Implementation of multivariate techniques for the selection of volatile compounds as indicators of sensory quality of raw beef

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Abstract This study was performed in order to select volatile compounds to predict the off-odour and overall assessment of raw beef's freshness Maronesa breed, using multivariate analysis. *M. longissimus dorsi* packed in vacuum and MAP (70 % O₂/20 % CO₂/10 % N₂) stored at 4°C were examined for off-odour perception as well as the overall assessment of freshness at 10 and 21 days post mortem. The results achieved in this study demonstrated that the selected volatile compounds could be considered as volatile indicators of beef spoilage, enclosing information for discrimination of Maronesa beef samples in sensory classes of odour corresponding to unspoiled and spoiled levels. Fifty-four volatile compounds were detected. A significant increase of aldehydes, ketones and

alcohols were observed during storage in MAP. 2 and 3-methylbutanal, 2 and 3-methylbutanol, 1-pentanol, 1-hexanol, 2,3-octanedione, 3,5-octanedione, octanal and nonanal were suggested as indicators of beef spoilage. 3-methylpentane was considered as a marker in the first stages of spoilage in beef, decreasing during storage. Data were examined using PCR and PLSR models for different optimal subsets of volatile compounds. The simplicity and usefulness of the technique in using 0/1 data in preserving high levels of accuracy was also prevalent. The powerful analytical methodologies for reducing variables and the choice of optimal subsets could be advantageous in both basic research and the routine quality control of chilled beef.

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Introduction

The evaluation of the degree of meat spoilage is usually made either subjectively, based on sensory assessment or by microbiological analyses (Dainty 1996; Ellis and Goodacre 2001; Nychas et al. 2008). Sensory analysis of fresh meat employs the human senses to provide information about appearance and odour which are the most important quality attributes that consumers use to judge meat quality and its acceptability (Han and Lee 2011; Soncin et al. 2007). Thus, the detection of off-flavour can be an ultimate quality factor determining consumer acceptance (Han and Lee 2011). In general, fresh raw meat has a very little odour (Moon et al. 2006), which is described as “bloody” as well as sweet. During storage the meat odour suffers changes as it degrades and could manifest in different forms, namely sweet, buttery, putrid, sour and rancid odours (Dainty 1996). Many studies have examined the relationships between off-flavour development and products formed during

inappropriate meat storage (Han and Lee 2011). The formation of odour and flavour are related to endogenous enzyme activities, microbial activities reported in *Pseudomonas* spp., lactic acid bacteria (LAB) and *Enterobacteriaceae* levels (Al-Bachir and Mehio 2001; Insausti et al. 2001; Nattress and Jeremiah 2000) and to chemical reactions between the natural components (Soncin et al. 2007). Lipid oxidation is a main factor which reduces beef quality (Campo et al. 2006). Several volatile compounds that rise during the storage have repercussions on the odour of the products, such as aldehydes, ketones (Insausti et al. 2002; Jackson et al. 1992; Jayasingh et al. 2002) and alcohols. Dainty et al. (1985) refer to various volatile compounds that are present from the beginning of decomposition in the first phases of storage, regardless of its connotation with typical odours of spoiled meat. Among those compounds, diacetyl (2,3-butanedione) and acetoin (3-hydroxy-2-butanone) were detected (Dainty 1996) and were suggested as useful indicators of quality/ microbial spoilage of pork stored in an atmosphere enriched with O₂ and CO₂ (Ordóñez et al. 1991; Pablo et al. 1989). Stutz et al. (1991) suggested that 2-propanone, 2-butanone, dimethyl sulphide and dimethyl disulphide can be indicators of beef spoilage.

It has been found that the most important source of volatile compounds is the lipid fraction of meat (Campo et al. 2006; Estévez et al. 2003; Gray et al. 1996), mainly phospholipids, which go through autoxidation phenomena, producing a large number of volatile compounds such as acids, aldehydes, ketones and alcohols (Estévez et al. 2003).

The solid-phase microextraction (SPME) is an isolation technique of the volatiles in foods based on sorption of analytes on or into a polymeric material that coats a silica fibre. This technique is gaining much interest, due to the fact that it is simple, low-cost, solvent-free, requires minimum sample pre-treatment and no specific consumables or reagents. It is a relatively fast and sensitive method (Balasubramanian and Panigrahi 2011; Brunton et al. 2000; Moon and Li-Chan 2004; Summo et al. 2010). SPME is a recognized sample preparation technique for the analysis of volatile and semi-volatile compounds in various matrices (Kataoka et al. 2000), useful in a diverse range of applications in the food industry. As far as volatile compound analysis is concerned, gas-chromatography associated to mass-spectrometry (GC-MS) is the most frequently used technique. There are several studies demonstrating the potential of GC-MS in providing information about specific compounds present in the headspace (HS) of a food sample including spoilage evaluation and shelf-life determination as well as quality assessment (Balasubramanian and Panigrahi 2011; Insausti et al. 2002). The usage of SPME-GC-MS for rapid quality control of beef is therefore of

interest. Basically, it is important to achieve a selection of volatile indicators which are characteristic of a food product at a given point in time; in this case the evaluation of the spoilage and the shelf-life determination.

Few publications are known for volatile profile in raw meat during refrigerated storage (Soncin et al. 2007) compared to the several papers concerning the volatile profile of meat products and cooked meat (Berdagué et al. 1991; Carrapiso et al. 2003; Elmore et al. 2000; García et al. 2000; Mataragas et al. 2003; Summo et al. 2010). Soncin et al. (2007) studied volatile profile of duck, pork and chicken raw meat using HS-SPME-GC-MS.

Nowadays, multivariate techniques have become a powerful tool in the evaluation of food quality. Berrueta et al. (2007) present a review of the principal techniques used in food analysis for pattern recognition and Mataragas et al. (2003) also provide a review of several multivariate data techniques useful for the modelling and prediction of meat product's spoilage. Multivariate techniques such as principal component analysis (PCA), principal component regression (PCR) and partial least square methods (PLS) are described as the main statistical tools used to estimate the interactions between the chemical parameters and sensory characteristics, particularly when there are more variables than observations and multicollinearity exists among variables. When a large number of variables are highly correlated, contributions to final results exist with similar information. The overweight of a set of highly correlated group of variables could take results to misleading interpretations. It is therefore advisable to reduce the number of variables before the implementation of the statistical techniques in order to reach correct conclusions about the overall quality of the evaluated food product (Jolliffe 2002). In this study, several statistical analysis were done with subsets of a reduced number of variables taking into account the package and storage time effects and correlations with off-odours (OFF) and overall assessment of freshness (OAF), along with optimal methods used for variable selection. To the best of our knowledge, the choice of optimal subsets of variables has not received the same amount of attention by authors and has not yet been applied to this kind of data. The dataset were presented for prediction models with 2 component predictors. Data was also transformed to absence/presence (0/1) matrix in order to simplify the problem of finding a predictive model for OFF and OAF and then compared through PCR and PLS techniques.

The purpose of the present study was the assessment of the volatile profile of beef *longissimus dorsi*, Maronesa breed, after 10 and 21 days post mortem (pm), using HS-SPME-GC-MS, in order to understand the biochemical changes that may occur during storage, with implications

in sensory meat quality. Given the complexity of dealing with high dimensional datasets, selected optimal subsets of volatile compounds were applied to predict the spoilage level of the beef samples. The simplicity and usefulness of the technique using 0/1 data while preserving high levels of accuracy, was also shown.

Material and methods

Experimental design

Sampling *M. longissimus dorsi* of bovine males of the Maronesa breed (9–11 months; 90–130 kg carcass weight) was used in this study. Maronesa is an autochthonous cattle breed from Northern Portugal, produced in a mountain system with the utilization of local agricultural resources (hay, cornflour, potatoes and grass).

Bovine males were slaughtered in an Industrial slaughterhouse in Northern Portugal and *ms. longissimus dorsi* were collected at 24 h pm, after excision from the 6th thoracic and 2nd lumbar vertebra on the left half of the carcasses ($n=8$) and then transported under refrigeration (4 °C) to the laboratory within 15 min. Muscles were sliced perpendicularly into steaks (thickness 1.5 cm; weight ± 80 g), and packed under vacuum (VP) and in modified atmosphere packaging (MAP). 32 samples were obtained, 8 for each cross level according to the two types of package (VP and MAP) and 2 days of storage time (10 and 21 pm) at 4 °C.

Packaging conditions In VP, steaks were placed individually in bags, COMBITHERM, 0.09 mm thick (WIPAK Walsrode, Hafri), having an O_2 transmission rate of $63 \text{ cm}^3 \text{ m}^{-2} \text{ d}^{-1} \text{ atm}^{-1}$ at 23 °C and 0 % RH and WVT of $1 \text{ g m}^{-2} \text{ d}^{-1}$ at 23 °C and 85 % RH. In MAP, steaks were put in bags, COMBITHERM XX, 0.115 mm thick (WIPAK Walsrode, Hafri), having an O_2 transmission rate of $1 \text{ cm}^3 \text{ m}^{-2} \text{ d}^{-1} \text{ atm}^{-1}$ at 23 °C and 0 % RH and WVT of $1 \text{ g m}^{-2} \text{ d}^{-1}$ at 23 °C and 85 % RH and completed with 70 % O_2 /20 % CO_2 /10 % N_2 in a ratio of gas/sample of approximately 3/1. The packing procedure was held in a SAMMIC V-420 EMS machine.

Sensory analysis

Sensory evaluation of steaks was performed by a trained ten-member panel (with a minimum of 6 members per session) and was carried out under the controlled light conditions in sensory booths. All the assessors were selected and trained before the final studies in accordance with ISO 8586-1 (1993) and were familiar with sensory assessment of meat. After the training period of the panel composed by three sessions of

different beef samples in which sensory descriptors were defined, assessors rated the descriptors on a structured scale extending from 1 to 7. The descriptive attributes were based on the perception of off-odour (OFF) (1=not very intense, 7=very intense) and overall assessment of freshness (OAF) (1=very spoiled, 7=very fresh). For OAF the aspects considered were odour evaluation and visual assessment.

Beef samples were overwrapped with polyethylene film and kept at 4 °C. Each evaluation was carried out once. Overall, eight sessions were scheduled throughout the experiment, its frequency being dependent of storage time.

Analysis of the volatile fraction

The evaluation of volatile compounds (VOCs) of meat samples using this technique includes the sequentially solid phase microextraction (SPME), gas chromatography (GC) and mass spectrometry (MS) techniques. The extraction of compounds was performed by analyzing the headspace by SPME, using a 75 μm thick carboxen/PDMS SPME fibre assembly (Supelco, Bellefonte, PA, USA) preconditioned in the GC injection port as indicated by the manufacturer. One gram of frozen meat was minced and placed in a 4 ml SPME vial sealed with a Teflon/silicone septum, drilled by the activated fibre injection system and exposed to the headspace of the vial. Vials (two thirds) were kept at right angles in a water bath at 37 °C for 30 min, keeping the water in agitation (adapted from Rodríguez-Carpena et al. 2012). After extraction, the fibre was transferred as quickly as possible to the gas chromatograph port (Agilent HP-6890 series II, Milan, Italy) equipped with mass-spectrometer (Agilent model HP-5973, Milan, Italy), operating at 280 °C in splitless mode. Volatiles were separated using a Supelco capillary column HP-5 (5 % phenyl methyl silicone, 50 m $\times$ 0.32 mm $\times$ 1.05 mm), under the following conditions: injection port temperature, 280 °C; helium gas carrier with a 1.4 ml/min flow rate; oven temperatures, 60 °C for 10 min then 10 °C/min to 150 °C and up to 250 °C in a gradient of 5 °C/min and final isothermal cycle for 10 min. The mass detector was set at the following conditions: detector voltage 1,756 V; interface temperature 280 °C; ionization energy 70 eV, collecting data at a rate of 1 scan s^{-1} over a range of m/z 30–300. Volatile compound's mass spectra were tentatively identified by comparing their mass spectra with those from the NIST/EPA/NIH and Wiley libraries as well as other linear retention indices (LRI) previously described (Kondjoyan and Berdagué 1996; Elmore et al. 2000; García et al. 2000; Carrapiso et al. 2003; Machiels et al. 2003; García-Esteban et al. 2004) or positively identified by comparing the mass spectra and retention time with those displayed by the standard compounds (Sigma-Aldrich, Steinheim, Germany). Solutions of alkanes (C5–C18) were submitted under the same

conditions for calculation of LRI. Results were expressed in arbitrary area units (AAU) $\times 10^6$ resulting in counting the total ion chromatogram.

Statistical analysis

For sensory evaluations, the means of the panelists' scores were calculated for each package/storage time combination. Means and standard deviations for abundances of VOCs were calculated by package and storage time. Due to the presence of many zero and asymmetry, variables were subjected to Mann-Whitney non-parametric tests using package and storage time as effects. Significance was assigned at $p < 0.05$. The Pearson correlation coefficients were calculated among volatiles and with sensory characteristics.

Univariate and multivariate analytical techniques were used in several steps for: exploration of the data, variable selection, dimensionality reduction as well as the prediction of spoilage level. The quality and accuracy of each model were evaluated by squared correlation coefficients (R^2) and root mean squared error prediction (RMSEP) values.

Statistical analysis was performed with R Cran 2.14.1. software (R Development Core Team 2011) using packages MASS (Venables and Ripley 2002), pls (Mevik et al. 2011), subselect (Cerdeira et al. 2012), lattice (Sarkar 2008) and car (Fox and Weisberg 2011).

Variables selection Duarte Silva (2002) proposed the choice of subsets as the direct application of algorithms, however he argues that there should be no reason for ignoring the knowledge of data itself in one first step of the variable selection process. In agreeing with this point of view, the relation with the predictor's variables and its significance of effects were evaluated previously. Currently, R statistical software package subselect accepts eight different criteria for measuring the quality of any given variable subset. Of those eight, three are useful in exploratory analysis or principal component analysis of a dataset.

In subselect, VOCs were chosen using optimal methods with RM criterion as described in Cadima et al. (2004) and Duarte Silva (2001, 2002). First, all multicollinearity between variables were analyzed using the correlation matrix. The significance between variables and package and storage time effects as well as the correlation with OFF and OAF were taken into account in order to select a first subset of 31 VOCs which was then used in subselect package to obtain the optimal subsets of size k (k -subset). For plotting and easier identification and manipulation of data, the VOCs were relabeled.

Modelling and prediction A Principal Component Analysis (PCA) was performed on the correlation matrix for the several subsets of original data and also for 0/1 data, and PC scores

were used as predictors in linear multiple regression models. Principal Component Regression (PCR) (Jolliffe 2002) was used to avoid high correlation between VOCs and to understand the complexity in interrelations to predict OFF and OAF. PCR was obtained from pls package in R (Mevik et al. 2011). PLSR is also another multivariate technique used as an alternative to PCR as an exploratory tool and also as a modelling technique. Both methods construct the new uncorrelated predictor variables as linear combinations of the original variables. In PCR, components are obtained concerning the variances derived from matrix of predictor variables without considering the response variable(s) at all. This method also uses the least squares regressions to maximize correlations between component predictors and the response variable(s). On the other hand, PLSR takes the response variable into account and combines correlation and variance to consider covariance, therefore often leading to models that are able to fit the response variable with fewer components. Therefore, in order to compare the results, a fixed number of retained components (two) were chosen, even though in the literature there is no reason why the PCR model should be restricted to the same number of components of PLSR models (Jolliffe 2002). Squared correlation coefficients (R^2) and the root mean squared error of prediction (RMSEP), for each subset submitted to PCR or PLSR, were used to compare the effectiveness of the two methods.

Results and discussion

Sensory analysis

The means obtained for OFF and OAF, according the package and storage time, are presented in Table 1. As expected, the lowest scores of OFF were observed in vacuum and the OAF scores were lower in MAP on day 21, corresponding to samples with a high OFF. These samples were considered spoiled by the panelists with unacceptable meat freshness of OAF (scores 1–3.5). On the other hand, samples packed under vacuum, and in MAP until days 10 were considered unspoiled with acceptable meat freshness (scores 3.5–7).

Analysis of volatile compounds

Table 2 lists the volatile compounds detected in samples by SPME-GC-MS expressed in AAU $\times 10^6$ and ordered by retention times and peak number due to its elution time in a HP-5 column. The significance of the effects (package and storage time) and the correlation between VOCs and OFF and OAF is also presented. In total, 54 VOCs were identified and 8 of them were detected as coelution of 2 compounds (2-propanone + penthane, 1-propanol + 2-

Table 1 Means±standard deviation of OFF and OAF of beef samples according to package and storage time (days pm)

Package Storage (days pm)	Vacuum		MAP	
	10	21	10	21
Off-odour (OFF)	1.87±0.61	2.27±0.90	2.30±0.59	4.80±1.41
Overall assessment of freshness (OAF)	5.59±0.31	5.22±0.62	4.51±0.87	2.30±1.52

methylpropanal, pentanal+heptane, hexanal+octane), showing the same retention times in the column.

Effects of package and storage time Fifteen compounds were significantly affected by package and eight compounds were affected by storage time. The time induced significant increases and decreases, which could be related to the oxidant or microbial enzyme activities.

In this study, the 3-methylpentane and the 1-propanol+2-methylpropanal presented significant differences according to the time of storage. The 1-propanol+2-methylpropanal showed higher amounts on day 21, while the 3-methylpentane presented higher amounts on day 10. The acetaldehyde was also detected in higher abundance on day 10. This compound results from the catabolic process of the threonine by the threonine-acetaldehyde-liase in general existent in LAB (Christensen et al. 1999). A significant increase of hexanal+octane was observed, suggesting the rise of lipid oxidation in MAP with high oxygen concentration. Octanal and nonanal presented higher levels in MAP on day 21, without showing any significant differences. Hexanal, octanal and nonanal have been considered by various authors (Brewer 2006; Ross and Smith 2006) as markers of lipid oxidation because they derive from hydroperoxide degradation (Frankel 1982). The 2 and 3-methylbutanal significantly increased during storage, but only the 2-methylbutanal presented changes according to the package, being higher in MAP on day 21 and undetected in vacuum samples. These two aldehydes can be oxidated or reduced to the corresponding acids and alcohols, respectively (Berdagué et al. 1991), and are derived from leucine and isoleucine catabolism, respectively, either through the Strecker reaction (Barbieri et al. 1992) or by microbial enzyme activities (Bailey et al. 1992). In aerobic package, *Brochothrix thermosphacta* produces acetoin, 2,3-butanediol, diacetyl, 3-methylbutanal, 2-methylpropanol and 3-methylbutanol in media with glucose, ribose (Dainty and Hibbard 1980). *Enterobacteriaceae* can produce acids, alcohols and acetoin/diacetyl, but not esters. The LAB heterofermentative (*Leuconostoc*, *Weissella*, *Carnobacterium*) produced lactate, acetate or CO₂ and ethanol from glucose (Dainty 1996; Dainty et al. 1985). The 2 and 3-methylbutanol also significantly increased during storage. These alcohols are derived from microbial activity or oxidation of the corresponding

aldehydes (Stanke 1995). Only the 2-methylbutanol presented significant changes between package, being visible in MAP but undetected in vacuum samples.

In relation to the package effect, several VOCs presented a high and significantly different amount in MAP samples, except the dimethyl sulphide, which showed higher abundance in vacuum. Dimethyl sulphide is a sulphur-containing compound that results from degradation of cysteine. Stutz et al. (1991) suggested that it is a good indicator of bovine meat spoilage. Insausti et al. (2002) also detected dimethyl sulphide in beef samples and in declined levels during storage. In general, MAP samples were characterized by significantly higher levels of hexane, 2-ethylhexene and 2-octene, hexanoic acid, alcohols like 1-pentanol, 1-hexanol, 1-penten-3-ol and 2-methylbutanol, aldehydes such as 2-methylbutanal and ketones specifically 2-pentanone, 2,3-pentanedione, 2-heptanone, 1-octen-3-one, 3-octanone, 2,3-octanedione. The VP samples were in general characterized by showing an absence or low levels of the majority of the identified volatiles in MAP, such as aldehydes, ketones and alcohols. According to Spanier et al. (1992), the storage of bovine meat under vacuum delays the development of the chemical markers of deterioration, such as hexanal and/or pentanal. Jackson et al. (1992) detected nonanal in meat samples packed in 4 conditions (vacuum, 100 % CO₂, 40 % CO₂/60 % N₂ and 80 % O₂/20 % CO₂) and Dainty et al. (1984) in the volatile profile from samples of sterile beef, inoculated with some lineages of *Pseudomonas* packed in air. Insausti et al. (2002) refers to hexanal and 2,3-octanedione, also detected in our study, as VOCs usually associated with lipid oxidation reactions and as marker compounds in monitoring the development of off-odour. Among the other identified VOCs, those derived from microbial activities (carbohydrate fermentation, amino acid catabolism, lipid β -oxidation and ethyl esters) were present at lower concentrations than those observed by other authors in similar products (Ansorena et al. 2001; Marco et al. 2008).

In this study, several identified VOCs can be imputable to microbial activities, namely ethanol, acetic acid, and 3-hydroxy-2-butanone (diacetyl). In particular, ethanol could rise from carbohydrate fermentation and from pyruvate microbial metabolism (Spaziani et al. 2009), while diacetyl might derive from the oxidation of 2,3-butanediol. Acetic acid could be originated from direct fermentation

Table 2 Compounds identified in the volatile fraction of beef by HS-SPME-GC-MS analysis and the multiple comparisons (Mann–Whitney test) performed on the analytical data (means and standard deviation, expressed as AAU $\times 10^6$) and r Pearson correlations with OFF and OAF

Peak number	Compounds (Code)	LRI ^a	Identification ^b	Package		Storage time (days pm)				Effects		r Pearson	
				Vacuum	MAP _{70/20/10}	t ₁₀	t ₂₁	t ₁₀	t ₂₁	PK	Storage time	OFF	OAF
1	Acetaldehyde (nn.Act)	<500	MS	171 (131)	218 (109)	204 (143)	185 (98)	ns	**	ns	ns	ns	ns
2	Ethanol (nn.Eth)	<500	MS	473 (457)	373 (208)	397 (217)	449 (458)	ns	ns	ns	ns	ns	ns
3	2-propanone+pentane (nn.pr_pe)	500	MS+KI	474 (260)	483 (262)	525 (253)	432 (8,260)	ns	ns	ns	ns	ns	ns
4	Dymethyl sulfite (dMS)	519	MS	66 (37)	32 (42)	57 (42)	41 (42)	**	ns	ns	0.380*	ns	ns
5	Carbon disulphide (nn.cDS)	542	MS+KI	359 (276)	352 (217)	370 (275)	341 (217)	ns	ns	ns	ns	ns	ns
6	1-Propanol+2-Methylpropanal (mPr2)	550	MS+KI	20 (45)	25 (45)	6 (24)	39 (54)	ns	**	0.360*	ns	ns	ns
7	2-Methylpentane (n.mp2)	560	MS+KI	62 (83)	97 (239)	122 (233)	36 (82)	ns	ns	ns	ns	ns	ns
8	Acetic acid (n.Ace)	572	MS	147 (366)	44 (70)	32 (44)	159 (366)	ns	ns	ns	ns	ns	ns
9	3-Methylpentane (mp3)	579	MS+KI	17 (33)	9 (17)	20 (26)	6 (24)	ns	**	ns	ns	ns	ns
10	2,3-Butanedione (bD23)	588	MS+KI+SC	171 (132)	315 (225)	204 (184)	282 (205)	ns	ns	0.474**	−0.477**	ns	ns
11	2-Butanone (n.Bu2)	595	MS+KI	57 (72)	52 (49)	54 (50)	55 (71)	ns	ns	ns	ns	ns	ns
12	Hexane (n.hX)	600	MS+KI+SC	50 (72)	80 (113)	83 (108)	47 (78)	**	ns	ns	ns	ns	ns
13	Ethyl acetate (n.EtA)	607	MS+KI	32 (32)	23 (45)	26 (41)	29 (38)	ns	ns	ns	ns	ns	ns
14	3-Methylbutanal (mB3)	647	MS+KI+SC	3 (9)	21 (36)	0 (1)	24 (36)	ns	**	0.512**	−0.424*	ns	ns
15	2-Methylbutanal (mB2)	657	MS+KI+SC	0 (0)	7 (12)	0 (0)	7 (12)	**	**	0.504**	−0.448*	ns	ns
16	Benzene (n.bZ)	660	MS+KI	0 (0)	1 (4)	0 (1)	1 (4)	ns	ns	ns	−0.368*	ns	ns
17	1-Penten-3-ol (p3ol)	676	MS+KI	4 (7)	36 (41)	14 (16)	26 (44)	**	ns	0.587**	−0.602**	ns	ns
18	2-Pentanone (pe2)	683	MS+KI+SC	1 (1)	15 (13)	7 (8)	9 (15)	**	ns	0.411*	−0.514**	ns	ns
19	2,3-Pentanodione (k.pe23)	691	MS+KI	0 (0)	42 (67)	11 (22)	31 (69)	**	ns	0.607**	−0.645**	ns	ns
20	Pentanal+Heptane (p_hp)	700	MS+KI+SC	1 (2)	19 (37)	2 (4)	18 (37)	ns	ns	0.639**	−0.624**	ns	ns
21	2-Ethyl furan (eF)	703	MS+KI	0 (2)	12 (29)	1 (2)	12 (29)	ns	ns	0.597**	−0.589**	ns	ns
22	3-Hydroxy-2-butanone (nn.hDbu)	708	MS+KI+SC	216 (232)	471 (555)	260 (311)	427 (534)	ns	ns	0.515**	−0.482**	ns	ns
23	3-Methylbutanol (mB3ol)	727	MS+KI	1 (2)	34 (64)	0 (0)	35 (64)	ns	**	0.668**	−0.645**	ns	ns
24	2-Methylbutanol (mB2ol)	734	MS+KI	0 (0)	14 (31)	0 (0)	14 (31)	**	**	0.644**	−0.641**	ns	ns
25	Piridine (n.pi)	741	MS+KI	5 (14)	0 (1)	0 (1)	5 (14)	ns	ns	ns	ns	ns	ns
26	1-Pentanol (Ptol)	770	MS	1 (2)	51 (94)	11 (20)	41 (97)	**	ns	0.577**	−0.587**	ns	ns
27	2-Ethyl-hexene (ethH)	790	MS	0 (0)	5 (6)	1 (3)	4 (6)	**	ns	0.382*	ns	ns	ns
28	2,3-Butanediol (n.bDiol)	782	MS+KI	1 (6)	1 (4)	2 (6)	1 (3)	ns	ns	ns	ns	ns	ns
29	Hexanal+octane (hxl_oc)	800	MS+KI+SC	18 (33)	200 (480)	23 (42)	196 (481)	ns	**	0.552**	−0.541**	ns	ns
30	2-octene (oct2)	809	MS+KI	0 (0)	2 (5)	2 (5)	1 (2)	**	ns	ns	ns	ns	ns
31	2-hexenal (k.2hex)	853	MS+KI+SC	0 (0)	1 (4)	0 (0)	1 (4)	ns	ns	0.451**	−0.449*	ns	ns
32	1-hexanol (hXol)	872	MS+KI	0 (0)	21 (50)	0 (0)	21 (50)	**	ns	0.650**	−0.605**	ns	ns
33	Ethyl-benzen (n.etB)	873	MS	0 (1)	2 (5)	1 (1)	1 (5)	ns	ns	ns	−0.383*	ns	ns
34	p-xylene (p_x)	879	MS+KI	2 (3)	8 (14)	4 (5)	6 (14)	ns	ns	0.472**	−0.469**	ns	ns
35	2-heptanone (hP2)	892	MS+KI+SC	0 (0)	7 (9)	3 (6)	5 (8)	**	ns	0.413*	−0.440*	ns	ns
36	Heptanal (k.hpl)	902	MS+KI+SC	0 (1)	8 (21)	1 (2)	8 (21)	ns	ns	0.516**	−0.512**	ns	ns
37	Gamma-Butyrolactone (n.g_bu)	914	MS+KI	1 (2)	1 (3)	0 (0)	2 (4)	ns	ns	ns	ns	ns	ns
38	Alpha-pinene (n.alpha_p)	945	MS+KI	1 (4)	2 (5)	0 (1)	3 (6)	ns	ns	0.369*	ns	ns	ns
39	Heptenal (k.hep)	962	MS+KI+SC	0 (0)	1 (2)	0 (0)	1 (2)	ns	ns	0.507**	−0.493**	ns	ns
40	Hexanoic acid (k.hexA)	974	MS+KI	0 (0)	10 (26)	1 (3)	9 (26)	**	ns	0.482**	−0.481**	ns	ns
41	1-Octen-3-ol (oct_o)	982	MS+KI	1 (2)	15 (22)	3 (5)	13 (24)	**	ns	0.461**	−0.420*	ns	ns
42	2,3-Octanodione (k.oc23)	984	MS+KI	2 (7)	64 (129)	8 (12)	58 (132)	**	ns	0.620**	−0.624**	ns	ns
43	3-Octanone (oc3)	989	MS+KI	0 (0)	13 (23)	2 (7)	11 (23)	**	ns	0.594**	−0.532**	ns	ns
44	2-Pentyl furan (k.pF2)	995	MS+KI	1 (3)	14 (42)	1 (3)	14 (42)	ns	ns	0.483**	−0.481**	ns	ns
45	Octanal (ocAl)	1,004	MS+KI+SC	0 (0)	4 (11)	0 (0)	4 (11)	ns	ns	0.599**	−0.548**	ns	ns
46	2-Nonanone (n.no2)	1,021	MS+KI	0 (1)	2 (4)	1 (2)	2 (4)	ns	ns	ns	ns	ns	ns
47	Delta-3-carene (n.delta_c)	1,040	MS+KI	1 (3)	1 (3)	1 (2)	1 (3)	ns	ns	ns	ns	ns	ns

Table 2 (continued)

Peak number	Compounds (Code)	LRI ^a	Identification ^b	Package		Storage time (days pm)						Effects		r Pearson	
				Vacuum	MAP _{70/20/10}	t ₁₀		t ₂₁				PK	Storage time	OFF	OAF
48	Limonene (n.lim)	1,093	MS+KI	3	(9)	1	(2)	1	(2)	3	(9)	ns	ns	ns	ns
49	3,5-Octanodione (k.oc35)	1,098	MS+KI	0	(0)	1	(4)	0	(0)	1	(4)	ns	ns	0.647**	−0.603**
50	Nonanal (k.nOal)	1,104	MS+KI+SC	1	(3)	10	(18)	2	(4)	9	(17)	ns	ns	0.613**	−0.593**

ns Non-significant

^a Linear Retention Index (LRI) calculated for HP-Innowax capillary column

^b Verified by retention index match with authentic standard compounds (SC); by Kovats index (KI) and by probability based matching with mass spectra (MS) in the Wiley Library (Hewlett Packard, Palo Alto, CA)

*, **, *** indicates significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

of amino acids, by Stickland reaction, favored by the decreased availability of sugar during storage. Diacetyl and the 2,3-butanediol isomer are derived from carbohydrate fermentation (Gottschalk 1986).

Hydroperoxides (ROOH) are often formed as primary products and may subsequently undergo breakdown, giving way to secondary products of oxidation, with lower molecular weight, including aldehydes, ketones and epoxides. Some examples of these include hexanal, propanal, heptenal. The oxidation can be initiated by light, heat, metals (iron and covers) as well as myoglobin, which contains iron and other factors (Brewer 2006). This author refers to the hexanal in bovine meat as an indicator of lipid oxidation.

Correlations between VOCs and sensory data Several VOCs had significant correlations with OFF and/or OAF. The VOCs with higher positive correlations ($r > 0.6$) with OFF are the aldehydes pentanal+heptane, heptanal+octane and nonanal, the ketones 2-pentanone, 2,3-pentanedione, 2,3-octanedione and 3,5-octanedione and the alcohols 2 and 3-methylbutanol and 1-hexanol. The 2-ethylfuran, 1-pentanol, hexanal+octane, 3-octanone and octanal presented positive correlations with OFF higher than 0.55. All of these VOCs presented a negative and significant correlation with OAF, higher than 0.5 in absolute values. On the contrary, only the dimethyl sulphide showed a positive and significant correlation with OAF ($r = 0.38$), without presenting any correlation with OFF. The spoiled samples were characterized by lower levels of methylpentane and acetaldehyde than the unspoiled samples, but higher levels of the most aldehydes, ketones and alcohols. These volatile compounds usually result from the oxidation of lipids during storage and may lead to rancid off-flavours (Byrne et al. 2002; Mottram 1998). In particular, the 3-methylbutanal and diacetyl confer a buttery and sweet odour with a very low sensorial threshold value (Stanke 1995).

The detection of furans in the raw meat indicated that this sample had suffered some degree of heating in the processes involved in the recovery of VOCs (Han and Lee 2011).

Correlations between VOCs Due to the difficulty in reading a correlation matrix 50 by 50, a grading colour plot of the absolute values of Pearson correlation matrix was presented in Fig. 1. The absolute correlation values goes from 1.0 (perfect correlation) plotted in black, graded until white, meaning no linear correlation (0.0). The diagonal of 1's runs from the lower left to the upper right and axis labels are shown two by two, 25 labels on/off by row and 25 labels off/on by column. As expected, the consistent shade of dark gray throughout the correlation plot suggests the presence of strong correlations among VOCs identified in beef samples, plotted near black.

Positive correlations between the aldehydes, ketones and alcohols were observed. mB2 and mB3 as well as the mB2ol and mB3ol had significant correlations ($r > 0.9$) with each other, associated to the regular occurrence in the same sample, resulting from interdependent origin. The same levels of correlations could be observed between p_hp and n.hex, k.hex2, hxl_oc, k.hpl, k.hep, k.nOal, k.pe23, k.oc23, k.oc35, p3ol, pTol, hXol, eF and k.pF2. p_hp also had significant correlations ($r > 0.8$) with ocAl and n.bZ. k.pe23 showed highly significant correlations ($r > 0.9$) with the hxl_oc, k.hpl, and k.nOal, k.oc23 and pTol and also ($r > 0.8$) with k.pF2, 1-p3ol and k.oc35. These aldehydes, ketones and alcohols presented similar positive and significant correlations between each other, showing their presence associated to lipid oxidation in beef samples. hXol also presented significant correlations ($r > 0.9$) with p_hp, hxl_oc, k.hpl, and k.oc23. Information presented in Table 2 and Fig. 1 allowed us to focus on the choice of VOCs for the final subsets.

Multivariate analysis

Variable selection and subsets Primarily the full set of variables was reduced to 31 descriptors by the selection of VOCs according to package and/or storage time effects and significant correlation coefficients with OFF or/and OAF. Within the 31 VOCs some were relabeled with k. due to the

Fig. 1 Correlation matrix (absolute values) of two by two volatile compounds identified in beef samples by HS-SPME-GC-MS

presence of a higher correlation with other chosen VOCs. The VOCs not chosen, presented in almost all samples (31 and 32,) were labeled with the prefix nn, and the ones with no significant effect and/or no correlation with OFF or OAF were labeled with n. Five subsets were chosen for this analysis, the referenced 31-subset and two others with 20 and with 10 variables, respectively. The 20 and 10-subsets were chosen by using subselect package of the referenced 31 dataset from original data and 0/1 data.

Principal component analysis Results derived from PCA with 31, 20 and 10 VOCs are synthesized in Fig. 2 by presenting the biplots for the six analyses performed with standardized data. The first row of Fig. 2 shows the biplots for data analysis with raw data, and second row shows the biplots for data analysis with 0/1 datasets. The retained variance within each PCA taken in the first two principal components (PCs), was respectively 69 %, 58.6 %, 61 % for 31, 20 and 10 of raw data and respectively 52.4 %, 52.4 %, 57.2 % for 31, 20 and 10 of 0/1 data. The internal structure of variables, within biplots, and its interrelations showed large similarities unless of direction of PCs, with a pattern for PC scores commonly known in multivariate analysis as horseshoe shape (the most spoiled and the most unspoiled samples on opposite sides of the horseshoe shape) (Nadeau et al. 2011). The single variable negatively correlated with all other variables (at least more evident) in the first component was

dimethyl sulphide (dMs), but this variable was not chosen for the two 10 subsets.

Modelling and prediction Table 3 shows the results of PLSR and PCR obtained with the different k-variable subsets (10, 20 and 31) for raw data and 0/1 indicators data to predict OFF and OAF.

In what concerns PLSR, the choice of 31 VOCs is better in 0/1 data, while in raw data analysis the choice of the subset with 10 VOCs could be an option because it leads to higher values of R^2 and lower values of RMSEP. PLS predictions of OFF and OAF are illustrated in Fig. 3, according to 0/1 indicators data for 31, 20 and 10-subset. It is noticed that the different PLS prediction values of OFF and OAF were similar and most of the values appear close to the diagonal which means good prediction. There are few cases with high differences between observed and predicted values. In relation to OFF, one sample originally classified with a value near to 1.0 and predicted by the three models with values near to 3.0 appeared.

Based on the dataset (not shown), one reason for this misclassification could be the presence of 3-methylbutanal and 3-methylbutanol, which commonly appear when a sample is spoiled or due the presence of the sweet and buttery attributes of odour which contribute for a lower OFF evaluation by the sensory panelists. In the right plot, two samples originally classified near the 1.5 OAF value, and with predicted values near to 3 appear. The absence of 1-propanol+2-methylpropanal, pentanal+heptane, heptanal and 2-

Fig. 2 Biplots (scores and loadings) of PC1 vs. PC2 corresponding to 31, 20, 10 subsets from raw data (first 3 graphs) and 0/1 data (last 3 graphs) of PCA model for Maronesa beef. Samples were labelled as 1 for Vt10, 2 for

MAPt10, 3 for Vt21 and 4 for MAPt21 and variables were coded as indicated in Table 1 with the suffix 01 for 0/1 data

methylbutanal in these two samples could have influenced the OAF results by PLSR since these VOCs are negatively correlated with OAF.

Looking at the R^2 and RMSEP, PLS with two components is hardly better than using PCR. R^2 values from the two regressions confirm that and the number of significant

variables needed to predict the OFF and OAF are higher in PCR than that observed in PLSR. Accuracy of prediction values of OFF and OAF by PCR was, within each choice of subset, lower when compared with PLS results. At same time, it was possible to notice that within the PCR results, the choice of 10 VOCs or 20 VOCs could perform as well as the PCR for

Table 3 Comparative results of R^2 and RMSEP for PLS and PCR models obtained with two PCs for OFF and OAF according to #10, 20, 31 raw and 0/1 data subsets

		OFF variables subsets						OAF variables subsets					
		# 10		# 20		# 31		# 10		# 20		# 31	
Volatile compounds		PLS	PCR	PLS	PCR	PLS	PCR	PLS	PCR	PLS	PCR	PLS	PCR
Raw data	R^2	0.726	0.663	0.702	0.599	0.710	0.654	0.689	0.596	0.644	0.592	0.660	0.586
	RMSEP	0.763	0.846	0.795	0.923	0.781	0.857	0.865	0.848	0.924	1.063	0.898	0.997
0/1 data	R^2	0.778	0.628	0.784	0.672	0.806	0.637	0.747	0.669	0.738	0.645	0.773	0.653
	RMSEP	0.687	0.888	0.678	0.834	0.641	0.877	0.779	0.891	0.793	0.923	0.738	0.913

Fig. 3 Scatterplots between observed and predicted values corresponding to different PLS models of OFF (left) and OAF (right), according to 0/1 data with 31, 20 and 10 subsets

31 subset variables. The significant variables in all data analyses allowed for the identification of the main VOCs which are related to the spoilage level of meat samples. Information from Table 2 and Fig. 1 permits us to focus our results in one final subset of set of VOCs where most of the chosen VOCs have correlation values with OFF and OAF higher than 0.6. In the beef samples, the main VOCs associated with high OFF and low OAF obtained by multivariate analysis are: 3-methylbutanal and 3-methylbutanol, highly correlated with 2-methylbutanal and 2-methylbutanol respectively, and with significant values concerning package and storage time effects. They are considered to be indicators in the evaluation of the spoilage level in beef samples packed in MAP with high level of O_2 , increasing during storage, in agreement with other spoilage meat studies. Other VOCs associated with OFF are some aldehydes, namely pentanal+heptane, octanal e nonanal; ketones like 3,5-octanedione, 2,3-octanedione 2,3-pentanedione and alcohols namely 1-hexanol. The 1-pentanol and 1-penten-3-ol should be considered important descriptors of spoiled samples. On the other hand, in Fig. 2 the 3-methylpentane and the dimethyl sulphide appear in the biplots associated with vacuum samples with 10 and 21 days (identified as 1 and 3, respectively) and some MAP samples with 10 days of storage (identified as 2), considered to be less spoiled. The 3-methylpentane decreases during storage and could be considered as one indicator in the first stages of spoilage in beef.

Conclusions

The results achieved in this study demonstrated that the selected volatile compounds could be considered as volatile indicators of beef spoilage, enclosing information for discrimination of Maronesa beef samples in sensory classes of odour corresponding to unspoiled and spoiled levels. 2 and 3-

methylbutanal, 2 and 3-methylbutanol, 1-pentanol and 1-hexanol, 2,3-octanedione, 3,5-octanedione, octanal and nonanal can be considered as indicators in spoilage of beef samples. Furthermore, 3-methylpentane could be considered an indicator in the first stages of spoilage progress in beef.

We concluded that 0/1 data leads to an easier model to implement than the models based on the original data analysis. The results also showed the simplicity of applying the technique and the usefulness of selecting optimal subsets to predict the spoilage level of samples. Also presented is how variable subsets can be used in the prediction analysis, preserving high levels of accuracy.

In addition, HS-SPME is a simple and versatile sampling technique that associated with gaseous chromatography and mass spectroscopy, along with multivariate statistical analysis may be a practical strategy to monitoring the sensory quality of beef and the shelf-life evaluation.

Despite using different mathematical analyses and data types, results showed large similarities. The powerful analytical methodologies for reducing variables and the choice of optimal subsets could be advantageous in both basic research and the routine quality control of chilled beef.

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Compliance with Ethics Requirements The authors declare that:

1. Cristina Saraiva has no conflict of interest.
Irene Oliveira has no conflict of interest.
José António Silva has no conflict of interest.
Conceição Martins has no conflict of interest.
Jesus Ventanas has no conflict of interest.
Carmen García has no conflict of interest.

2. The present work does not contain any studies with human or animal subjects.

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