



The role of the canonical biplot method in the study of volatile compounds in cheeses of variable composition

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SUMMARY: The canonical biplot method (CB) is used to determine the discriminatory power of volatile chemical compounds in cheese. These volatile compounds were used as variables in order to differentiate among 6 groups or populations of cheeses (combinations of two seasons (winter and summer) with 3 types of cheese (cow, sheep and goat's milk). We analyzed a total of 17 volatile compounds by means of gas chromatography coupled with mass detection. The compounds included aldehydes and methyl-aldehydes, alcohols (primary, secondary and branched chain), ketones, methyl-ketones and esters in winter (WC) and summer (SC) cow's cheeses, winter (WSh) and summer (SSh) sheep's cheeses and in winter (WG) and summer (SG) goat's cheeses. The CB method allows differences to be found as a function of the elaboration of the cheeses, the seasonality of the milk, and the separation of the six groups of cheeses, characterizing the specific volatile chemical compounds responsible for such differences.

KEYWORDS: *Canonical biplot; Cheeses; Seasonality; Type of milk (cow, sheep, goat); Volatiles*

RESUMEN: *Papel del método biplot canónico en el estudio de compuestos volátiles en quesos de composición variable.* El método biplot canónico (CB) se utiliza para determinar el poder discriminatorio de compuestos químicos volátiles en queso. Los compuestos volátiles se utilizan como variables con el fin de diferenciar entre los 6 grupos o poblaciones de quesos (combinaciones de dos temporadas (invierno y verano) con 3 tipos de queso (vaca, oveja y cabra). Se analizan un total de 17 compuestos volátiles por medio de cromatografía de gases acoplada con detección de masas. Los compuestos incluyen aldehídos y metil-aldehídos, alcoholes (primarios de cadena, secundaria y ramificada), cetonas, metil-cetonas y ésteres. Los seis grupos de quesos son, quesos de vaca de invierno (WC) y verano (SC); quesos de oveja de invierno (WSh) y verano (SSh) y quesos de cabra de invierno (WG) y verano (SG). El método CB permite la separación de los seis grupos de quesos y encontrar las diferencias en función del tipo y estacionalidad de la leche, caracterizando los compuestos químicos volátiles específicos responsables de tales diferencias.

PALABRAS CLAVE: *Compuestos volátiles; Estacionalidad; Quesos; Tipo de leche (vaca, oveja, cabra)*

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1. INTRODUCTION

The aroma of a cheese is an important factor when it is acquired. The typical flavor of each variety of cheese is the result of a complex balance between volatile and non-volatile compounds originated during the ripening process from milk fats, proteins and carbohydrates. (Pillonel *et al.*, 2003; Fox and Wallace, 1997).

The importance of volatile compounds is due to their correlation with the flavor, which depends on the ripening, time, cheese technology, seasonality, etc. The volatile fractions of some Iberian Peninsula cheeses such as Manchego (Gómez-Ruiz *et al.*, 2002), Roncal (Izco and Torre, 2000), Zamorano (Barron *et al.*, 2005a, Fernández-García *et al.*, 2004a), Serra da Estrela (Dahl *et al.*, 2000; Tavaría *et al.*, 2004; Tavaría *et al.* 2006) have been studied as a response to the growing interest in the characterization of traditional products protected by a Denomination of Origin.

Canonical biplot (Vicente-Villardón, 1992), when it is oriented to the discrimination between groups or MANOVA-biplot (Gabriel, 1971; Galindo, 1986), when the aim is to study the variables responsible for the discrimination, are two effective methods. The main advantage to the biplot version of the technique is that it is possible not only for establishing the differences between groups but also to characterize the variables responsible for them.

Classic multivariate techniques, such as Principal Component Analysis and Canonical Correlation have been applied, among other areas, in fresh fruit (King *et al.*, 2012), in the characterization of the sensory properties and texture of French cheeses (Antoniou *et al.*, 2000), and to study the effects of mixtures of fats in the texture and sensory properties of cheeses (Lobato-Calleros *et al.*, 1997). The Canonical biplot method has been used in the conservation of historical buildings and monuments (Varas *et al.*, 2005), in civil engineering (Iñigo *et al.*, 2005; Iñigo *et al.*, 2013) and, in the case of foods, in the study of margarines (Rui Alves and Beatriz Oliveira, 2003), but no reference has been found regarding its application to the characterization of cheeses using volatile compounds as variables.

Taking into account that the volatile composition of cheese shows significant changes during the ripening period (Fernández-García *et al.*, 2004a; Fernández-García *et al.*, 2004b; Innocente *et al.*, 2013), cheeses ripened from 0 to 6 months have been analyzed to obtain the characteristic profile of the samples throughout the ripening stage.

Here we used the Canonical biplot method to study which volatiles from the cheeses studied were most affected by each of the factors explored: the type of milk used to elaborate the cheeses (cow, sheep, goat) and the seasonality of the milk (winter, summer).

2. MATERIAL AND METHODS

2.1. Samples and cheese-making procedure

To perform the present study a total of 48 cheeses of known composition were elaborated and controlled. Cheeses were prepared in the laboratory, according to the following procedure: raw milk (40 L), not standardized, was incubated with 15 mg·L⁻¹ direct-vat-set starter made of *Streptococcus lactis*, *cremoris* and *diacetylactis* (MA400, Arroyo Laboratories, Santander, Spain) at 30 °C. After 10 min at 32 °C, 12.5 mg·L⁻¹ of calf rennet (90% chymosin, 10% trypsin and 1:150,000 strength) were added to each vat. Coagulation was allowed to take place over 20–70 min. When the curds had developed the desired firmness, evaluated subjectively, they were cut with a cheese harp until pieces similar in size to a grain of rice were obtained. Then, the curd was stirred for 30 min, and heated for 10–20 min at 37 °C until it had reached the desired consistency to improve its drainage with sieves. The curd was packed in round hoops (1 kg) and pressed for 6 h at 1.5 kg cm⁻² at 20 °C. After pressing, the cheeses were salted by soaking them in a sodium chloride brine (18%) at 18 °C for 6 h. The cheeses were then moved to a drying chamber, where temperature (15 °C) and relative humidity (70%) were controlled. They were made of milk collected directly from farms in winter and summer; bovine, ovine and caprine raw milks were obtained directly from the producers in Zamora (Spain). Cheeses with 16 different compositions were elaborated, prepared with known, varying amounts of milk from cows, sheep and goats, with percentages ranging between 0, 25, 75 and 100%. These cheeses were cylindrical, with an initial diameter of 10 cm and a thickness of 5 cm and they were monitored over 6 months (at 0.2, 1, 2, 3, 4, 5 and 6 months) using one of the pieces each time. With respect to all the cheeses elaborated, Table 1 shows the number of samples of the 48 cheeses analyzed, their composition, the ripening time and the season (summer or winter) when the milk was collected (González-Martín *et al.*, 2007).

2.2. Analysis of volatile compounds

Sample preparation, concentration of volatile compounds and gas chromatography coupled with mass detection were carried out at the Estación Tecnológica de la Leche (Instituto Tecnológico Agrario, Junta de Castilla y León, Palencia, Spain) in accordance with the following procedure: a 25-g piece of cheese without rind was homogenized in an analytical grinder. 1 g of the ground sample was weighed in an assay tube with 2 g of Na₂SO₄ and 0.2 mL of cyclohexanone 50 mg·mL⁻¹ as internal standard and the mixture was homogenized. The sample was heated at 40 °C and afterwards purged with helium (40 mL·min⁻¹ for 20 min).

TABLE 1. Composition and number of samples of cheeses analyzed

Composition	N° of samples	Month of ripening	Seasonality	% Fat	
				S	W
100% Cow	6	1,2,3,4,5,6 (1 sample)	3(W), 3(S)	30.33	32.6
100% Sheep	6	1,2,3,4,5,6 (1 sample)	3(W), 3(S)	44.6	50.0
100% Goat	6	1,2,3,4,5,6 (1 sample)	3(W), 3(S)	38.6	51.3
25% Cow 75% Sheep	6	1,2,3,4,5,6 (1 sample)	3(W), 3(S)	34.6	47.0
25% Cow 75% Goat	6	1,2,3,4,5,6 (1 sample)	3(W), 3(S)	34.0	46.0
25% Sheep 75% Goat	6	1,2,3,4,5,6 (1 sample)	3(W), 3(S)	36.3	45.6
75% Cow 25% Goat	6	1,2,3,4,5,6 (1 sample)	3(W), 3(S)	31.3	38.0
75% Ewe 25% Goat	3	4,5,6 (1 sample)	2(W), 1(S)	41.0	49.0
75% Cow 25% Sheep	3	4,5,6 (1 sample)	2(W), 1(S)	36.0	38.0

Winter: W, Summer: S.

Volatile compounds were concentrated in a Tenax/Charcoal trap (Tekmar, Cincinnati, USA) and then desorbed using a helium flux ($40 \text{ mL}\cdot\text{min}^{-1}$) for 20 min at $40 \text{ }^\circ\text{C}$. The volatile compounds were separated and detected in a gas chromatograph (HP 7695, Hewlett-Packard, Palo Alto, USA) coupled with a mass detector (G1800A, Hewlett-Packard) fitted with a 19091N-136, HP INNOWAX column ($60 \text{ m}\times 0.25 \text{ mm}$ o.d., film thickness $0.5 \text{ }\mu\text{m}$, Agilent Technologies, Las Rozas, Spain). The helium flux was $1.0 \text{ mL}\cdot\text{min}^{-1}$ and the temperature gradient started at $45 \text{ }^\circ\text{C}$, increasing by $4.0 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ up to $110 \text{ }^\circ\text{C}$, then remaining at this temperature for 10 min, after which the temperature was raised to $240 \text{ }^\circ\text{C}$ at $18.0 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$.

Detection was performed with the mass selective detector (HP5973) operating in the scan mode, 2.6 scan s^{-1} , m/z range 33–250, with 70 eV IE, and a detector temperature of $250 \text{ }^\circ\text{C}$. Analyses were carried out in duplicate. Peak identification was accomplished by comparison of retention times with authentic standards (Sigma Aldrich Química) and comparison of spectra with bibliographical data from the Wiley 275 library (Wiley and Sons, Inc., Germany). Quantification was measured as the sum of the abundance of all the ions (TIC), with reference to the cyclohexanone, which was added as the internal standard. Data are presented as relative abundances, the compounds' quantified compiling functional groups were aldehydes (acetaldehyde, propanal), methyl aldehydes (3methyl-butanal), primary alcohols (ethanol, propanol, butanol), secondary alcohols (2-butanol, 2-pentanol, isopropanol, 2-heptanol), branched-chain alcohols (3-methyl-1-butanol), ketones (acetone), methyl ketones (2 butanone, 2 heptanone, 2-nonanone, 2-pentanone), and ester (ethyl acetate). Although the number of volatiles detected was higher, we only considered those that appeared in all the samples (The results of the composition of volatile are shown in the work of Gonzalez-Martín *et al.*, 2014).

2.3. Statistical analyses (Canonical biplot)

Canonical biplot is a method of multivariate analysis which permits simultaneous plots of the different groups to be compared and the different variables under analysis to be obtained, but with the intrinsic characteristics of biplot methods (Gabriel 1971; Galindo 1986; Amaro *et al.*, 2004).

To implement the technique, we started from a matrix of dimension ($n \times p$), where the n rows were divided into k groups. After the corresponding decomposition into single values (SVD) of the matrix of the means of the groups, we built a biplot representation (in dimension r) (Vicente-Villardón 1992, 2013). We represented the row markers as points (stars for the average values of the different groups; + for the different elements of the groups) and the column markers as vectors in a scatter diagram. The Euclidean distance between two row markers approximates the Mahalanobis distance between groups and elements, i. e. the row markers are the coordinates of the group means on the canonical subspace with maximum discriminatory power.

Canonical biplot is oriented towards the discrimination between groups obtained with the MANOVA-biplot in order to study the variables responsible for the discrimination. The main advantage of the Canonical biplot version that uses this technique is that it offers the possibility not only of establishing the differences between groups but also of characterizing the specific variables that cause those differences. The results are usually summarized on different factorial planes that depend on the absorption of variance or dimensions that we retain (r). The description of Canonical biplot method is described in the literature (Varas *et al.*, 2005).

The Canonical biplot Analysis offers different analyses in a single plane and the interpretations can be directed to answer the issues of the study by

comparing and contrasting groups and variables and focusing on their outstanding differences and similarities. This statistical method is not yet widely used, mainly because it is still not available in the major statistical packages. Generally, the biplot method includes t-tests based on Wilk's Lambda distribution, a probability distribution used in multivariate hypothesis testing. It is a multivariate generalization of the univariate F-distribution similar to Student's t-distribution. ANOVA and MANOVA tests in a single numerical table can be analyzed easily by following the graphic representation of the results. For statistical analysis we used a free application specifically constructed for biplot (Vicente-Villardón, 2013). The Canonical biplot analysis was applied to a matrix formed of 17 variables and 48 rows in 6 groups accounting for combinations of two seasons (winter and summer) with three types of cheese (goat, cow and sheep's milk); that is, Winter Cow (WC), Summer Cow (SC); Winter Sheep (WSh), Summer Sheep (SSh), and Winter Goat (WG), Summer Goat (SG).

3. RESULTS AND DISCUSSION

The application of individual ANOVAs to the variables analyzed, (Table 2), shows that the variables isopropanol, 2-butanone, 2-butanol, propanol, 3-methyl-1-butanol, propanal, 3-methylbutanal and butanol did not have discriminatory power since they gave a result of $p > 0.05$.

The results of the Canonical biplot are shown in Table 3, where it may be seen that the variance of the

three main axes accounts for 89.175% of the variance explained. The global contrast based on Wilk's Lambda test has a value of 2.5647, with $p < 0.01$, indicating that, globally, differences are present between the 6 groups of populations of cheeses to be compared (WC), (SC), (WSh), (SSh), (WG), (SG).

On the factorial planes (Figure 1a) Axis 1 vs. Axis 2; b) (Figure 1b), Axis 1 vs. Axis 3; c) (Figure 1c), Axis 2 vs. Axis 3), the labels with poor, acceptable and good quality of representation appear respectively in grey, normal font and in bold. The interpretation of the labels depends on a series of measurements such as the quality of representation for the different planes (variance absorption of the planes, the goodness of the projections of the measurements on the variables for the dimensions selected, etc.) (Gabriel, 1971; Gabriel and Odoroff, 1990; Galindo, 1986).

In general, the quality of representation of the projected groups and variables on the first three planes is good, with the exception of that of SC, which is acceptable, and butane, which is poor. Upon analyzing the first two factorial planes (Figure 1a, b) it may be seen that the group of cheeses from winter cow's milk (WC) is clearly separated from the rest of the groups. Analyzing the variables responsible for this separation, it may be seen that this difference is due to the fact that the samples take higher values in ethanol, ethyl acetate, acetaldehyde and 2-butanol, together with lower values in 2-heptanone, 2-pentanone, isopropanol, 2-pentanol, acetone and 2-heptanol. This result is in agreement with previous works indicating that ethanol is present at higher concentrations in cow's cheese than in

TABLE 2. Individual ANOVAs

Variable	Total	Explained	Residual	F	Sign.
Acetaldehyde	48	14.781	33.219	3.738	0.00688
Acetona	48	18.706	29.294	5.364	0.00067
isopropanol	48	5.321	42.679	1.047	0.40298
acetatodeetilo	48	21.577	26.423	6.859	9e-005
2-butanone	48	5.711	42.289	1.134	0.35735
Ethanol	48	21.125	26.875	6.603	0.00013
2-pentanone	48	13.704	34.296	3.356	0.0122
2-butanol	48	10.731	37.269	2.419	0.05149
Propanol	48	8.853	39.147	1.9	0.11478
2-pentanol	48	16.245	31.755	4.297	0.00302
2-heptanone	48	18.137	29.863	5.102	0.00096
3-methyl-1-butanol	48	6.215	41.785	1.249	0.3036
2-nonanone	48	14.512	33.488	3.64	0.00796
Propanal	48	8.673	39.327	1.853	0.1234
3-methylbutanal	48	4.208	43.792	0.807	0.55111
Butanol	48	4.859	43.141	0.946	0.46146
2-heptanol	48	19.055	28.945	5.53	0.00053

The individual F statistics follow a Snedecor's F with 5 and 42 d.f.

TABLE 3. Eigenvalues and explained variance

Dimension	Eigenv.	% Expl.	Cumm.	TSS	ESS	F	p-val
1	2.039	43.572	43.572	5.156	4.156	34.911	0
2	1.637	28.09	71.662	3.679	2.679	22.506	0
3	1.292	17.513	89.175	2.67	1.67	14.032	0
4	0.737	5.7	94.875	1.544	0.544	4.567	0.002
5	0.699	5.125	100	1.489	0.489	4.106	0.004

Global contrast based on Wilk's Lambda: 2.5647.

The F statistics follows a Snedecor's F with 85 and 130 d.f.

p-value: 6.241e-007.

sheep's cheese (Molina *et al.*, 1999), while acetaldehyde is not one of the more important aldehydes in sheep's cheese (Barron, *et al.*, 2005b; Fernández-García *et al.*, 2004a, b) and it could not be found in goat's cheese (Castillo *et al.*, 2007).

There is a clear difference between the WC and WG winter cheeses and the rest of the cheeses made in summer. This difference is hardly noticeable in the sheep cheeses (WS and SSh). The sheep cheeses (Figure 1a) were characterized by higher contents of methyl-ketones (2-pentanone, 2-heptanone and 2-nonanone), confirming earlier results indicating that methyl-ketones are compounds that appear in greater amounts and proportion in sheep cheeses (Barron *et al.*, 2005a).

Regarding goat cheeses, these were characterized (Figure 1b) by 3-methyl-1-butanol and 3-methyl-1-butanol. The presence of this alcohol is a consequence of the reduction in the aldehyde and it has previously been reported that it is present at a higher level in goat's milk cheese than in cow and sheep's milk cheeses (Molina *et al.*, 1999).

Regarding the variables, propanal clearly separates the winter from the summer cheeses ($p < 0.05$) since it takes the lowest values in winter in comparison with the higher values found for summer. When the mean values of the six groups of cheeses are projected onto the propanal variable, it can be observed that winter milk cheeses WC, WG, WSh show lower values than their summer equivalents

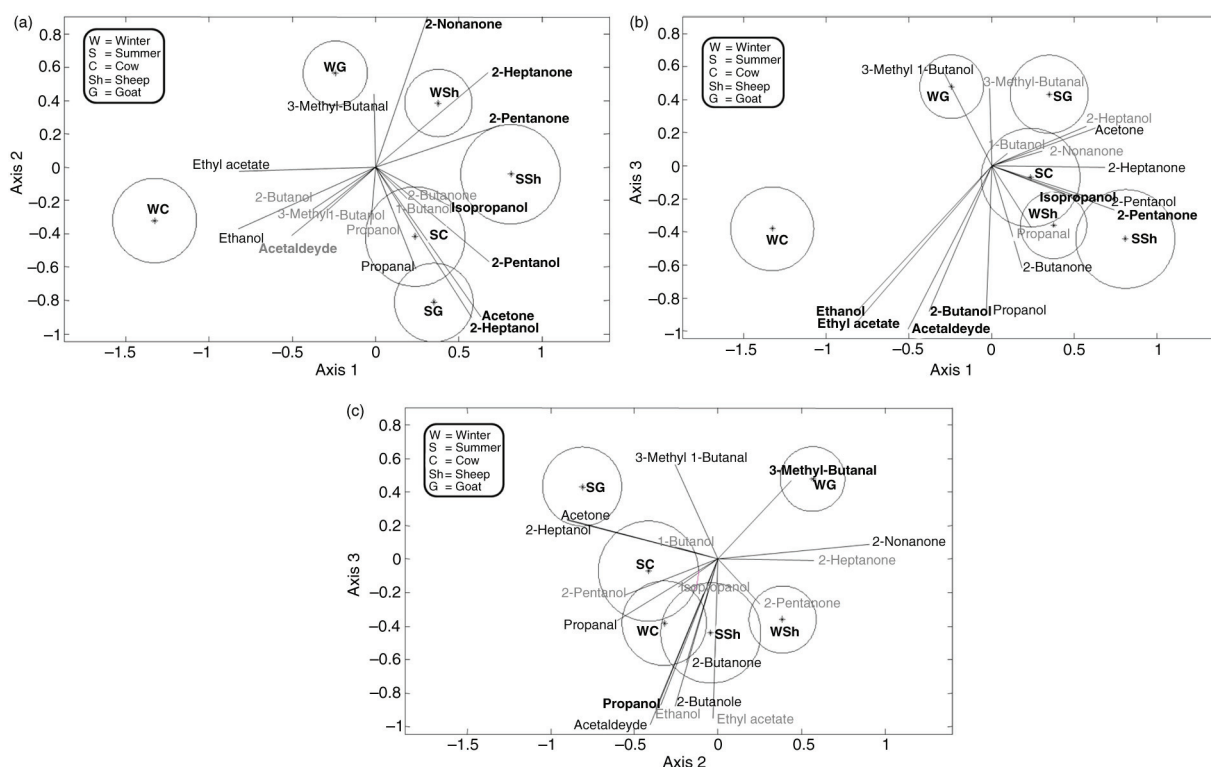


FIGURE 1. (a) Canonical biplot representation: axes 1 and 2. (b) Canonical biplot representation: axes 1 and 3. (c) Canonical biplot representation: axes 2 and 3.

(SC, SG, SSh). The same results have been obtained in Zamorano cheeses by Fernández-García *et al.*, (2004a), who found the highest values of propanal in summer cheeses, with lower values in autumn and winter. This compound, as all the aldehydes, appears in very low concentration since it is an intermediate and unstable compound which is usually reduced to alcohols.

The same difference, although less pronounced, can be seen in the variables 2-pentanol, acetone, 2-heptanol and isopropanol (Figure 1a). These results are in agreement with those reported previously. Thus, Fernández-García *et al.* (2004a, b), found lower values of propanal in winter cheeses while 2-pentanol and 2-heptanol were significantly lower in autumn cheeses and no significant differences were observed for 2-propanol. In Figure 1b it may be seen that the parameters ethanol, ethyl acetate 2-butanol, acetaldehyde and propanol are detectors of winter cow's cheese samples owing to their higher mean values than the rest. The parameters 3 methyl 1butanol, 3 methyl butanal separate the goat (WG, SG) and sheep samples (WSh, SSh), (Figure 1b, Axis 1-Axis 3). The parameter 2-butanone separates goat's cheese since it was not possible to detect it in a large number of the samples of this cheese. In Figure 1c, Axis 1 vs. Axis 3, the parameter 3 methyl butanal is responsible for the statistically significant differences between the winter and summer goat's cheeses, together with acetone and 2-heptanol, which also show differences between winter and summer with the rest of the samples.

With respect to the seasonality of the cheeses, a comparative analysis between the same cheeses made at different times of the year (W and S) afforded the following results: the WC group vs. SC differs ($p < 0.05$) in all the variables, but mainly in ethanol, acetaldehyde, 2-pentanone, 2-heptanone and 2-butanol. On comparing WG and SG, these differ ($p < 0.05$) in most of the variables, but mainly in the variables 3-methyl butanal (Figure 1c) and acetone, 2-heptanol and 2-pentanol. (Figure 1a, b). Fedele *et al.*, 2005, found that goat winter milk had smaller amounts of ketones and more alcohols than summer milk. However, they did not find significant variations in the volatile compounds of goat cheeses from one season to another. The WSh group vs. SSh only differed ($p < 0.05$) in the variables isopropanol, 2-pentanol, acetone, 2-heptanol and propanal. 2-propanone has been found to be higher in winter-autumn sheep cheeses, while heptanol, isopropanol and 2-pentanol are less abundant in these cheeses (Fernández-García *et al.*, 2004a, b).

These results are due to the fact that the concentration and composition of various milk components differ according to season. Differences in the amount and composition of milk fat have been reported (Johansen *et al.*, 2002, Nudda *et al.*, 2005) owing to variations in feed factors. During the first months of the year the forage consumed tended to

be mainly hay (98% of dry matter) with a small contribution from silages, less than 30% of the ration. From spring to summer the contribution of silage, first cuts, green forages etc., increased even if the animals remained in the pens. This type of diet reduces the dry matter intake. Afterwards, from summer to winter the diets were standardized and were composed of 40% hay and a 30% silages. When summer and winter diets are compared, although the composition was similar, the ration changes, in fact, because the animals tend to eat more in winter than in summer due to the number of daylight hours and temperatures. Indeed, the rumen metabolism is also affected because of the contributions of melatonin in the blood that affect both the ruminal bacteria populations and rumen parasympathetic regulation. Therefore, there are modifications in food intake, food composition, rumen activity, ruminal flora and circadian rhythms that affect cheese composition.

Thus, the fat contents (% by weight) of the winter cheeses analyzed in this work had higher values than those of the summer cheeses (González-Martín *et al.*, 2011). Moreover, important properties such as the protein:fat ratio and the casein:whey protein ratio are seen to be affected by time of year (Barron *et al.*, 2001; Sitzia *et al.*, 2015; Addis *et al.*, 2015). Since volatile compounds are formed through the degradation of amino acids, leading to amines, aldehydes, alcohols, acids and sulfur compounds, and the breakdown of fatty acids, which produces esters, methyl ketones and secondary alcohols, the above changes in milk composition will affect the volatility profile.

4. CONCLUSIONS

The present results show that the volatile chemical compounds used as variables in the Canonical biplot method are suitable for establishing relationships between the significant variables (graphically and quantitatively) and the six groups of cheese: Winter Cow, Summer Cow, Winter Sheep, Summer Sheep, Winter Goat, Summer Goat. Winter cow's milk cheeses group (WC) is clearly separated from the rest of the groups because these cheeses have higher values of ethanol, ethyl acetate, acetaldehyde and 2-butanol, together with lower values of 2-heptanone, 2-pentanone, isopropanol, 2-pentanol, acetone and 2-heptanol. There is a clear difference between the WC and WG winter cheeses and the rest of the cheeses made in summer but this difference is hardly noticeable in the sheep cheeses. The variables propanal together with 2-pentanol, acetone, 2-propanol and isopropanol, although to a lesser extent, clearly separate winter and summer cheeses owing to their lower mean values in winter. Goat's and sheep's milk cheeses were separated by 3-methyl-1-butanol and 3-methyl-1-butanal compounds. This last compound together with acetone and 2-heptanone allow for the differentiation

between winter and summer goat's milk cheeses. All this indicates that the Canonical biplot is a suitable method for explaining this type of population, in which many different factors are involved.

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