are provided in Supplementary material, Fig. S1.

2.3. ^{1}H HR-MAS NMR measurements

Three representative sections originating from three different sausage units for each ripening time were analyzed. Samples of lean meat were obtained from the inner part of the *salchichón* units. A D_2O solution (15 μL) containing trimethylsilyl 3-propionic acid sodium salt (TSP, 0.1 mM) was added to the 30 μL disposable Kel-F HR-MAS inserts (Bruker Biospin, Rheinstetten, Germany) together with the meat tissue sample (10 ± 2 mg). TSP was used as internal chemical shift reference (0.00 ppm). The insert was placed into a 4 mm zirconium rotor. Proton NMR spectra were recorded on a Bruker Avance 600 spectrometer, operating at a ^{1}H frequency of 600.13 MHz, equipped with a HR-MAS probe (Bruker BioSpin, Rheinstetten, Germany). A water-suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin echo sequence [90°-(t-180°-t)$_{n-acquisition}$] (Mannina, Sobolev, & Viel, 2012) was used to eliminate signals from lipids and macromolecules with an effective echo time of 300 ms. This effective echo time was previously optimized in our laboratory (Lamichhane et al., 2015). Proton spectra were acquired at 4 °C with a spin rate of 5 kHz. Two hundred and fifty-six scans over a spectral region of 10 kHz were collected into 32k data points with a relaxation delay of 5 s. The free induction decay (FIDs) obtained were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz before Fourier transformation. The spectra were phased, baseline-corrected and referenced to TSP using the Bruker Topspin software (version 2.1).

2.4. Statistical analysis

One-way ANOVA was performed, using Statgraphics, version 5.1., to determine the effects of ripening time on physicochemical parameters. Bartlett's test was used to examine the null hypothesis that the standard deviations of data were the same. Duncan's test for multiple mean comparisons procedure was used to determine which means are significantly different from others (at 95% of confidence level). The analyses were conducted across six different ripening times and data are presented as mean values and their standard deviation. A simple regression model was also obtained to predict pH values from carnosine chemical shift data.

Multivariate statistical analysis was conducted on NMR data. A total of 18 *salchichón* spectra were subjected to PCA using AMIX software (version 3.9.11, Bruker BioSpin). Prior to the multivariate analyses, each individual spectrum was data reduced over the 9.03–0.76 ppm range by dividing it into spectral regions (buckets) of variable width. These variable widths were chosen to account for the chemical shift variations due to working with non-buffered samples. Regions with spectral artifacts (i.e. unsuppressed water region) were excluded from the bucketing. Therefore, a total of 161 buckets were defined and the integral of each bucket was calculated. The variable buckets used for the multivariate analysis, including their midpoint and their width are provided in Supplementary material, Table S1. In order to account for the variable concentration of each reconstituted sample, its bucket intensities were normalized to the total spectral intensity over the whole spectrum. For the unsupervised PCA analysis a 161 × 18 matrix was constructed, its rows representing the different *salchichón* samples (cases) and its columns the integrated buckets (variables). These columns were scaled to unit variance prior to PCA calculations, resulting in all the buckets becoming equally important in the final analysis; in short, case clustering, if evidenced, should be explained by differences in the metabolite profile of the cases rather than in their metabolite absolute levels. The number of principal components (PCs) employed for PCA was established as the minimum required to explain 95% of the total variance. Significance analysis of variables (buckets) was based on the procedure of Goodpaster, Romick-Rosendale, & Kennedy, 2010 using a confidence level of 95%.