Antioxidant status and odour profile in milk from silage or lucerne-fed cows

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Abstract

In order to determine the effect feeding differences on natural antioxidant vitamin delivery, two contrasting diets, lucerne (ALF) and sorghum silage (SS), were analysed. Diets showed different profiles for fat-soluble vitamins. ALF was higher in α-tocopherol, β-carotene and retinol than the SS diet. On the contrary, SS diet was higher in γ- and δ- tocopherol, due to the soy expeller contribution in these isomers. The ALF diet favoured higher milk production and protein yield in comparison to SS, whereas the fat content was the opposite ($p<0.01$). The composition of the diets was partially reflected in milk. The ALF diet favoured the incorporation of α-tocopherol, retinol, β-carotene and vitamin D_3 into raw milk. Meanwhile, a less abundant isomer of vitamin E, γ-tocopherol, was significantly higher in the SS than the ALF milk and δ-tocopherol was not detected. In addition, the ALF diet also promoted higher antioxidant activity. The ferric reduction antioxidant power (FRAP) was significantly higher in ALF than in SS milk, however oxidation determined by thiobarbituric acid reactive substances (TBARS) was similar for both types of milk. Also, no differences in ascorbic acid concentration were detected in either type of milk. The ALF and SS milks were clearly separated (100%) by their antioxidant status and odour profiles as a function of feeding into SS and ALF groups when the biochemical variables and sensor LY2/gCTI were analysed together. Two linear Fischer's discriminant functions were defined according to dietary treatments using β-carotene and sensor LY2/gCTI. The success rate of correct classification of each sample was 100%, either for the original cases or after cross-validation ($p<0.0001$). The use of an electronic nose proved to be a useful instrumental method to discriminate the odour profile of milk samples with a different antioxidant status.

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The health benefits of milk and its natural character are appreciated by consumers. The nutrient composition of milk and dairy products is strongly influenced by the nature of the diet ingested by the cows. Among these nutrients, antioxidants and polyunsaturated fatty acids compromise the oxidative stability of these products. A way of improving the natural antioxidant status in milk is through dietary delivery of the antioxidants contained in fresh pasture.

Grazed lucerne (*Medicago sativa* L., also known as alfalfa) is one of the most common feeds in the diets of lactating dairy cows in Argentina (Castillo *et al.* 2006) and constitutes a vital component of organic (ecological) dairy systems.

Fat-soluble vitamins and micronutrients are incorporated in plasma and milk from dairy cows fed lucerne-based diets (Calderón *et al.* 2007). Some of these compounds influence the sensory properties of milk and milk products. For example, carotenoids confer a yellow colour to milk that is perceived as a positive standard factor indicating pasture feeding. Forage diets improved the content of carotenoids and vitamins (such as vitamins A and E) in milk (Meglia *et al.* 2006; Nozière *et al.* 2006; Calderón *et al.* 2007) when compared to grain-based diets. Grigioni *et al.* (2007) evaluated the changes of colour in whole milk powder (WMP) in relation to the milking season and the thermal treatment applied during milk processing. The authors related the seasonal behaviour observed for the L* and b* parameters in WMP to the chemical composition of the raw milk.

In addition, carotenoids can act as non-enzymatic antioxidants, and together with vitamin E and ubiquinol operate as radical scavengers in the lipid phase, whereas vitamin C and flavonoids are active in the water phase (Lindmark-Månsson and Åkensson 2000).

The mechanisms involved include a complex interplay of pro- and anti-oxidants consisting of low-molecular-weight compounds. Oxidative deterioration of milk and the consequent development of off-flavours and off-odours are related also to fatty acid composition (particularly long chain polyunsaturated fatty acids), and the presence of transition metal ions, which are known to propagate lipid oxidation and can give rise to several secondary oxidation products (Ford *et al.* 1986; Leland *et al.* 1987; Rao and Murthy 1987).

The complexity of lipid and protein oxidation events and the counteracting activity of antioxidants make it difficult to describe with a single biochemical measurement, and several

approaches are needed to describe the whole picture. The use of an electronic nose (EN) proved to be a useful instrumental method to discriminate the odour profile of meat samples with different antioxidant status. Moreover, in a ruminant system, it contributed to differentiate vitamin E supplemented from non-supplemented grain-produced meat (Descalzo *et al.* 2007). Biolatto *et al.* (2007) applied electronic nose methodology with SPME-GC to evaluate the seasonal changes in whole milk powder odour characteristics. Applying linear discriminant analysis, seasonal variations in the odour profiles assessed by electronic nose were observed. A better classification outcome was obtained when volatile compounds and electronic nose data were analysed together, obtaining a success rate of 89.5% and 70.7% of the original cases and after cross-validation, respectively.

In the present study, cows were fed either grazed lucerne (*Medicago sativa*) or sorghum silage. It was expected that milk from cows fed lucerne would have a higher concentration of antioxidant vitamins, mainly α-tocopherol, and β-carotene than milk from cows fed silage, and that the antioxidant status could be related to the odour profile.

Materials and methods

Animals, design and feeding

Diets were isoenergetic (National Research Council 2001) and were formulated in order to assure differences concerning the presence of lucerne pasture. In the first diet, grazed lucerne (ALF) assured at least 60% of dry matter (DM) on dietary basis. Grazing consisted of rotational strip-grazing systems within daily paddocks at high herbage allowance (more than 30 kg DM/head/day). Herbage allowance was determined by cutting 1 m^2 squares at a stubble height of 4-5 cm with scissors (Meijs 1981). The strips were determined daily in order to maintain the herbage yield over the set levels. In the second diet, grain sorghum silage (SS) constituted at least 50% of forage. The SS diet also contained soy expeller and sunflower pellets (3.5 and 1.1 kg/d per cow, respectively) and hay (1.5 kg/d per cow). Concentrates were offered during milking at a fixed rate of 9.5 and 6 kg/d per cow for the ALF and SS diets, respectively, into two equal feeds during milking. The DM herbage intake was estimated daily as the difference between pasture offered and refused. Estimated intake of concentrate, hay and silage was calculated as the difference between the amount offered and rejected, based on the daily supply of each dietary ingredient.

The experiment was conducted during spring at the National Institute of Agricultural Technology in Rafaela (Santa Fe province, Argentina: 31º11'S; 61º30'W). During a first pre-experimental period of four weeks, 10 Holstein cows were fed only the SS diet. This pre-experimental period served as covariate and thereafter five cows were randomly assigned to the ALF diet, and the other group remained as SS control during 60 experimental days (spring period). Cows on the SS diet were maintained in dry-lot pens and all cows had access to shade, feed and water.

The ALF diet resembles productive conditions applied within the central region of Argentina in the spring-summer period, whereas the SS diet responds to a typical winter productive system, without access to fresh lucerne.

Milk samples

Individual milk samples were collected at 0, 20, 40 and 60 days after the initial switch to the ALF or SS diet. Milk was collected once at each sampling period for all the analyses performed, except for protein and fat content, total solids and non-fat solids, which were performed weekly.

Milk samples were stored at -20°C for approximately one month until analysis for α- and γ-tocopherol, β-carotene, retinol, vitamin D_3, and E-Nose odour profile. Samples for TBARs and FRAP were stored at -80 ºC for approximately one month until analyses were performed.

Feed samples

Samples of sorghum silage, lucerne, hay, concentrate, soybean pellets and sunflower expeller were taken at the same experimental times, pooled and stored at -20°C for approximately two months until analyses for α- and γ-tocopherol, β-carotene and retinol were completed.

Lipid oxidation measurement

To assess the amount of lipid oxidation, the content of thiobarbituric acid reactive substances (TBARS number) was used. The acid precipitation technique described by Havemose *et al.* (2004) was applied. Briefly, triplicate aliquot samples of milk (3 mL), were homogenised with the addition of 2.4 mL of 0.25% thiobarbituric acid (Sigma Aldrich, Buenos Aires, Argentina) in trichloroacetic acid (Merck, Darmstadt, Germany) 10% solution with vortex for 2 min. After 30 min centrifugation at 6,000 g at 4ºC, supernatants were filtered (Whatman 0.2µm filters) and derivatised for 1 h at 70ºC. TBARS were determined at maximum absorption (530 nm) and concentrations were calculated using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, St. Louis, USA) as standard within the range from 0 to 0.5 µM. Results were expressed as mg of malonaldehyde (MDA) equivalents/kg fresh milk.

Fat-soluble vitamins determination

Fat-soluble vitamins were extracted as described by Buttriss and Diplock (1984). Sample aliquots (3 mL) were mixed with 1% pyrogallol (Sigma-Aldrich, St. Louis, USA) in ethanol. Saponification was performed for 30 min at 70ºC with 10 N KOH (Merck Química Argentina, Buenos Aires). Samples were then extracted twice with n-hexane (J.T. Baker, Phillipsburg, USA; HPLC grade), evaporated under nitrogen flow, dissolved in absolute ethanol (J.T. Baker, Ecatepec, Mexico; HPLC grade) and filtered through a 0.45 µm pore nylon membrane before injection. All samples were analysed by reverse phase high-performance liquid chromatography (HPLC).

Ten grams of milled feed samples were homogenised in 20 mL potassium phosphate buffer pH 7.2 and 1 g of homogenised samples was extracted as explained above.

HPLC conditions for electrochemical detection

An HPLC pump (model P4000) with a membrane vacuum degasser and a 20 µL loop injector were used (Thermo Separation Products, San Jose, USA), connected to an Alltima C18 column (250 mm x 4.6 mm), 5 µm particle size (Alltech, Des Plaines, USA). The electrochemical detector (Decade, Antec Leyden, Zoeterwoude, The Netherlands) was equipped with a flowcell with Ag/AgCl and glassy carbon reference and working electrodes, respectively.

The mobile phase used for electrochemical detection was modified from the technique described by de Rijke *et al.* (1997). The flow-rate was 1 mL/min and the reference cell was set at + 700 mV. Recovery of α-tocopherol, γ-tocopherol and β-carotene was 98%. Calibration curves were performed with DL-α-tocopherol (Merck, Darmstadt, Germany), γ- tocopherol and β-carotene (Sigma-Aldrich, St. Louis, USA) standards diluted in absolute ethanol.

HPLC conditions for diode array detection

The HPLC system (Thermo Separation Products) was equipped with a quaternary pump (model P4000), membrane vacuum degasser and autosampler (AS4000), with a 100 µl loop injector connected to an Alltima C18 column (250 mm x 4.6 mm), 5 µm particle size (Alltech). The diode array detector (UV600LP Spectrasystem; Thermo Separation Products) was used with two simultaneous channels set at 325 nm and 265 nm for retinol and vitamin D_3 detection, respectively.

The mobile phase consisted in an isocratic mixture of acetonitrile in methanol (75:25) at 1 mL/min. Recovery of retinol and vitamin D_3 was 98% and 97%, respectively. Calibration curves were performed with all-trans-retinol and vitamin D_3 standards (purchased from Sigma-Aldrich, Buenos Aires, Argentina) freshly prepared in absolute ethanol.

FRAP assay

Antioxidant compounds such as α-tocopherol, trolox, vitamin C, uric acid and billirubin, among others, are able to reduce ferric- to ferrous-tripyridyltriazine which develops a blue colour (Benzie and Strain 1996) with an adsorption maximum at 593 nm.

For milk samples, this assay was adapted from Descalzo *et al.* (2007) to measure endogenous ions that could react with TPTZ developing blue colour (i.e. endogenous Fe^{+2}). Milk samples (1 g) were centrifuged at 10,000 x g at 4ºC and 83 µL aliquots were mixed with 2500 µL of FRAP reagent containing 10 mM TPTZ (Sigma Aldrich, Buenos Aires, Argentina) in 40 mM HCl and 20 mM $FeCl_3$ (Sigma Aldrich) added to 300 mM acetate buffer. Samples were incubated for 10 min at 37ºC for end-colour development, placed on ice, filtered through 45 µm nylon membrane and absorbance measured at 593 nm. For determination of ascorbic acid content (FRAPC), samples were incubated with 4 U/mL of ascorbate oxidase (Sigma-Aldrich, Buenos Aires, Argentina) for 15 min at 30ºC, before incubation with the assay buffer. Endogenous Fe^{+2} content (FRAPo) was determined with a TPTZ/HCl solution without the addition of $FeCl_3$ to the reaction mixture and considered as blank for each measurement.

FRAP activity was calibrated with a ferrous sulphate ($Fe_2SO_4.7H_2O$, Sigma-Aldrich, Buenos Aires, Argentina) curve within the range from 100 to 1000 µM and results were expressed as Fe^{+2} equivalent in µM.

Odour profile determination

The odour profile of milk samples was measured by an electronic nose coupled with a mass spectrometer system (NE-MS, Alpha Prometheus, Alpha MOS, Toulouse, France). Samples were defrosted and blended by shaking in a water bath at 40ºC during 5 min. For headspace analysis, 3 mL of sample were placed in a 10 mL vial and sealed with magnetic caps, including septum for headspace analysis. The methodology applied to milk samples involves incubation at 50ºC for 10 min with an agitation speed of 500 rpm (Autosampler HS100, Alpha MOS). For electronic nose analysis (Alpha Fox 4000, Alpha MOS), 1 mL of headspace sample was injected and the acquisition was obtained with 18 semi-conductor oxide metallic sensors (MOS). The acquisition time was 120 s, with a frequency of 0.5 s. For statistical analysis, the value of the maximal resistance regarding to the base resistance of each sensor was used.

For MS analysis, an aliquot of 4 mL of headspace was injected in a quadrupole mass spectrometer (Alpha Kronos, Alpha MOS) with an electronic impact (70 eV) ion source. The resulting spectrum was obtained of an average of 90 scans measured during the headspace injection. The range of mass/charge (m/z) ratios studied was 50-200 amu.

Statistical analysis

Milk concentrations of fat-soluble micronutrients were related to the milkfat content determined in the corresponding sample and expressed as micrograms per gram of fat. Data were analysed statistically as repeated measurements using the MIXED procedure of the SAS software package (v8e; SAS Institute, Cary, USA), with group (ALF and SS), time (d 0 to 60) and their interactions as fixed effects, and animal as random effect. After the pre-experimental period, at day 0, the diet effect was not significant and, therefore, it was included in the model. Means separation was performed using the PDIFF option of the LSMEANS procedure. The experimental unit used for intake, milk yield and milkfat, and protein contents was mean per animal per week. The experimental unit used for vitamin concentration, FRAP, FRAPC, TBARS and odour profile (sensor values), in milk was the data per animal per day (0, 20, 40 and 60).

In all milk samples, the relationship between biochemical measurements and E-Nose sensor array values was evaluated using Pearson correlation. After that, hierarchical clustering (Ward method) was performed to describe the relationship between samples corresponding to the ALF or SS variables as a result of the combination of variables with significant correlation ($p<0.05$). Furthermore, data were subjected to a linear discriminant analysis (LDA) as a classification procedure. This method maximises the variance between and within categories. A stepwise method was applied for

variable selection. The criterion used was the significance of F with a maximum of 0.05 to enter and a minimum of 0.10 to exit. First, the variables x_i were transformed into standardised (normalised) z_i values where $z_i = (x_i - \bar{x})/s$. SPSS Advanced Statistics 12 software (SPSS, Chicago, USA) was used.

Results and discussion

Dietary differences in the incorporation of antioxidants into milk

In order to determine feeding differences on natural antioxidant vitamins delivery, the two contrasting diets were analysed.

The ALF and SS diets were isoenergetic (2.46 and 2.47 Mcal/kg DM, respectively), with a higher content ($p<0.05$) of protein in ALF (16.3 vs. 14.3 % DM) and higher fibre ($p<0.05$) in the SS diets (35.7 vs. 33.9% DM for neutral detergent fibre and 19.3 vs. 16.7% DM for acid detergent fibre).

Diets showed different profiles for fat-soluble vitamins (Table 1). ALF had higher concentration of α-tocopherol, β-carotene, and retinol than the SS diet. The silage diet showed higher levels of the γ- and δ-tocopherol isomers due to the contribution of soy expeller.

The composition of the diets was partially reflected in the milk. Daily production of α-tocopherol, β-carotene, retinol and vitamin D_3 was higher for the lucerne-produced milk when compared with their silage counterparts and lower for γ- tocopherol. In addition, the lucerne diet favoured higher milk production and protein yield in comparison to the silage diet, whereas the fat content was higher in the SS than in the ALF milk (Table 2).

As shown in Tables 1 and 2, the relationship among vitamins offered through dietary delivery and their incorporation into milk, varied among diets and the vitamin considered.

For retinol, 0.91% of the theoretical offer of dietary retinol was incorporated into milk for the SS treatment, whereas for the ALF diet the percentage was 0.25%. For vitamin E isomers, α-tocopherol was more efficiently incorporated (7.11% in ALF and 4.61% in SS) than γ-tocopherol (0.09% in ALF and 0.25% in SS) and δ-tocopherol was not detected either in the SS or ALF milk samples. The incorporation of β-carotene was similar for both diets, with values of 2.87% and 3.43% for the SS and ALF treatments, respectively.

After the pre-experimental period, at the beginning of the experiment (t0) all cows showed similar concentrations of antioxidant vitamins in milk. After a 20-day feeding period, significant differences within vitamin concentrations were found between ALF and SS groups ($p<0.001$) and these differences persisted through time until day 60. These results are shown in Table 3, demonstrating a clear discrimination which differentiated milk samples regarding the contrasting diets.

Clearly, the lucerne diet favoured the incorporation of α-tocopherol, retinol, β-carotene and vitamin D_3 into raw milk. Meanwhile, a less-abundant isomer of vitamin E, γ-tocopherol, was significantly higher in SS than in ALF milk, probably due to the presence of soy expeller in the SS diet. Soy expeller contributed with the highest proportion of γ- and δ-tocopherol. However, the different isomers were selectively incorporated in the milk. This result is in accordance with the selectivity of tocopherol isomers in mammals, which is $\alpha > \gamma > \delta$. In our system, δ- tocopherol was not detected in milk.

A higher incorporation of vitamin D_3 in the ALF milk could be associated with the higher retinol content. Both vitamins could be transported by beta-lactoglobulin into the mammary gland and thereafter incorporated into the milk. Binding of vitamin D and retinoids to beta-lactoglobulin was demonstrated by Wang *et al.* (1999).

Figure 1 shows a significant positive relationship between these vitamins (Pearson correlation coefficient R= 0.8379; $p<0.05$).

Antioxidant activity was measured by the FRAP assay. Statistical analysis showed significant differences for diet effect ($p<0.0001$), but not for time and interaction effects. Means and standard deviation corresponding to each dietary group are indicated for the ALF and SS milks: 540.25 ± 67.93 and 426.27 ± 49.01, respectively. These results show that the ALF diet promoted higher antioxidant activity in milk.

However oxidation determined by thiobarbituric acid reactive substances (TBARS) was similar for both types of milk. This could be due to the higher percentage of long chain polyunsaturated fatty acids (C 18:2 trans, CLA and C 18:3) in ALF milk (Páez *et al.* 2007), which are more susceptible to oxidation. Indeed, the peroxide value in ALF milk was 0.06, whereas the SS milk showed 0.03 (Páez *et al.* 2007).

Table 1: Average intake of antioxidant vitamins for lucerne (ALF) or sorghum silage (SS) diets.

Diet	ALF			SS					
	Conc.	Lucerne	Total	Conc.	Sunflower pellets	Soybean expeller	Sorghum Silage	Hay	Total
DM (kg/day/cow)	8.10	13.42	21.52	5.38	1.03	3.13	9.31	1.38	20.23
Retinol g/day/cow	0.83	18.28	19.11	0.55	0.01	0.07	1.85	0.34	2.82
δ-tocopherol mg/day/cow	0.00	0.00	0.00	0.00	0.00	105.18	174.86	0.00	280.04
γ-tocopherol mg/day/cow	680.27	292.08	972.35	452.17	12.32	822.46	46.50	25.29	1358.7
α-tocopherol mg/day/cow	147.94	270.22	418.16	98.33	19.80	57.04	94.77	69.12	339.06
β-carotene mg/day/cow	8.78	110.43	119.21	5.84	2.88	2.44	0.00	22.28	33.44

Notes:
Conc.: concentrate feed *DM: dry matter* *ALF: lucerne diet* *SS: sorghum silage diet*

Ascorbic acid was estimated in the ALF and SS milk by the FRAPC method and both types of milk showed similar concentrations (148.4± 37.7 vs. 149.7± 29.1 µg/L for the ALF and SS milk, respectively), indicating that the differential antioxidant activity in lucerne-produced milk could not be attributed to this compound.

Dietary differences and aroma profile

The E-Nose approach was successfully used to discriminate groups of samples in relation to their odour profile. In order to determine whether the antioxidant status in milk could be related to the odour profile as determined by EN/MS, the relationship between milk vitamins, FRAP activity and the patterns generated from the sensor array were analysed using Pearson Correlation coefficients. The sensor called LY2/gCTI correlated positively with α-tocopherol, β-carotene and retinol, and negatively with γ-tocopherol ($p<0.05$). The sensor P30/1 correlated positively with retinol and the sensor P10/1 showed a positive correlation with γ-tocopherol ($p<0.05$).

Table 2: Milk production and composition.

Daily production	ALF	SS	Std error
Milk (L/day/cow)	31.78a[1]	26.14b	0.833
Fat (%)	2.85b	3.25a	0.076
Protein (%)	3.22	3.20	0.043
NFS (%)	8.77	8.79	0.025
TS (%)	11.63b	12.03a	0.066
Lactose (%)	4.78	4.95	0.021
Yield (kg fat/animal/day)	0.899	0.851	0.0307
Yield[2] (kg PB /day per cow)	1.019a	0.841b	0.0249
Vitamins[3] (mg/animal/day)			
Retinol	47.63a	25.71b	2.916
Vitamin D_3	24.69a	15.04b	3.198
α-tocopherol	29.72a	15.61b	1.593
γ-tocopherol	0.88b	3.46a	0.361
β-carotene	4.09a	0.96b	0.212

Notes:
1. Means with different letters within the same row are significantly different ($p<0.05$).
2. PB, Protein Balance
3. Values are means for 5 cows per group and 4 repetitions each.

Table 3: Fat-soluble vitamins in raw milk from lucerne or silage-fed cows. Results are expressed in *µg*/g of milkfat.[1]

Diet	Day 0	Day 20	Day 40	Day 60
α-tocopherol (*µg*/ g fat)				
ALF	17.93±3.61 c	44.26±5.77 a	37.44±2.17 ab	35.83±4.50 b
SS	15.59±3.07 c	16.46±1.93 c	17.47±2.32 c	21.89±2.01 c
β-carotene (*µg*/g fat)				
ALF	2.94±1.09 b	5.19±1.25 a	4.96±0.83 a	5.61±0.89 a
SS	2.41±0.42 bc	1.08±0.16 c	0.91±0.26 c	1.42±0.43 bc
γ-tocopherol (*µg*/ g fat)				
ALF	4.69±1.16 ab	1.13±0.29 d	1.04±0.36 d	1.20±0.16 d
SS	4.62±1.31 ab	3.76±0.59 bc	5.80±1.03 a	2.96±0.33 c
Vitamin D_3 (*µg*/ g fat)				
ALF	15.30±1.71 d	34.17±9.66 abc	37.78±13.27 ab	40.71±10.84 a
SS	14.96±3.15 d	13.93±3.38 d	18.89±2.49 cd	24.96±6.62 bcd
Retinol (*µg*/ g fat)				
ALF	31.31±6.67 b	62.54±15.40 a	59.99±11.48 a	60.65±8.50 a
SS	28.64±6.95 b	22.77±4.02 b	33.30±8.70 b	36.71±2.96 b

Notes:
1. Values are means for 5 cows per group and time.
2. Means with different letters within the same row are significantly different for each vitamin ($p<0.05$).

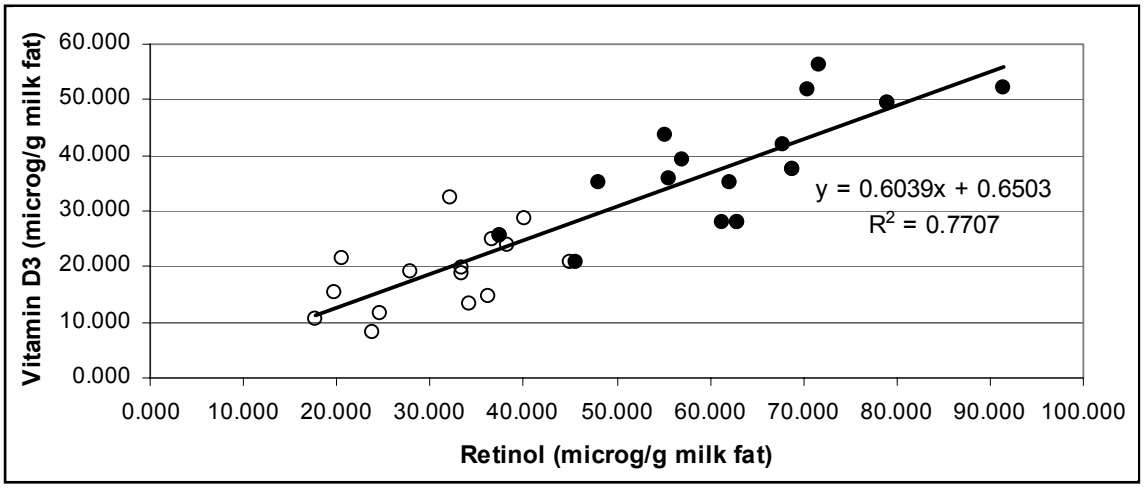

Figure 1: Relationship between Retinol and vitamin D3 in milk samples.

● ALF milk, ❍ SS milk.

In order to describe the relationship among dependent experimental variables and their influence over the milk samples, principal component analysis (PCA) was applied to the standardised values of fat-soluble vitamins, FRAP and the individual values of EN/MS sensors. Figure 2 shows two clusters corresponding to the ALF or SS milks that were clearly separated (100%) by their antioxidant status and odour profiles. The separation was obtained as a function of feeding when biochemical variables and sensor LY2/gCTI were analysed together.

Discriminant analysis was used for:

- detecting the variables that allow the researcher to discriminate between different (naturally occurring) groups;
- for classifying cases into different groups with better accuracy.

Therefore, this approach was applied to the complete set of measurements in raw milk.

The Stepwise method selected β-carotene and sensor LY2/gCTI as the variables that contributed the most to the classification of milk into their dietary treatment. Two linear Fisher's discriminant functions were defined according to dietary treatments with a success rate of correct classification of each sample of 100% either for the original cases or after cross-validation ($p<0.0001$). As shown in Figure 3, the SS or ALF milk were clearly differentiated among each other. In agreement with Laguerre *et al.* (2007) there is not a unique method to describe the antioxidant capacity of biological samples. The complexity and diversity of mechanisms that contribute to the onset of oxidation and the mechanisms that counteract oxidative reactions involve multiple pathways. Therefore, it is difficult to determine the exact weight to be allocated to each variable in order to ensure the consistency and interpretation of this diverse set of data. The multivariate approach applied to a set of biochemical variables related to a defined set of samples pictures the global understanding of complex biochemical mechanisms occurring in milk.

The use of an electronic nose proved to be a useful instrumental method to discriminate the odour profile of milk samples with different antioxidant status.

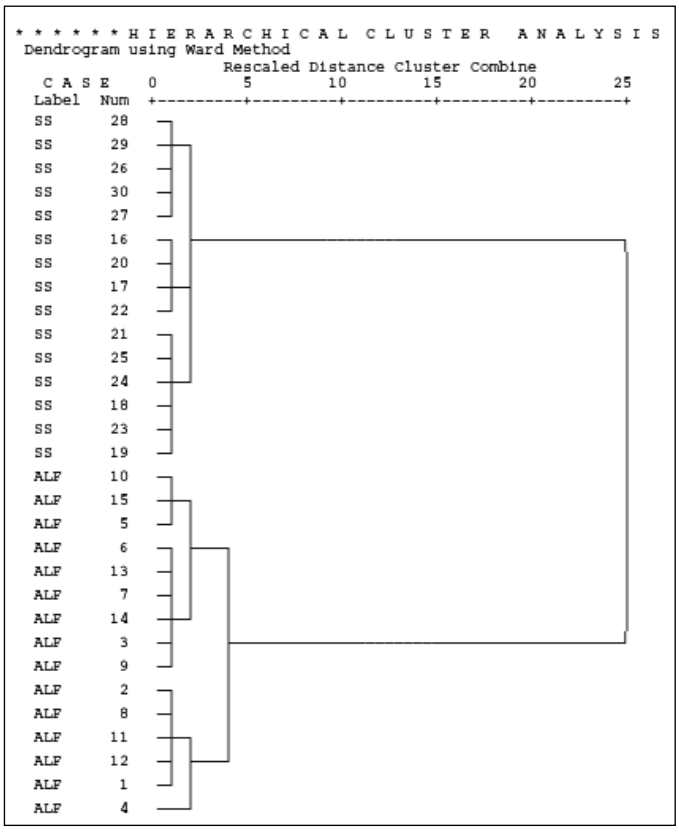

Figure 2: Hierarchical clustering analysis of milk samples. Variables: standardised (zi) values for: α-tocopherol, β-carotene, γ-tocopherol, retinol, vitamin D_3, FRAP, LY2/gCTI.

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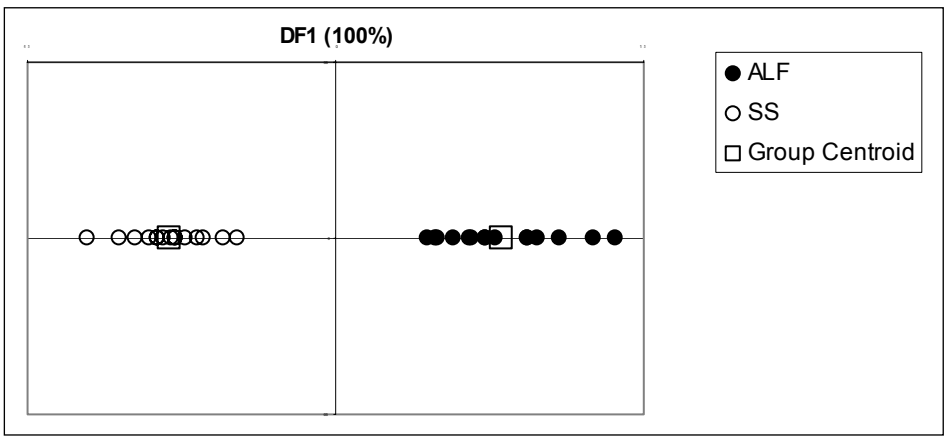

Figure 3: Linear Discriminant Analysis of milk samples obtained with the Stepwise method.

Fisher's Discriminant Functions ($p<0.0001$)

ALF = -6.459 -3.124 zi(Ly2sgCTI) +10.319 zi(β-carotene)

SS = 6.459 +3.124 zi(Ly2sgCTI) -10.319 zi(β-carotene)

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