A CHEMOMETRICS APPROACH TO ANALYSE VOLATILE MOLECULES RELEASED BY POST-MORTEM BOVINE FAST-TWITCH MUSCLES

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Abstract. It is well known that beef produces volatile molecules. In this work, the detection of volatiles released by *post-mortem* bovine fast-twitch muscles (*Musculus longissimus dorsi* and *Musculus cutaneus trunci*) was done using GC/MS-SPME (gas chromatography/mass spectrumsolid-phase microextraction). The releases of volatile molecules were modeled against three factors (*rigor-mortis*, animal age and oxidative capacity) using a chemometrics approach (experimental design and partial least squares regression). The GC/MS-SPME technique produced more than 30 reproducible chromatographic peaks, but only 13 were associated significantly with two factors (*rigor-mortis* and animal age). The volatile profile was composed mainly of alcohols, aldehydes and alkanes. The factor "animal age" was the main variable related to the release of volatile molecules. The results strongly suggest that the release of volatile molecules change according to *post-mortem* metabolism and the animal age.

1. Introduction

It is well known that volatile composition influences the odorant characteristics of foods. Different kinds of volatile molecules are present in meat and beef (Elmore et al., 2004; Insausti et al., 2005; Osorio et al., 2008; Vasta et al., 2007, 2010), which can be classified into several groups according to their functional groups such as hydrocarbons, aldehydes, ketones, alcohols, esters, aromatics and others (Rivas-Cañedo et al., 2009). Several of these molecules are produced by meat maturation. However, fresh meat has a volatile profile before maturation, hence the question: "How does muscle produce these volatile molecules?" The information about the biochemistry and metabolic source of these odorants is not totally available in scientific literature.

Many factors and biological processes affect the production of volatile compounds and their releases. It has been reported that environmental factors like feeding (Elmore et al., 2004), weather (Zhang et al., 2005) and animal age (Santander et al., 2013; Arredondo et al., 2014) affect the behavior of volatile profiles. Different kinds of beef also present differences in the volatile profiles (Watanabe et al., 2008). Moreover, biological processes like rigor-mortis or post-mortem glycolytic fluxes might modify the volatile release of fresh meat (Acevedo et al., 2012). Considering the importance of odors in food science, it is very important to understand the

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origin of volatile compounds, because this information could be used to develop technologies to certify beef and to enhance the flavor of fresh meat.

When an animal is slaughtered, the glycolytic flux of its muscles immediately increases. The anaerobic glycolytic activity of the *post-mortem* muscle is the main mechanism of energy production and it has been reported that glycolysis might be a key pathway in the release of volatiles by mammal cells (Acevedo et al., 2010) and raw meat (Acevedo et al., 2012). Since muscles can be classified according their metabolic characteristics into slow-twitch (essentially oxidative metabolism maintaining energy levels aerobically) and fast-twitch (essentially glycolytic metabolism maintaining ATP supply anaerobically) (Ylä-Ajos et al., 2006), we hypothesized that *post-mortem* fast-twitch muscles might be a good scientific model to use as an exploratory pattern analysis of volatile profiles by chemometrics analysis.

In this work, we analyzed volatiles released during *rigor-mortis* (fresh meat) in two bovine fast-twitch muscles by using GC/MSSPME (gas chromatography/mass spectrum-solid-phase micro extraction). Then, we analyzed the complex data system by using chemometrics tools based on partial least square regression to obtain a relationship between the volatile release and *post-mortem* metabolism of beef carcasses

2. Materials and Methods

2.1. **Samples of beef carcasses.** Fresh meat samples were obtained from male bovines (Holstein, *Bos taurus*). After slaughtering, the animals were skinned, eviscerated and split into two half carcasses. The half carcasses were stored at 0°C. Six half carcasses of newborn calves (< 3 days old) and 110 six half carcasses of 2 year old bovines were used.

2.2. **Gas chromatography.** Release of volatile molecules from beef was analyzed by means of a GC/MS-SPME. A headspace vials were loaded with 2.5 g of sample and equilibrated at extraction temperature (50°C) for 30 minutes. Then, a DVB/CAR/PDMS fiber (50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane) (Supelco, USA) (Martin et al., 2009; Santander et al., 2013) was introduced inside the headspace vial to extract the volatiles, through a silicon septum for 30 minutes. Analyses were made in triplicate.

Targeted analyses loaded in the fiber were analyzed in a Hewlett-Packard (USA) HP 6890 gas chromatography (with a HP MD5973 quadrupole mass spectrometer) in splitless mode (5 minutes). Desorption and cleaning of fiber was carried out at 250°C for 20 minutes. Helium at a constant flow (1.3 mL/min) served as a carrier gas.

Separation was conducted in a 60 m length × 0.32 mm i.d. × 1.0 μm film BPX5 column (SGE, Australia). The oven was programmed as follows: The temperature program was 40°C for 10 minutes, then raised to 200°C at a rate of 5°C/min (maintained 200°C for 1 minute) and then raised to 250°C at a rate of 20°C/min (maintained 250°C for 5 min).

Operation of the GC/MS was done by using the Chemstation software (Agilent Technologies). Scan mode was used.

Potential emanations were analyzed by matching sample mass spectrums with those of the National Institute of Standards and Technology (NIST) MS spectral library (USA) for peaks presented in the chromatograms. Chromatographic peaks were considered "unknown", when their mass spectral match quality (MQ) was less than 85%, and discarded in this identification process (Acevedo et al., 2010, 2012). MQ value is referred to the degree the target spectrum matches the standard spectrum in the NIST library (100% indicates a perfect fit). Chromatographic peaks with mass spectral fit value $> 85\%$ were confirmed with their respective commercial chemical standards.

2.3. **Experimental design.** In order to maximize the experimental information, the experimental factors studied were arranged as informed in Table 1. The sex of the animals was not taken into consideration (all bovines were male).

TABLE 1. Description of samples and factors.

Samples			Factors		
			X_1	X_2	X_3
New born calves (< 3 days old)	M. Cutaneus trunci	Pre rigor-mortis	0	0	1
		Rigor-mortis	1	0	1
	M. Longissimus dorsi	Pre rigor-mortis	0	0	0
		Rigor-mortis	1	0	0
Bovines of two years old	M. Cutaneus trunci	Pre rigor-mortis	0	1	1
		Rigor-mortis	1	1	1
	M. Longissimus dorsi	Pre rigor-mortis	0	1	0
		Rigor-mortis	1	1	0

The influence of *rigor-mortis* (X_1) was analyzed taken samples before rigor-mortis and advanced *rigor-mortis*. For that, the carcasses was sampled twice: the first sampling (45 minutes post-mortem) was considered as pre *rigor-mortis* and the second sampling (36 hours *post-mortem*) was considered as advanced *rigor-mortis*.

To analyze influence of animal age (X_2), we sampled beef obtained from newborn calves (< 3 days old) and animals of 2 years old.

To analyze the influence of the oxidative capacity of the fast-twitch muscles (X_3), two tissues were used: *M. longissimus dorsi* (essentially glycolitic fast-twitch) (Hwang et al., 2010; Ylä-Ajos et al., 2006) and *M. cutaneus trunci* (fast-twitch with oxidative capacity) (Devine et al., 1984).

To obtain an adequate number of experimental run repetitions, six half carcasses of newborn calves (< 3 days old) and six half carcasses of 2-year-old bovines were used. Each sample of tissue taken was sectioned into three parts to obtain triplicates of each experimental run.

2.4. **Chemometrics analysis.** The three predictors studied (X_1, X_2 and X_3, see Table 1) were associated with relative chromatographic areas of the volatile molecules present in chromatograms (response variables). The purpose of the statistical analysis was to relate the chromatograms to the proposed predictors. Our analysis was based on the multivariate linear regression model, given by:

$$\boldsymbol{Y} = \boldsymbol{X}\boldsymbol{B} + \boldsymbol{U},$$

where $\boldsymbol{Y}$ is the response matrix of order $n \times q$; $\boldsymbol{X}$ is an $n \times p$ design matrix; $\boldsymbol{B}$ is an unknown $p \times q$ matrix of parameters called regression coefficients; and $\boldsymbol{U}$ is $n \times q$ matrix of random disturbances, with n being the sample size, q the number of Y variables and p represent the number of X variables.

The estimation of the regression coefficients matrix $\boldsymbol{B}$ was carried out using partial least square (PLS) (Frank & Friedman, 1993). Before using the PLS regression, a simple pre-processing method of the variables X and Y was done based on autoscale of the values (Procopio et al., 2013). The selection of the number of components was based on cross-validation procedure (Wold et al., 2001). As suggested, for instance in Wold et al. (2001), the standard errors were estimated from the data by using Jackknife formula (Efron & Gong, 1983). Thus, we tested the hypothesis that individual coefficients b_{rs} are zero, for $r = 1, \ldots, p$; $s = 1, \ldots, q$ by applying an approximate t statistic. Importance of the factors was studied by mean of variable importance on projection (VIP) and shown as a VIP plot. This approach has been used to model volatile compounds in food systems (Acevedo et al., 2015; Procopio et al., 2013).

All computations were done using software SIMCA-P (Umetrics, Sweden) (Eriksson et al., 2006).

3. Results and Discussion

3.1. **Chromatographic profile.** Application of the GC/MS-SPME technique to measure volatiles in samples produced 30 chromatographic peaks. Figure 1 shows a selected chromatogram where several of these peaks can be distinguished. This later strongly suggests that the fast-twitch muscles of beef carcasses release volatile molecules and their profile can be identified by using GC/MS-SPME.

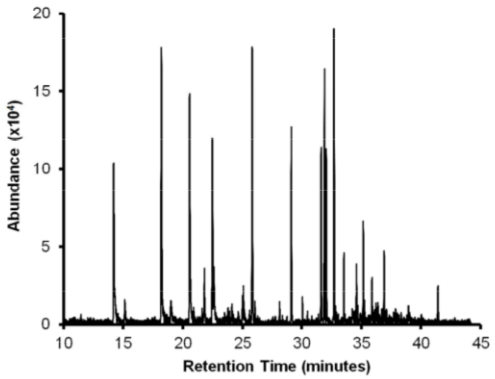

Figure 1. Selected chromatogram of *M. longissimus dorsi*.

3.2. **Analysis of chromatographic data.** The putative volatile compounds or chromatographic peaks of the chromatographic profile were modeled against all factors (see Table 1), by using the multivariate regression model previously described. It was used to represent each response (Y variable) against each factor (X variable).

Figure 2 shows a bar plot for the estimated coefficients of matrix $\boldsymbol{B}$. When applied the t test, there is a big group of volatiles (12 types of molecules) that have no significant ($p > 0.05$) relationship with any X variable, and the presence can be attributed to random behavior or other factors. The volatiles with significant regression coefficient in the matrix $\boldsymbol{B}$ ($p < 0.05$) and their respective confidence interval (95%) are indicated in the Figure 2. Only 18 volatiles have relationship with two factors (X_1 and X_2). In particular, the oxidative capacity (X_3) was not directly associated with the release of volatile molecules, suggesting likely a similar behavior of the *post-mortem* volatile metabolism among fast-twitch muscles.

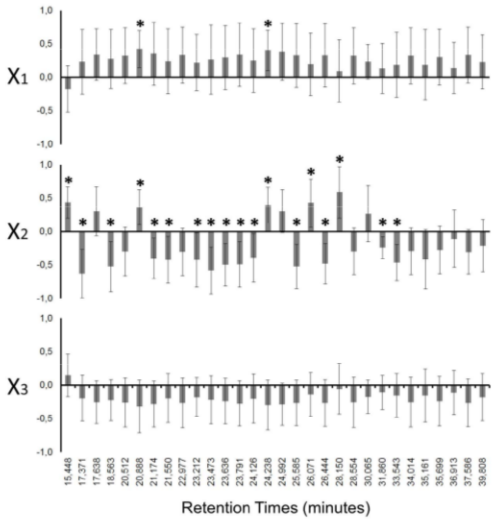

FIGURE 2. Coefficients of $\boldsymbol{B}$ matrix. Error bars correspond to confidence interval (95%). Asterisks (*) indicate significant coefficients ($p < 0.05$).

Figure 3 shows the VIP plot (which indicates the importance of X variables). *Rigor-mortis* (X_1) and animal age (X_2) have large and significant VIP values (larger than 1 and $p < 0.05$, respectively). Previous works (Santander et al., 2013; Arredondo et al., 2014) have reported that volatiles molecules release by fresh meat can be used as biomarker related to animal age. In our case, the factor animal age was the most important of the variables studied, indicating that the volatiles could be used to identify the animal age.

3.3. **Volatile molecules identified.** Eighteen chromatographic peaks display dependence with some of the factors considered in this study. Thirteen of these 18 volatiles were chemically identify by using mass spectrometry and chemical standards (Table 2). One of these 13 identified compounds was related with both main factors (X_1 and X_2). Only xylene - which presents a characteristic sweet and pungent odor - shows a strong dependence with animal age and *rigor-mortis*, simultaneously.

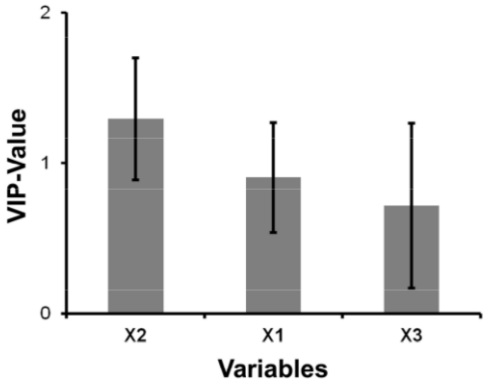

FIGURE 3. VIP-plot of X variables. Error bars correspond to confidence interval (95%).

TABLE 2. Volatile molecules selected by chemometrics analysis and identified using mass spectrometry and chemical standards.

Volatile Compound	Retention Time (min)	Associated with X_1 (rigor mortis)	Associated with X_2 (animal age)	Associated with X_3 (oxidative capacity)
1-Pentanol	17.371	-	Yes	-
Hexanal	18.563	-	Yes	-
Xylene	20.888	Yes	-	-
Heptanal	21.550	-	Yes	-
Heptanol	23.212	-	Yes	-
1-Octen-3-ol	23.473	-	Yes	-
Octanal	24.126	-	Yes	-
1-Octanol	25.585	-	Yes	-
Undecane	26.071	-	Yes	-
Nonanal	26.444	-	Yes	-
Dodecane	28.150	-	Yes	-
Tetradecane	31.860	-	Yes	-
Hexadecane	33.543	-	Yes	-

Xylene released by mammal cells (*in vitro*) has been correlated to glycolytic activity (Acevedo et al., 2010). It has been reported in raw lamb meat (Vasta et al., 2007, 2010), cooked beef (Insausti et al., 2005), grilled bovine and lamb meat (Elmore et al., 2004, 2005) and human blood (Deng et al., 2004).

In addition, four alcohols (1-pentanol, heptanol, 1-octen-3-ol, 1-octanol), four aldehydes (hexanal, heptanal, octanal, nonanal) and four alkanes (undecane, dodecane, tetradecane, hexadecane) were identified and associated strongly with the variable animal age, being this very important to perform further chemometrics studies such as classification algorithms.

3.4. **Alcohols identified.** Identification of 1-pentanol is associated with the presence of lactic acid bacteria (Jääskeläinem et al., 2013; Ercolini et al., 2009; Holm et al., 2013a; Hernández-Macedo et al., 2012; Muriel et al., 2004) and grass intake in

the diet (Vasta et al., 2012). Regarding 1-heptanol, this compound appears in lipid thermal oxidation of beef and pig meat (Elmore et al., 2004, 2005; Shi et al., 2012), 1-octen-3-ol is associated with lactic bacteria as well as monounsaturated lipid oxidative reactions in different species such as sheep, cattle and pigs (Elmore et al., 1999, 2004, 2005; Osorio et al., 2008; Watanabe et al., 2008; Ercolini et al., 2009; Holm et al., 2013a,b; Resconi et al., 2013). Concerning 1-octanol, it is reported as a lipid oxidation product generated during fat thermal oxidation (Shahidi, 1998; Elmore et al., 1999, 2005, 2004; Osorio et al., 2008; Watanabe et al., 2008; Shi et al., 2012).

The alcohol 1-octanol has been used a marker to identify beef obtained from OTM (over 30 months) cattle (Arredondo et al., 2014). Interestingly, the main result of this study is that the volatiles (including 1-octanol) are strongly associated with factor X_2 (animal age).

3.5. **Aldehydes identified.** Aldehyde compounds such as hexanal, heptanal, octanal and nonanal are related to lipid oxidation (Shahidi, 1998; Elmore et al., 1999, 2004; Muriel et al., 2004; Elmore et al., 2005; Watanabe et al., 2008; Shi et al., 2012; Jääskeläinem et al., 2013; Resconi et al., 2013) and regarding hexanal and nonanal they were found as products associated with the growth of lactic acid bacteria. Insausti et al. (2005) and Ercolini et al. (2009), have referred to hexanal, octanal and nonanal as the predominant volatiles compounds in meat, being hexanal one of the most abundant volatiles in beef because it is originated during linoleic acid (C18:2 n-9) oxidation (Elmore et al., 2005). In addition, other reports have used the nonanal as a putative marker related with the animal age with high accuracy (Arredondo et al., 2014).

3.6. **Alkanes identified.** The analysis of possible routes for obtaining alkanes proposed by Shahidi (1998) is only associated with lipid degradation, especially for saturated fatty acid degradation. Some alkanes, such as tetradecane and hexadecane, have been implicated as potent markers of grass intake in the diet (Sivadier et al., 2008) and linked by other authors as products provided by feeding and deposited in fat (Meynier et al., 1999; Tejeda et al., 2001).

It has been informed that the alkanes as undecane and tetradecane released by beef have relationship with the animal age (Arredondo et al., 2014), confirming the results obtained in the chemometrics analysis.

4. Conclusion

Our results show that bovine fresh meat from fast-twitch muscles release volatile molecules. The chromatograms obtained by GC/MS-SPME showed 30 peaks, but only 18 were associated with two of the three factors studied (*rigor-mortis* and animal age).

The factors *rigor-mortis* and animal age were the main variables associated with the whole chromatographic profile. The factor oxidative capacity was not associated with the release of volatile molecules, suggesting a similar behavior of the *post-mortem* volatile metabolism among fast-twitch muscles.

The factor animal age was the most important of the variables studied. The volatile profile associated with animal age was composed mainly of alcohols, aldehydes and

alkanes, indicating that these volatiles could be used to discriminate the animal age.

The evidence strongly suggests that the release of these volatile molecules change according to *post-mortem* metabolism and animal age.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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