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Pre-selection of potential molecular markers for the geographic origin of dried beef by HR-MAS NMR spectroscopy

L. Shintu [a,1], S. Caldarelli [a,*], B.M. Franke [b,c,d]

[a] JE 2421 TRACES Aix Marseille Universitè, Site de Saint Jérôme, F-13013, France
[b] Institute of Animal Science, Animal Nutrition, ETH Zurich, CH-8092 Zurich, Switzerland
[c] Agroscope Liebefeld-Posieux Research Station, CH-3003 Berne-Liebefeld, Switzerland
[d] Swiss Federal Office of Public Health, CH-3003 Berne-Liebefeld, Switzerland

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Abstract

The complexity of meat composition and the requirement of high analytical rates to prevent frauds and, in general, to assess food quality are stimuli to find analytical methods able to detect appropriate indicators of a given quality. We present here an evaluation of 1D ^{1}H HR-MAS NMR spectroscopy for the selection of potential molecular markers of one specific quality, geographic origin. Dried beef samples of certified origin were tested, and preliminary results on a limited sample set confirmed the good aptitude of this method for rapid food analysis, demonstrated earlier on dairy products. Fat content as well as specific metabolites, probably linked to feeding system, are shown to be good candidates for markers of origin. These results are in agreement with recent studies on the relative impact of genetic and dietary conditions on the metabolic profile of animal specimens. ^{1}H HR-MAS is thus an interesting tool for pre-screening potential markers, that can complement standard chemical and biochemical analysis.

Keywords: HR-MAS NMR; Geographic origin; Authenticity; Beef

1. Introduction

As shown by several studies, the origin of food, especially meat and meat products is a very important issue for consumers (McKean, 2001; Swiss Federal Office of Public Health, 2003) and a correct labeling of the country of production is mandatory by law in the European Union and Switzerland. Furthermore, some producers of foods go beyond the simple requirement of mentioning the geographical origin and claim a link with a local origin and even with local traditional manufacturing processes. This is the case of some regional specialties bearing either a protected designation of origin (PDO) such as prosciutto di Parma, or a protected geographical indication (PGI) such as Bresaola della Valtellina. Typical Swiss examples of PGI are the dried beef products Bündnerfleisch and viande séchée du Valais. The PGI label means that a geographical link with the region of origin "must occur in at least one of the stages of production, processing or preparation" (European Commission, 2006). In the case of the viande sÈchÈe du Valais, the product has to be manufactured in Valais using a local traditional process and Swiss raw meat exclusively (Anonymous, 2002). In the case of B¸ndnerfleisch, the production process is traditional to this region and the drying must take place in the canton of Grisons at least 800 m above sea level, but the origin of the raw meat is not prescribed (Anonymous, 2000). For these kinds of products, it is useful to have efficient ways to identify the origin of the raw materials or final products in order to prevent fraud. As correctly stated by the European

* Corresponding author. Tel.: +33 4912128295; fax: +33 491289147.
E-mail address: s.caldarelli@univ-cezanne.fr (S. Caldarelli).
[1] Current address: Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, United Kingdom.

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Commission "when a product acquires a reputation extending beyond national borders it can find itself in competition with products which pass themselves off as the genuine article and take the same name. This unfair competition not only discourages producers but also misleads consumers" (European Commission, 2006).

To achieve this goal, food authorities can rely on paper traceability, and/or physical and chemical analyses. The first has recently been given mandatory status by the EU regulation 178/2002, the so-called food law. Accordingly, food should be traceable with respect to all steps of production, transformation and distribution (Commission of the European Communities, 2000).

Different studies of chemical or physical analysis have been done to trace meat back to its origin (reviewed by Franke, Gremaud, Hadorn, & Kreuzer, 2005). Among these methods, different kinds of NMR procedures were carried out to analyse various agricultural products. ^{1}H High resolution Magic Angel Spinning Nuclear Magnetic Resonance (HR-MAS NMR) was used for this purpose with meat (Brescia, Jambrenghi, et al., 2002; Renou et al., 2004), wheat (Brescia, Di Martino, et al., 2002), cheese (Shintu & Caldarelli, 2005; Shintu & Caldarelli, 2006; Shintu, Ziarelli, & Caldarelli, 2004), olive oil (Mannina, Patumi, Proietti, Bassi, & Segre, 2001) and instant coffee (Charlton, Farrington, & Brereton, 2002).

NMR is a non-destructive technique, which requires little sample preparation and is rapid. Therefore it can be used to build databases (Guillou, 2003), in a situation where many samples need to be analysed in short time. Brescia, Jambrenghi, et al. (2002) believed there was potential for determining the geographic origin of foods using ^{1}H HR-MAS NMR. The potential of NMR for determining the geographic origin of meat was confirmed for beef through characterization of the diet by analyzing fatty acid contents (Renou et al., 2004).

In this study a selection of meat samples of certified origin was analyzed to test the capability of using 1D ^{1}H HR-MAS NMR for potential molecular markers of the geographic origin of dried beef. As the type of samples under scrutiny (dried beef) had not previously been analyzed by common techniques for complete molecular profiling, the results of the NMR analysis should be taken as a guide to further quantitative investigations targeted at specific molecules.

2. Materials and methods

2.1. Samples

Twenty-three raw dried-meat (21 dried beef and two Bresaola) sample from five countries (Australia (AU): $n = 4$; Brazil (BR): $n = 7$; Canada (CA): $n = 2$; Switzerland (CH): $n = 8$ and USA (US): $n = 2$) were studied. All the samples produced in Switzerland (8 from Swiss beef and 5 from Brazilian beef) were obtained from local producers. Australian, Canadian and USA samples were from the producers. Two samples were produced in Austria from Brazilian beef.

From each sample, about 30 mg of meat were packed into a 4 mm magic angle spinning (MAS) rotor of 50 μl total volume. To prevent a pH-dependent variation of some signals, about 30 μl of 1 M pH 7 phosphate buffer (Shintu et al., 2004; Shintu & Caldarelli, 2005, 2006) were used. This mixture was homogenized directly in the rotor with a spatula.

2.2. NMR spectroscopy

All spectra were acquired on a Bruker AVANCE 400 MHz spectrometer (Rheinstetten, Germany) equipped with a 4 mm HR-MAS ^{1}H/^{13}C probe head (Bruker, Rheinstetten, Germany).

A typical proton HR-MAS NMR spectrum consisted of 512 scans using 16 K data points over a 4800 Hz spectral width with a 6.9 μs 90_ pulse (Fig. 1a). A recycle time of 2 s was used. All 1D ^{1}H HR-MAS spectra were performed with presaturation of the water peak at 4.86 ppm (Gueron, Plateau, & Decorps, 1991). The samples were spun at 4000 Hz. Each spectrum was phased and baseline corrected using a polynomial function. For each sample, three ^{1}H HR-MAS NMR spectra from three independently prepared rotors were recorded. The assignments of the NMR signals were carried out based on the study of ^{1}H–^{1}H TOCSY (Fig. 1b), ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC HR-MAS NMR spectra and the literature (Al-Jowder et al., 2001; Brescia, Jambrenghi, et al., 2002; Fan, 1996; Govindaraju, Young, & Maudsley, 2000; Sharma, Atri, Sharma, Sarkar, & Jagannathan, 2003).

2.3. Data processing of 1D ^{1}H HR-MAS NMR spectra for statistical analysis

The frequency regions of each spectrum between 0.8–5.80 ppm, 6.80–7.45 ppm and 8.20–8.60 ppm, which correspond to regions with observable signals, were split into "buckets" of 0.05 ppm width using MectReC software (Mestrelab Research, La Coruña, Spain). Each bucket was integrated and normalized to the whole bucket integration. The 69 spectra × 111 buckets data matrix was imported into STATISTICA v.7.1 software (Statsoft Inc., Tulsa, OK, USA) for statistical analyses.

2.4. Chemometrics

2.4.1. One-way ANOVA

One-way ANOVA was used to reduce the number of initial variables by selecting the ones statistically significant at $p < 0.01$ and the ones being most discriminant by computing the F factor, which is the ratio of the between-group variance over the within group variance. The larger this ratio, the larger is the discriminant power of the corresponding variable. We chose to keep the variables having

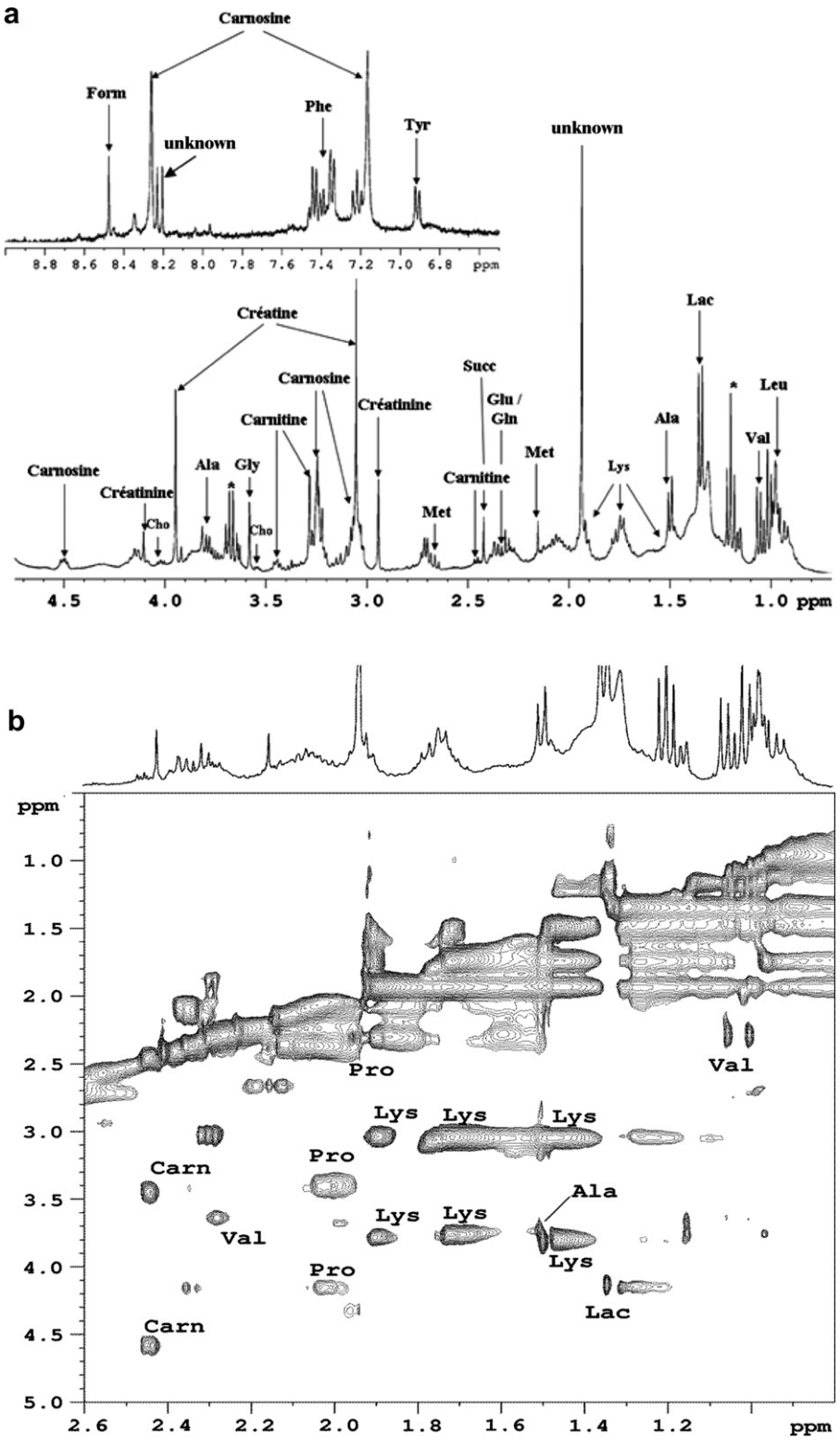

Fig. 1. (a) Portion of a 1H HRMAS spectrum of a Swiss dried meat sample, with the labeling of some signals (see text for the assignment procedure). Stars indicate residual ethanol from rotor washing. (b) Portion of the high-field region of a TOCSY spectrum of a dried meat sample, with some assignments.

a *F* factor greater than 10, which is the limit yielding a good compromise between the number of variables and the power of their discrimination in principal component analysis (PCA). The selection of the variables was carried out on the basis of the discrimination of the five groups (CH, BR, CA, US and AU).

2.4.2. Principal component analysis

This unsupervised method allows obtaining linear combinations of the selected variables which point along the directions of maximum variability. A first PCA on the whole (69×111) data matrix was performed to find possible outliers. A second PCA was performed on the remaining objects and some variables were selected by ANOVA to reduce the dimensionality of multivariate data and to permit a first overview of the data. In this work a correlation matrix as the basis for PCA was used.

2.4.3. Stepwise discriminant analysis (DA)

This is a supervised method in which *a priori* the group membership of the observations is known. The discriminant power of the variables is evaluated using Wilks' lambda, F and p-level parameters. The Wilks' lambda is computed as the ratio of the determinant of within-group variance–covariance matrix to the determinant of the total variance–covariance matrix. Its value ranges from 1 (no discriminant power) to 0 (high discriminant power). To select these variables, we used the forward stepwise algorithm which retains the variables with the lowest Wilks' lambda. Taking into account the small number of samples, we chose to test the robustness of the statistical model by the "leave-one-out" cross-validation method. All replicates of one sample were considered as indissociable and the three spectra of each sample were removed and reclassified together. The prediction ability of our model is proportional to the percentage of correct reclassified spectra.

Then, a graphical representation was performed using canonical variates which are linear combinations of the selected initial variables so that the ratio of the variance between groups to the variance intra-group is maximized. It is possible to visualize, on a plane formed by two canonical variates, the difference between the groups. Moreover the standardized coefficients within the canonical variates show the contributions of each initial variable to the discrimination. The higher the coefficient, on a given canonical axis, the more discriminating is the variable (Mark & Tunnell, 2006).

3. Results

3.1. Physico-chemical meat quality traits

PCA showed that one Swiss sample was an outlier with respect to all the others samples. Consequently, one-way ANOVA was performed for the 22 remaining samples and it allowed selection of the 45 statistically most significant or most discriminant variables. A PCA based on the correlation matrix of these variables showed that the first four principal components (PC) were significant (i.e., with an eigenvalue greater than 1) and explained 91% of the total variance. The sample projections on the first two principal component axes yielded the best discrimination of the different groups as shown in Fig. 2.

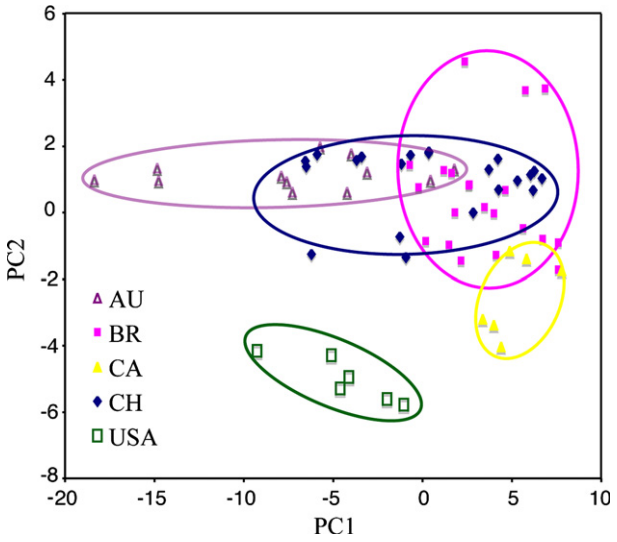

Fig. 2. Result of the principal component analysis. Projections of 66 observations of the five groups on the PC1 vs. PC2 plane.

These two PC axes represented 84.57% of the total variance, but only the American (USA) and Canadian (CA) sample groups were well separated with respect to the others, being the only samples with negative scores on the PC2 axis. Conversely, Swiss dried-meat samples (CH) were very dispersed in this representation and so it was difficult to define precisely their correlation with the first two PCs. The other sets had intermediate behaviour. American (USA) and Australian (AU) samples had mainly negative scores on the PC1 axis, whereas almost all Brazilian (BR) and Canadian (CA) groups had positive ones on this axis. AU samples lay mostly on the positive side of the PC2 axis.

The study of the PC1 and PC2 loadings (Table 1) enabled us to determine the compounds with the highest impact on the variance.

Table 1 showed that the main contributors to the first principal component, and thus to the variance, were (unsaturated and saturated) fatty acids and glycerol, negatively correlated to PC1 (from −0.86 to −0.98), while some amino acids (alanine, glutamic acid, lysine, phenylalanine, and proline), carnosine, carnitine and succinate and some unknown compounds had a positive correlation with this same principal component. It is noteworthy that the signal of threonine appeared with inconsistent values into different regions, and so was not included in this first conservative analysis. In detail, American and Australian dried meat samples were more concentrated in fat than the other samples and Canadian and Brazilian samples were more concentrated in amino acids, carnosine, carnitine, and succinate. The case of methionine is ambiguous because it participated, together with other compounds, in two spectral regions with opposite contributions to the PC1, so nothing could be inferred about the relevance of this amino acid as a marker. As far as the PC2 axis was concerned, the most significant compounds (methionine, valine and/or threonine and an unknown compound A) had negative correlation

Table 1
Significant spectral portions and associated compounds with the highest correlation coefficients (loadings) between initial variables and principal components (most relevant ones in italics)

Bucket (in ppm)	Assignment	PC1 loading	PC2 loading
5.30–5.35	Unsaturated fatty acids (UFA)	*−0.96*	0.00
4.25–4.30	Glycerol (Triglyceride) + threonine	*−0.86*	−0.30
3.85–3.90	Methionine	0.55	*−0.77*
3.65–3.70	Unknown compound A	0.61	*−0.70*
3.60–3.65	Valine + threonine	0.68	*−0.66*
3.35–3.40	Proline	*0.97*	0.12
3.30–3.35	Phenylalanine	*0.97*	0.05
3.20–3.25	Carnosine + tyrosine	*0.91*	0.12
3.15–3.20	Phenylalanine	*0.86*	0.16
3.10–3.15	Phenylalanine	*0.95*	0.08
2.85–2.90	Unknown compound B	*0.92*	0.25
2.70–2.75	Carnosine	*0.90*	0.16
2.65–2.70	Methionine + carnosine	*0.94*	0.24
2.60–2.65	Methionine	*0.92*	0.31
2.50–2.55	Unknown compound C	*0.95*	0.22
2.45–2.50	Carnitine	*0.92*	0.26
2.40–2.45	Carnitine + succinate + unknown compound B	*0.89*	0.29
2.35–2.40	Glutamic acid or glutamine+proline	*0.86*	−0.02
2.25–2.30	Fatty acids + valine	*−0.88*	0.27
2.20–2.25	Fatty acids + methionine	*−0.96*	0.08
2.15–2.20	Methionine	*0.91*	0.21
2.10–2.15	Glutamic acid + proline + methionine	*0.90*	0.03
2.00–2.05	Proline + UFA	*−0.97*	0.09
1.85–1.90	Lysine	*0.91*	0.20
1.80–1.85	Lysine	*0.93*	0.27
1.70–1.75	Lysine	*0.92*	0.06
1.65–1.70	Lysine	*0.94*	0.25
1.55–1.60	Fatty acids	*−0.96*	0.07
1.50–1.55	Alanine + lysine	*0.87*	0.21
1.45–1.50	Alanine + lysine	*0.85*	0.12
1.25–1.30	Fatty acids	*−0.98*	−0.03
0.85–0.90	Fatty acids	*−0.96*	−0.03

coefficients (from −0.66 to −0.77), and so these molecules were good candidates for discriminating American and Canadian meat samples, where they should be found in higher relative concentrations. Group discrimination through PCA was clearly incomplete, and so a stepwise discriminant analysis on the 45 previously selected variables, which were centered, was carried out. The first step in this procedure was selection of the most discriminant variables. The nine variables with the lowest Wilks' Lambda were kept (Table 2).

Table 2
Wilks' Lambda for the 9 most discriminant variables

Bucket (in ppm)	Compound	Wilks' lambda
3.20–3.25	Carnosine + tyrosine	0.002494
2.70–2.75	Carnosine	0.002518
2.35–2.40	Glutamine (or glutamic acid) + proline	0.002602
2.40–2.45	Carnitine + succinate + unknown compound B	0.003686
2.45–2.50	Carnitine	0.004396
3.80–3.85	Alanine	0.004696
3.30–3.35	Phenylalanine	0.005119
3.65–3.70	Unknown compound A	0.005494
3.40–3.45	Proline + carnitine	0.005653

Incidentally, a comparison of Tables 1 and 2 stresses the relevance of the unknown compound A in labeling samples of North-American origin.

The squared Mahalanobis distances reported in Table 3 show that the nearest groups (20.50) were the AU and BR groups whereas the US and AU groups were the farthest away (202.03).

The nine selected variables allowed correct classification of 99.3% of the studied samples. A "leave-one-out" cross-validation gave a prediction ability of the built statistical model of 80.3%.

Following this positive result, a canonical analysis was carried out on the dataset matrix (66 spectra × 9 buckets), to further explore the differences in the dried meat's chemical composition according to its geographical origins. The first two canonical variates (explaining 88.5% of the total variance) led to a good discrimination of all groups. The projections of the spectra on the CV1 vs. CV2 plane are shown in Fig. 3.

The standardized coefficients of correlation used to build the statistical model are reported in Table 4.

Table 3
Squared Mahalanobis distances for the dried beef meat samples

	CH	BR	CA	US	AU
CH	0.00	30.78	56.669	119.25	56.97
BR	30.78	0.00	56.264	174.70	20.50
CA	56.67	56.26	0.000	52.61	93.02
US	119.25	174.70	52.609	0.00	202.03
AU	56.97	20.50	93.022	202.03	0.00

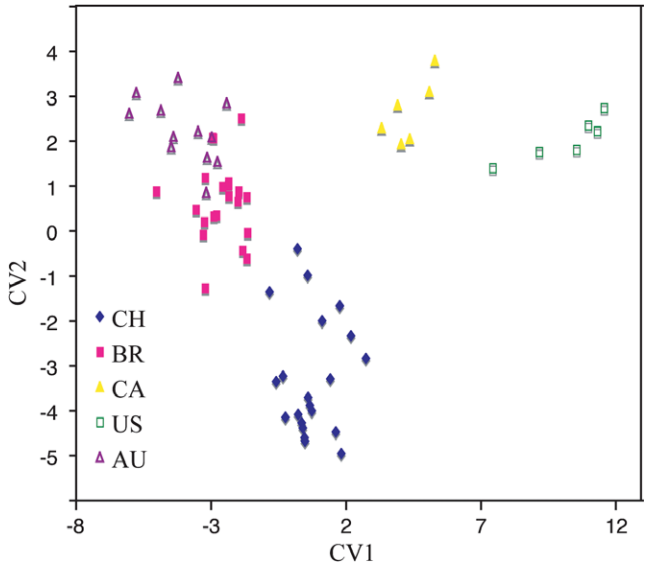

Fig. 3. Main canonical variates representation of the group classification. Projections of the 66 spectra on the CV1 vs. CV2 plane.

Table 4
Standardized coefficients of correlation used to built the statistical model (most relevant ones in italics)

Bucket (in ppm)	Compound	Loadings of CV1	Loadings of CV2
3.20–3.25	Carnosine + tyrosine	−1.20	*3.38*
2.70–2.75	Carnosine	−1.35	−2.99
2.35–2.40	Glutamine (or glutamic acid) + proline	0.42	0.53
2.40–2.45	Carnitine + succinate + unknown compound B	*3.33*	−1.41
2.45–2.50	Carnitine	*−4.15*	2.94
3.80–3.85	Alanine + methionine	0.93	*3.05*
3.30–3.35	Phenylalanine	−0.60	*−3.83*
3.65–3.70	Unknown compound A	1.69	−0.20
3.40–3.45	Proline + carnitine	2.28	−1.58

The samples from USA, Switzerland and Canada had positive scores on the CV1 axis, whereas Brazilian and Australian samples were located on the negative side. This axis was mainly correlated with the buckets at 2.40–2.45 ppm, with a positive coefficient of 3.33, and with the region at 2.45 and 2.50 ppm, with a negative coefficient of −4.36. These were neighbouring regions of the spectrum, and the carnitine signal was spread over both buckets, being the only compound in the low-field region and overlapping with two more signals in the high-field one. Since the buckets had opposite sign contributions to the CV1, the discriminatory effect of carnitine should be inferred by the bucket where this molecule appeared alone. US, CH and CA groups were thus expected to have lower relative amounts of carnitine than the two other groups and a higher relative concentration of succinate and/or unknown compound B, whichever determined the statistical change of sign with respect to the neighbouring bucket.

The second canonical variates allowed separation of the Swiss samples (with negative scores) from the other groups (with positive scores). The spectral domains at 3.30–3.35 ppm (phenylalanine signals), 3.20–3.25 ppm (carnosine and tyrosine signals) and at 3.80–3.85 ppm (alanine and methionine signals) contributed mainly to the discrimination of the Swiss samples. This suggests the dried meat specimens of Swiss origin were characterized by higher relative concentrations of phenylalanine (coefficient of −3.85) and lower ones of alanine and/or methionine, carnosine and/or tyrosine (coefficients of 3.05 and 3.38, respectively) than the other analyzed meats.

4. Discussion

The overall chemometric analysis, taken at the most conservative level, suggests further investigation of a few molecules. The content of fatty acids was observed to be higher in American and Australian sample (although for this latter large variations of this variable were observed), the concentration of several small metabolites (proline, phenylalanine, glutamic acid or glutamine, to name a few), were relatively more concentrated in the Canadian and Brazilian samples. The Swiss sample showed a discriminatorily high content of phenylalanine, and a relatively low amount of alanine and/or methionine. A lower content of carnitine and a higher content of succinate and/or an unknown compound (both resonating at 2.40–2.45 ppm) discriminated between North American samples and Australian and Brazilian ones.

Possible sources of such variations in the molecular constituents of the beef samples investigated can be found the literature. The content and profile of fatty acids in beef can be influenced by feeding regime (Felton & Kerley, 2004; French et al., 2000, French, O'Riordan, Monahan, Caffrey, & Moloney, 2003; Ponnampalam, Mann, & Sinclair, 2006; Scheeder et al., 2001) as well as castration (luteinizing hormone-releasing hormone fusion protein vaccine, surgerical castration) (Ruiz et al., 2005) and ambient temperature makes a contribution to the fatty acid composition (Kelly, Tume, Newman, & Thompson, 2001). For example cattle grown in tropical environment were fatter and showed higher percentages of saturated fats than cattle from a temperate environment (Kelly et al., 2001). This is not confirmed by our data where US and AU samples were more concentrated in fat, than Brazilian ones, but reasons for it were not clear. Breed can also have an influence (do Prado, Moreira, Matsushita, & de Souza, 2003; Itoh, Johnson, Cosgrove, Muir, & Purchas, 1999; Moreira, de Souza, Matsushita, do Prado, & do Nascimento, 2003) but differences are probably small and likely to have been caused by different breed-specific feeding intensities.

The content of amino acids in meat is normally genetically fixed. The content of both essential (lysine, threonine, valine, isoleucine, and phenylalanine) and non-essential amino acids (glutamic acid, asparagines, proline, serine, and alanine) was significantly different in beef from different breeds (Mont-Beliard, Mainc-Anjou, Hereford, Aberdeen-Angus) (Subrt, Kracmar, & Divis, 2002). Production system influenced the content of carnosine in beef, but for reasons that were not clear (Purchas & Busboom, 2005). Different beef muscles had different carnosine contents (Purchas, Rutherfurd, Pearce, Vather, & Wilkinson, 2004; Purchas & Busboom, 2005). However, this would not affect the determination of the geographic origin of dried beef as this product is always produced from the same muscle (*m. biceps femoris*). Lysine and byproducts of its production as well as threonine, methionine, valine and tryptophane can be supplemented in feed for various reasons (Henrique, Leme, Lanna, Alleoni, & Sampaio, 2005), but would probably not have any effect on the determination of the geographic origin as the amino acid profile of meat is not influenced by the amino acid profile of the feed, (Roth, Kirchgessner, Ristic, Kreuzer, & Maurus-Kukral, 1990).

Succinate is used to produce pharmaceutical products and can be used as a substitute for salt in foodstuffs. It is also synthesized in the citric acid cycle. Carnitine is involved in lipid metabolism and is contained in membranes of the mitochondria of mammals. Therefore both

substances were found in the meat samples, but why their content varied in samples of different origins is not clear.

Although dried beef is a processed meat product, as it is just made by curing (addition of salt and herbs) followed by several cycles of drying and pressing, influences on fatty and amino acid profile are unlikely.

The potential of HR-MAS NMR has been demonstrated, under similar conditions to those used in this work, for cheeses for identification of molecular markers associated with qualities such as age and geographic origin (Shintu et al., 2004; Shintu et al., 2005, 2006). In these previous studies, HR-MAS NMR indicated as markers the same molecules as found in a larger data set. Thus, the present HR-MAS analysis suggests good candidates for markers of the origin of the raw meat in dried beef.

5. Conclusions

The relatively small size of the data set analysed in this preliminary study allows only a determination of the discriminating power of ^{1}H HRMAS NMR analysis. Notwithstanding the small number of samples and their high intrinsic heterogeneity, the percentage of correct reclassification is encouraging and the method should be tested on larger sets of samples, taken all year round.

The compounds identified as being most discriminating for dried beef made from meat from different geographic areas were probably either metabolites specific for a given breed or those caused by characteristic feeding or housing conditions. However, any difference in amino acid contents in different muscles is not relevant to this study, because dried beef is always made from the *M. biceps femoris*. Local feeding practices, different animal husbandry conditions and differing breeds can influence the parameters (secondary indicators) and may change over time thus making the determination for whole geographic regions difficult. On the other hand, parameters steaming from different climatic conditions are less easy to influence, and can therefore be considered as a better choice to prove the geographic origin (primary indicator).

As all of the above factors, some of which cannot be clarified exactly, might cause variations in the amino and fatty acid contents, the real potential of the discriminating features suggested needs to be further investigated, for any given property, and in conjunction with other analysis. The advantage of HR-MAS is the speed of analysis due to the limited pre-treatment of the sample and to the sensitivity of the method.

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