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RESEARCH PAPER

Potential of *n*-alkanes as biomarkers in grass-feeding steers

María Eugenia Martínez[1], Aldo Benavente[2], and Rodrigo Morales[1]
[1]Instituto de Investigaciones Agropecuarias, INIA Remehue, Ruta 5 Norte km 8. P.O.Box 24-0, Osorno, Chile
[2]Universidad Austral de Chile, Campus Isla Teja, Facultad Ciencias Agrarias, Escuela de Graduados. Casilla 657, Valdivia, Chile

Abstract

M.E. Martínez, A. Benavente, and R. Morales. 2017. Potential of *n*-alkanes as biomarkers in grass-feeding steers. Cien. Inv. Agr. 44(3): 239-251. The objective of this study was to evaluate the effect of diet (grazing vs. pasture silage with concentrate) and changes in diet on *n*-alkane concentrations in cattle feces. The experiment lasted 35 d (15 d of adaptation and 20 d of sampling). Thirty Holstein-Friesian steers were divided into three groups of ten, and each group was randomly allocated to one of the following three treatments: GZ, diet consisting of 100% pasture; SC, diet consisting of pasture silage: concentrate in a 60:40 ratio; and MX, diet consisting of a gradual decrease in pasture and a gradual increase in SC. Fecal samples were taken daily from every animal, and their *n*-alkane content was analyzed using gas chromatography. The data obtained in this study showed detectable changes in *n*-alkane concentrations in steer feces. The changes were noticeable within two to three d after a change in diet, whereas concentrations of *n*-alkanes remained stable in the feces of animals that did not experience any change in diet. These results can be used as the basis for developing a tool that can determine the type of feed bovines received prior to slaughter.

Keywords: Authentication, beef, feedlot, grazing, traceability.

Introduction

Beef production in southern Chile is based on pasture feeding. There is substantial evidence in the literature indicating that grass-fed beef has higher levels of certain nutritionally desirable components, e.g., *n*-3 fatty acids, conjugated linoleic acid (CLA) and α-tocopherol (vitamin E), when compared to meat from animals fed in feedlot systems, i.e., receiving large quantities of concentrate (Morales *et al*., 2012, 2015). At the same time, consumers consider grass-fed beef to be healthier (Morales *et al*., 2013), and this production system is also considered more amenable to animal welfare and more environmentally sustainable. Consumers are increasingly interested in the authenticity of the food they purchase. To differentiate among similar products, consumers base their opinion on attributes such as geographical location, environmental stewardship and sensory quality or functionality. Confirming dietary history is necessary to authenticate the geographic origin of beef; therefore, non-invasive authentication methods that are rapid, economic, reliable and easy to use are needed (Osorio *et al*., 2013).

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Corresponding author: rmorales@inia.cl

To date, the available and most reliable markers or tracers developed by research groups in this field are of two types: a) indirect metabolic markers that derive from the animal's metabolism and b) direct markers in the animal's feed. Efforts have been made to use the profiles of stable isotopes, fatty acids and other organic compounds in the meat and adipose tissue of animals (Serrano *et al*., 2011), DNA (Nicoloso *et al*., 2013), carotenoids and vitamin A in plasma; additionally, other biological fluids (Álvarez *et al*., 2015) could be used as discriminators of geographic origin, type of diet or production system of meat. Indirect authentication systems usually require slaughtering the animal to take samples, and to some degree, they are subject to adulteration in the results. In this case, strategies to detect such adulterations are needed (Röhrle *et al*., 2011).

There have been attempts to develop methods that do not require samples from slaughtered animals, such as indirect metabolic markers in urine or the analysis and comparison of stable C, N and S isotopes in tail hairs (Monahan *et al*., 2012). Despite the utility of these studies for origin certification and type of feed, it is not possible to detect short-term changes in diet using these procedures.

Plant biomarkers are compounds that the animal cannot metabolize. Their presence in animal tissue and/or animal products is undoubtedly related to the feed the animal has consumed. A marker that detects short-term changes and does not require slaughtering would complement the techniques to authenticate grass-fed beef. According to Prache *et al*. (2005), the low persistence of the marker is of interest to discriminate short-term changes in diet.

There is vast literature supporting the use of alkanes as a tool to determine the composition of diets in large and small ruminants (Martínez and De la Barra, 2014). The *n*-alkane profile in feces reflects the diet, and it varies according to changes made in diet. Given this, a fecal *n*-alkane profile can be related to a specific type of feed. Benvenutti *et al*. (2014) found that *n*-alkanes were effective in monitoring the progressive reduction of hay in the diets of oxen in the context of detecting pasture feeding versus feeding in confinement systems. Currently, an analysis of fecal concentrations of *n*-alkanes has not been used to determine the type of diet animals have been consuming (grazing vs. concentrates). To the best of our knowledge, no research has yet focused on *n*-alkanes as a tool for certifying meat by differentiating its origin and type of system used in the rearing of animals. Additionally, no research has focused on confirming the value of these markers to complement other biomarkers to identify the origin and feeding system.

To use *n*-alkanes as indicators, it is necessary to be able to determine the time that has passed since the change in the diet was made, and the moment of this change is reflected in fecal *n*-alkane concentrations. The objectives of this study were to determine the effect of diet (grazing vs. pasture silage with concentrates) on *n*-alkane concentrations in cattle feces and to determine the amount of time after a change in the diet, which is reflected in *n*-alkane fecal concentrations.

Material and Methods

Animals and feeding

The experiment was carried out in autumn (May and June 2013). A group of 30 Holstein-Friesian steers of similar age (autumn birth and 14 mo old) were selected from the animal production unit of the Instituto de Investigaciones Agropecuarias (INIA) Remehue (40°31'S; 73°3'W; altitude, 73 m.a.s.l.; annual rainfall, 1,300 mm). The initial average live weight was 283.5 ± 27.2 kg, and the initial mean body condition score was 3.0 according to the five-point system of Edmonson *et al.* (1989). The experiment lasted 35 d, including 15 d as an adaptation period for animals to the experimental conditions and 20 d for data collection. For the first 15 d, the steers grazed in the same pasture used for the experimental adaptation period. After this

time, animals were ranked according to their live weight and categorized by weight into one of the three treatments. Treatments were then randomLy assigned to each of the groups (10 steers per treatment) as follows: a) Grazing Treatment (GZ): the steers received a 100% grazing diet in the same pasture in a strip-grazing system; b) Silage and Concentrate Treatment (SC): the steers were placed in two collective pens (five steers each) and received a diet consisting of pasture silage and a commercial pelleted mixture concentrate in a ratio of 60:40; and c) Mixed Treatment (MX): in this group, pasture treatment was gradually replaced by the SC treatment. For the first five d, steers were 100% grazing; from d 6 to d 10, they received 4 kg of dry matter (DM) daily from pasture grazing, the same as that of the GZ treatment, and 2 kg of DM of pasture silage: concentrate at a 60:40 ratio, the same as that of the SC treatment (66% and 33% from the GZ and SC diets, respectively). From d 11 to d 15, they received 2 kg of DM d^{-1} from the GZ treatment and 4 kg of DM d^{-1} from the SC treatment (33% and 66% from the GZ and SC diets, respectively). Finally, from d 16 to d 20, they received a feed of 100% SC treatment. Animal dry matter intake (DMI) was set at 6 kg d^{-1}. The steers' initial and final weights were recorded, and the average daily gains were calculated. The composition of the commercial concentrate (Concentrados Cisternas®, Osorno, Chile) was (in g kg^{-1}, as-fed basis): wheat bran (500), sifted oats (250), oat ground beans (60), rice bran (50), marigold seeds (50), peanuts (50) and flax expeller (40). The pasture silage offered to animals was harvested the previous spring in the same sown grassland that was utilized by the grazing animals.

The pasture consisted of sown grassland with an approximate yield of 12 tons DM ha^{-1} $year^{-1}$. The botanical composition from paddock was 97% perennial ryegrass (*Lolium perenne*) and 2.9% white clover (*Trifolium repens*). A paddock of 3.5 ha was subdivided into 2 sub-paddocks of similar size. Both the GZ and MX groups individually grazed the sub-paddocks under strip grazing, using temporary electric fencing. The grazing animals were kept in the same paddock all d. At the beginning, the GZ and MX groups were offered the same total herbage allowance of 6 kg of DM, i.e., more than 3 cm per animal per d. Similar herbage allowances per animal were obtained by adjusting the grazing surface of each treatment according to the pre-grazing and post-grazing herbage mass. On d 6, the MX treatment was supplemented with the SC diet on the pasture, and the herbage allowance was adjusted based on the quantity of SC supplemented in order to complete the DMI set for each steer. During the last five d of the MX treatment, steers were placed in two collective pens (five steers each) and received SC treatment. The pre-grazing sward height was recorded daily using a rising plate meter (F200, Farmworks®, Feilding, New Zealand) in 80 random locations within the area to be grazed the next d. Post-grazing sward heights were recorded daily in 20 locations randomly chosen within each treatment area grazed the previous d. Mean herbage DMI per animal was estimated daily according to the difference between the pre- and post-grazing herbage mass at ground level, divided by the number of steers per treatment. Herbage mass above ground level was estimated from compressed herbage heights by a linear equation developed for naturalized and sown pastures in southern Chile (Canseco *et al*., 2007).

The offers and refusals of supplementary feed for MX and SC treatments were weighed daily, and this information was used to calculate DMI. A mineral mix (100 g d^{-1}, Vetersal Pastoreo, Veterquimica®, Santiago, Chile) and clean fresh water *ad libitum* were offered throughout the experiment. The procedures used in this experiment were approved by the INIA Animal Care and Use Committee.

Sampling

To analyze *n*-alkane concentrations, samples of feces were taken directly from the rectum of every animal (at 9:00 am) on a daily basis, from d 0 until the end of the experiment on d 20. Representative samples

of the feed consumed by the animals (pasture, silage and concentrates) were collected on d 1, 6, 11 and 16. Simulated grazed pasture samples were hand-plucked from the pre-grazing herbage strips. All samples were weighed fresh and then dried in a forced air oven (RE 115; Redline; Binder®, Tuttlingen, Germany) at 60 ºC until constant weight.

The chemical content of feed samples was analyzed at the INIA Remehue Laboratory. Dry matter to 105 ºC (DM), crude protein, and ash were determined by the methods of AOAC (2005), whereas neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by the methods of Van Soest *et al.* (1991). Digestible organic matter was estimated from *in vitro* digestibility (Tilley and Terry, 1963). Data on the chemical composition of feeds are shown in Table 1.

N-alkane analysis

Part of the dry fecal samples and feed samples (approximately 5 g) were ground to a particle size of 10 µm in a micro-grinder (MM 200, Retsch®, Germany). *N*-alkanes in samples of feed and feces were extracted following the technique proposed by Mayes *et al*. (1986). Afterward, *n*-alkanes were quantified using gas chromatography (GC-2010 Plus; Shimadzu®, Kyoto, Japan) equipped with an automatic injector and a flame ionization detector. A capillary RTX-1 column (Restek®, Bellefonte, Pennsylvania, USA) of 30 m × 0.53 mm i.d. and a film thickness of 1.50 µm, using helium as a carrier gas, were used. The purified extract was re-diluted in heptane, and 0.4 µl was injected. Oven conditions were as follows: initial temperature of 230 ºC for 0.2 minute and 300 ºC for 18 min at an increase of 6 ºC per minute. The temperature of the injector was 233 ºC; the temperature of the detector was 350 ºC. The air flow was 420 mL per minute, hydrogen flow was 42 mL per minute, make-up gas was 30 mL per minute of helium, column flow was 10 mL per minute, and the split ratio was 1 mL per minute. *N*-alkanes were identified by calibration with a solution of *n*-heneicosane (C_{21}), *n*-docosane (C_{22}), *n*-tricosane (C_{23}), *n*-tetracosane (C_{24}), *n*-pentacosane (C_{25}), *n*-hexacosane (C_{26}), *n*-heptacosane (C_{27}), *n*-octacosane (C_{28}), *n*-nonacosane (C_{29}), *n*-triacontane (C_{30}), *n*-hentriacontane (C_{31}), *n*-dotriacontane (C_{32}), *n*-tritriacontane (C_{33}), *n*-tetratriacontane (C_{34}), *n*-pentatriacontane (C_{35}) and *n*-hexatriacontane (C_{36}). The *n*-alkanes C_{22} and C_{34} were used as internal standards, and they were added to the sample at the beginning of the extraction process. All the standard *n*-alkanes were purchased (Sigma Aldrich, St. Louis, Missouri, USA). The fecal concentrations of *n*-alkanes were corrected using the recovery data reported by Sánchez Chopa *et al*. (2012) for steers of the same breed. *N*-alkane concentrations for the different diets and feeding periods are shown in Table 2.

Statistical analysis

Live weight and daily gain were analyzed using an ANOVA, having the treatment as the main effect. *N*-alkane data from feces were analyzed by

Table 1. Chemical composition of the feeds used in the study.

	Pasture	Silage	Concentrate
Dry matter (g kg^{-1})	121±12.5	267±8.0	846±3.2
Crude protein (g kg^{-1} DM)	267±4.4	140±8.0	163±1.1
Digestible organic matter (g kg^{-1} DM)	809±15.3	788±0.7	702±8.8
Neutral detergent fiber (g kg^{-1} DM)	493±32.4	504±7.4	451±6.7
Acid detergent fiber (g kg^{-1} DM)	267±23.4	353±36.6	169±25.5
Ash (g kg^{-1} DM)	117±3.0	87±0.7	51±0.4

a mixed model procedure for repeated measures with SAS®, including treatment and time (from d 0 to d 20), and their interaction as fixed effects. The animal was included as a random effect. Least-square means were separated by Tukey's studentized range test.

Principal component analysis (PCA) was carried out based on data from the feces' *n*-alkanes, using the PRINCOMP procedure of the SAS®.

Results

The DM consumption of the three treatments was approximately 2% of the live weight of the steers (Table 3). Daily weight gain was greater (P<0.05) under the SC treatment than under the GZ treatment. Under the SC treatment, animals were confined, and therefore, energy loss by thermoregulation and movement was lower. In addition, the SC treatment included concentrates

Table 2. *N*-alkane concentrations (mg kg^{-1} of DM) in the feeds used in the study

n-alkanes	Treatment			
	GZ	MX		SC
		6-10 d	11-15 d	
C_{23}	1.41±0.435	2.86±1.181	4.55±2.021	6.09±2.883
C_{25}	10.54±0.677	13.86±3.331	15.62±4.826	17.51±7.369
C_{27}	30.53±2.070	26.54±3.883	27.61±5.363	28.40±8.242
C_{28}	8.16±0.927	6.31±0.134	5.44±0.286	4.77±0.592
C_{29}	117.54±18.212	87.90±3.690	84.31±4.207	78.69±3.512
C_{30}	16.67±2.436	11.44±0.295	9.54±0.618	7.56±0.752
C_{31}	225.84±53.24	149.71±6.467	133.49±9.937	115.25±13.115
C_{32}	12.83±1.529	9.09±0.324	6.98±0.393	4.88±0.534
C_{33}	168.02±14.85	114.47±3.693	77.66±7.649	41.64±10.323
C_{35}	21.04±2.335	14.53±0.764	9.71±0.567	5.13±0.705
C_{36}	7.26±1.80	5.18±0.230	4.98±0.085	4.81±0.062

GZ: Daily intake of 6 kg of DM d^{-1} of pasture; MX: Mixed treatment, d 1-5: 6 kg of DM d^{-1} of pasture; d 6-10: 4 kg of DM d^{-1} of pasture + 2 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40); d 11-15: 2 kg of DM d^{-1} of pasture + 4 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40); d 16-20: 6 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40); SC: Daily intake of 6 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40).

Table 3. Dry matter intake, initial and final live weight and daily weight gain of steers under the different dietary treatments

	Treatment			SEM
	GZ	MX	SC	
Dry matter intake (kg d^{-1})	5.5±0.25	5.6±0.53	5.5±0.39	
Initial body weight (kg)	287.0	284.0	279.5	4.97
Final body weight (kg)	308.5	313.5	319.5	4.77
Daily weight gain (kg d^{-1})	1.02^{b}	1.40^{ab}	1.90^{a}	0.132

^{ab}No letter or the same letter in a row indicate that the means are not significantly different (P > 0.05).

GZ: Daily intake of 6 kg of DM d^{-1} of pasture; MX: Mixed treatment, d 1-5: 6 kg of DM d^{-1} of pasture; d 6-10: 4 kg of DM d^{-1} of pasture + 2 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40); d 11-15: 2 kg of DM d^{-1} of pasture + 4 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40); d 16-20: 6 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40); SC: Daily intake of 6 kg of DM d^{-1} of pasture silage:concentrate (ratio 60:40).

and had greater energy density than that of the GZ treatment having the same DM content.

Table 4 shows the average concentrations of each *n*-alkane obtained in the feces for the three treatments. Significant differences were found in all cases considering time and treatment × time interaction ($P<0.05$). Regarding treatment effect, the highest concentrations of C_{23} occurred under the SC treatment ($P<0.05$), whereas no differences between the GZ and MX ($P>0.05$) treatments were observed for C_{27}. Both C_{23} and C_{27} showed higher concentrations under the GZ and MX treatments than under the SC treatment ($P<0.05$). The GZ treatment showed higher concentrations of *n*-alkanes from C_{27} to C_{35} than those under the MX and SC treatments ($P<0.05$).

Figures 1 to 3 present the mean *n*-alkane concentrations for odd alkanes (except C_{25}) for the three treatments in the study over time. Significant differences between the SC treatment and the other two treatments were observed in the concentration of C_{23} (Figure 1A), starting from d 1 and continuing until d 20, presenting a progressive increase during the first 5 d ($P<0.05$). The concentrations under the GZ and MX treatments showed differences at d 7 and 9 and from d 15 to d 20 ($P<0.05$). *N*-alkane C_{27} (Figure 1B) showed significant differences between treatments MX and SC from d 2 until d 8, as C_{27} had a higher concentration during this period under the MX treatment than under the SC treatment ($P<0.05$). The tendency changed from d 10 until d 20, the period in which the GZ treatment showed a higher C_{27} concentration than that of the MX and SC treatments ($P < 0.05$). From d 11 to d 20, the MX and SC treatments had similar concentrations of C_{27}.

The concentrations of C_{29} and C_{31} (Figure 2A and B) under the SC treatment decreased by d 2, and by d 10, the concentration of C_{29} did not differ between the SC and MX treatments, whereas by d 11, C_{31} concentrations were similar between these two treatments. For C_{33} and C_{35} (Figure 3A and B), there were significant differences among the three

Table 4. Effects of dietary treatment and time (daily sampling from d 1 to 20) on *n*-alkane concentrations (mg kg^{-1} of DM) of steer feces

n-alkanes	Average per treatment (feed type)			SEM	*P* value		
	GZ	MX	SC		Treat	Time	T*Treat
C_{23}	**2.70^{b}**	**4.03^{b}**	**8.09^{a}**	**0.124**	**< 0.001**	**< 0.001**	**< 0.001**
C_{25}	**24.8**	**24.6**	**25.6**	**0.226**	**0.079**	**< 0.001**	**< 0.001**
C_{27}	**76.5^{a}**	**66.1^{a}**	**54.3^{b}**	**0.772**	**< 0.001**	**< 0.001**	**< 0.001**
C_{28}	**14.4^{a}**	**11.9^{b}**	**7.76^{c}**	**0.156**	**0.005**	**< 0.001**	**< 0.001**
C_{29}	**220.9^{a}**	**193.6^{b}**	**163.6^{c}**	**2.021**	**< 0.001**	**< 0.001**	**< 0.001**
C_{30}	**31.0^{a}**	**24.0^{b}**	**15.4^{c}**	**0.364**	**0.05**	**< 0.001**	**< 0.001**
C_{31}	**442.2^{a}**	**352.8^{b}**	**271.0^{c}**	**4.651**	**< 0.001**	**< 0.001**	**< 0.001**
C_{32}	**36.0^{a}**	**25.9^{b}**	**14.1^{c}**	**0.476**	**0.05**	**< 0.001**	**< 0.001**
C_{33}	**496.8^{a}**	**331.6^{b}**	**130.6^{c}**	**7.951**	**< 0.001**	**< 0.001**	**< 0.001**
C_{35}	**72.3^{a}**	**49.2^{b}**	**19.4^{c}**	**1.147**	**< 0.001**	**< 0.001**	**< 0.001**
C_{36}	**10.7**	**10.3**	**10.3**	**0.066**	**0.422**	**< 0.001**	**< 0.001**
Σ *n*-alkanes	**1428.5**	**1094.2**	**720.5**	**16.2**	**< 0.001**	**< 0.001**	**< 0.001**

Treat: Treatment; T: Time

[ab]Means with the same letter in the same row are not significantly different ($P > 0.05$).

GZ: Daily intake of 6 kg DM d^{-1} of pasture; MX: Mixed treatment, d 1-5: 6 kg DM d^{-1} of pasture; d 6-10: 4 kg DM d^{-1} of pasture + 2 kg DM d^{-1} of pasture silage:concentrate (ratio 60:40); d 11-15: 2 kg DM d^{-1} of pasture + 4 kg DM d^{-1} of pasture silage:concentrate (ratio 60:40); d 16-20: 6 kg DM d^{-1} pasture silage:concentrate (ratio 60:40); SC: Daily intake of 6 kg DM d^{-1} of pasture silage:concentrate (ratio 60:40).

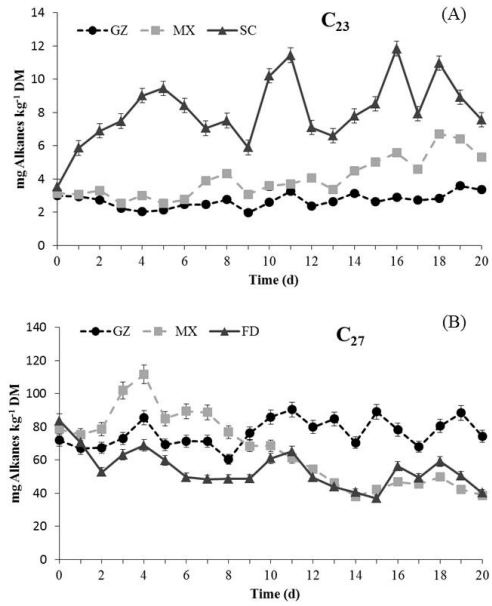

Figure 1. Average concentrations over time of the n-alkanes C23 (A) and C27 (B) in steer feces samples for the three treatments (P<0.05). GZ: Daily intake of 6 kg d^{-1} DM of pasture; MX: Mixed treatment, d 1-5: 6 kg d^{-1} DM of pasture; d 6-10: 4 kg d^{-1} DM of pasture + 2 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 11-15: 2 kg d^{-1} DM of pasture + 4 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 16-20: 6 kg d^{-1} DM pasture silage: concentrate (ratio 60:40); SC: Daily intake of 6 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40).

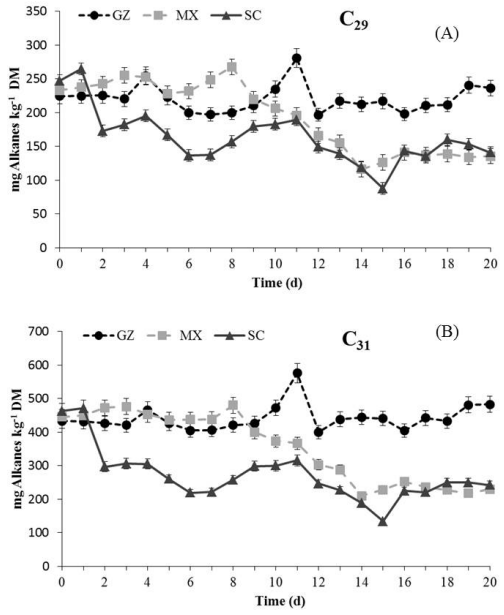

Figure 2. Average concentrations over time of the n-alkanes C29 (A) y C31 (B) in steer feces samples for the three treatments (P<0.05). GZ: Daily intake of 6 kg d^{-1} DM of pasture; MX: Mixed treatment, d 1-5: 6 kg d^{-1} DM of pasture; d 6-10: 4 kg d^{-1} DM of pasture + 2 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 11-15: 2 kg d^{-1} DM of pasture + 4 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 16-20: 6 kg d^{-1} DM pasture silage: concentrate (ratio 60:40); SC: Daily intake of 6 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40).

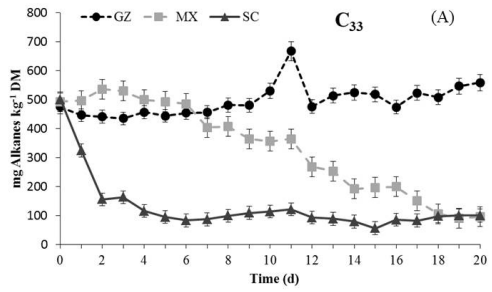

Figure 3. Average concentrations over time of the n-alkanes C33 (A) and C35 (B) in steer feces samples for the three treatments (P<0.05). GZ: Daily intake of 6 kg d^{-1} DM of pasture; MX: Mixed treatment, d 1-5: 6 kg d^{-1} DM of pasture; d 6-10: 4 kg d^{-1} DM of pasture + 2 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 11-15: 2 kg d^{-1} DM of pasture + 4 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 16-20: 6 kg d^{-1} DM pasture silage: concentrate (ratio 60:40); SC: Daily intake of 6 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40).

treatments from d 7 to d 17. From d 18 to d 20, there were no differences between the MX and SC treatments regarding the concentrations of C_{33} and C_{35} (P<0.05). The tendency was the same between d 10 and 20 for C_{29}, C_{31}, C_{33} and C_{35}, which presented their highest values under the GZ treatment (P<0.05).

The relationship among the observations of *n*-alkanes of the three treatments for d 0, 2, 7, 11, 18 and 20 using PCA are shown in Figure 4. At d 0, the observations of the three treatments are mixed, and there is no clear differentiation among treatments (Figure 4A). Between d 2 (Figure 4B) and

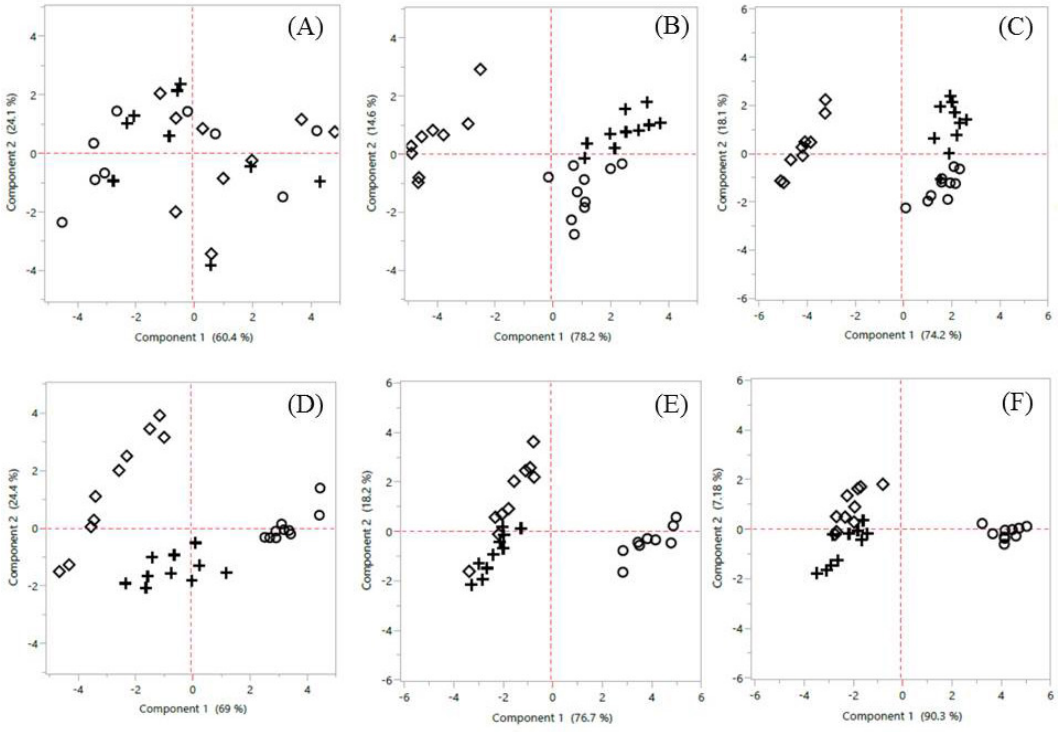

Figure 4. Projection of the observations of the three treatments for d 0 (A), 2 (B), 7 (C), 11 (D), 18 (E) and 20 (F) in the plot defined by two principal components: (○) GZ: Daily intake of 6 kg d^{-1} DM of pasture; (+) MX: Mixed treatment, d 1-5: 6 kg d^{-1} DM of pasture; d 6-10: 4 kg d^{-1} DM of pasture + 2 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 11-15: 2 kg d^{-1} DM of pasture + 4 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40); d 16-20: 6 kg d^{-1} DM pasture silage: concentrate (ratio 60:40); (◊) SC: Daily intake of 6 kg d^{-1} DM of pasture silage: concentrate (ratio 60:40).

d 7 (Figure 4C), the SC treatment observations are on the left side of the PC1 axis, whereas the GZ and MX treatment observations are on the right side of the PC1 axis. Starting on d 8 (no data shown), the MX observations begin to move to the center of the PC1 axis as shown in Figure 4D, and they remain there until d 14. From d 15 onward, the MX treatment observations are mixed with those of the SC treatment. On d 18 (Figure 4E), the MX and SC treatment observations are both on the left side of axis PC1, and they remain steady until the end of the experiment on d 20 (Figure 4F).

Discussion

Effect of feed on n-alkane concentrations

It is widely documented in the literature that odd-numbered carbon-chain *n*-alkanes (C_{25} to C_{35}) are present in higher concentrations than even-numbered *n*-alkanes in plants (Dove *et al*., 1996). In the present study, the most abundant *n*-alkanes for all treatments were C_{33}, C_{31}, C_{29}, C_{27} and C_{35}. As expected, *n*-alkane concentrations were higher in fecal samples than in the feed for all cases. Similar results were reported by Sánchez Chopa *et al*. (2012). The grass used as a dietary source for the GZ treatment and, in different proportions, for the MX treatment was composed mainly of perennial ryegrass (97%) and, to a lesser extent, (2.9%) white clover. The *n*-alkane concentrations observed in the feces of animals fed with grass in the present study were in the same range as those concentrations obtained in other studies performed with the same grass species, although the range can be quite wide depending on the literature consulted (Lewis *et al*., 2003; Dove and Mayes, 2005; Ferreira *et al*., 2007; Sánchez Chopa *et al*., 2012). Regarding this

concern, the concentration and profile of *n*-alkanes can vary widely within a species and among species according to the time of year, climatic and agricultural conditions, growth stage, and leaf/stem ratio (Dove and Mayes, 1991). Differences in the literature consulted could be due to sampling and laboratory conditions (e.g., temperature of drying) and/or the time of the year in which the samples were collected (phenological state). Regarding this, oven-drying at 60 ºC could affect the *n*-alkane concentrations in the present study, e.g., *n*-alkanes with a shorter chain length. Sánchez Chopa *et al*. (2012) found lower *n*-alkane concentrations in samples of feces oven-dried at 60 ºC than in those samples freeze-dried; e.g., concentrations of the C_{29} *n*-alkane were 24% less, of C_{31} were 9% less, of C_{33} were 14% less, of C_{27} were 12% less, and of the remaining *n*-alkanes were from 2% to 6% less. However, the low number of replicates (*n*=4) used by Sánchez Chopa *et al*. (2012) limits the conclusions regarding significant differences in *n*-alkane concentrations between the two drying methods (oven-dried at 60 ºC or freeze-dried). In addition, Elwert *et al*. (2006) found no differences in fecal *n*-alkane concentrations for oven-dried samples at 65 ºC or freeze-dried samples of ovine feces.

On the other hand, the contribution of white clover in the pasture used in the present study, despite its minor presence, could have also affected the concentrations of some of the markers. This result is important to consider in the experimental context in order to determine precisely the variations in *n*-alkane patterns during different seasons and in different pasture types.

The decrease in dietary and fecal concentrations of the *n*-alkanes C_{27} to C_{36} as the quantity of concentrates in the diet increased (Tables 2 and 4) is consistent with the fact that these markers are found mainly in the aerial parts of plants (leaves, stalks, and inflorescences) but not in the grains (Moshtagi Nia and Wittenberg, 2002). However, the concentrations of C_{23} and C_{25} in the diet and feces of animals that received the SC treatment were higher than those of the other treatments, although they were present in all three treatments in low concentrations (Table 2). Regarding this result, the concentrate used in this study was composed mainly of seed and cereal derivatives and sub-products. Oliveira *et al*. (2007) also found higher concentrations of C_{23} and C_{25} in concentrates based on ground corn, fish meal, soybean meal, wheat bran, and a vitamin-mineral mix rather than in the *Cynodon nlemfuensis* grass or in the leaf and stalk fractions of this species. The high mean dietary and fecal concentrations of the *n*-alkanes C_{23} and C_{25} under the SC treatment do not concur with the results reported in the literature, where these markers are usually found in lower concentrations than other *n*-alkanes. Valiente *et al*. (2003) found markers C_{23} and C_{25} in the diet of sheep consuming four distinct proportions of grain and barley straw; C_{23} was not present in the feces under any of the treatments. Although C_{25} was found in the ovine feces, the difference was not significant under any of the treatments. These results were attributed to the low concentration of C_{23} and C_{25} markers in the offered diets. However, if the C_{23} and C_{25} concentrations were consistent and not attributable to technical errors, *n*-alkanes could be used as discriminators of concentrate diet (Boland *et al*., 2012). Further studies need to be performed to confirm if the concentration of the *n*-alkanes C_{23} and C_{25} could indicate whether the animal had been receiving a diet composed of pasture silage and concentrates (60:40).

The highest fecal *n*-alkane concentrations occurred among odd-numbered carbon atom chains (C_{27}, C_{29}, C_{31} and C_{33}) (Table 4), reflecting the pattern of *n*-alkane concentrations ingested in the diet (Table 2). This tendency has been observed before in studies of large (Oliván *et al*., 2007) and small ruminants (Ferreira *et al*., 2005).

Changes in fecal n-alkane concentrations over time

From d 1 (C_{33} and C_{35}) or d 2 (C_{27}, C_{29} and C_{31}) of the experiment, and continuing until the end of

the study, the SC treatment had significantly lower concentrations (Figures 1B to 3) compared to the other two treatments. This was also confirmed in a multivariable analysis (Figure 4). This result indicates that the *n*-alkane profile in feces changes rapidly after a change in diet, and the changed profile remains over time according to the type of feed consumed. The feed passage rate can be modified by feed particle size, shape and density (Warner *et al*., 2013). The passage rate is higher in feeds having lower quantities of long fiber, as for the MX and SC treatments in the present study. The time lag between a change in diet and the reflection of this change in feces is expected to be shorter in high-quality diets compared to poor-quality diets due to the faster passage rate (Poppi *et al*., 1981). The faster passage rate under the MX and SC treatments could reflect more rapidly in feces the changes made to the diets. From d 10 of the experiment, concentrations of C_{27}, C_{29} and C_{31}, and from d 18, concentrations of C_{33} and $C_{35,}$ under the MX treatment, were similar to those found under the SC treatment. Both the MX and SC treatments maintained similar profiles until the final d of the experiment, considering that the last change in the diet was made on d 16.

The concentrations of the *n*-alkanes C_{27}, C_{29} and C_{31} between the MX and GZ treatments start to be significantly different at d 10 (the end of the second diet change). Consequently, there were no differences between the first and third change in the diet. Additionally, fecal samples did not discriminate animals consuming a diet including 66% of pasture silage and concentrates from those animals under the SC treatment. This result was also observed in the PCA, where on d 11 (Figure 4D), the MX treatment observations are separated from those of the GZ treatment. The MX treatment observations were on the left side of the PC1 axis next to the SC treatment observations on d 18. In the case of C_{23}, the concentrations were too variable to be reliably interpreted. However, C_{23} concentration under the MX treatment was significantly higher compared that under the GZ treatment on d 15.

Under the conditions of the present study, the *n*-alkanes C_{33} and C_{35} were able to detect changes in diet from the first- or second-d post-diet change (Figure 3). These results could indicate that the fecal concentrations of some *n*-alkanes, such as C_{33} and C_{35}, allow one to differentiate animals that were fed 100% on pasture (6 kg DM d^{-1}) from those that were consuming 100% a diet of a 60:40 ratio of silage:concentrate or a mixed diet with 33% of the diet being a 60:40 ratio of silage:concentrate and 66% being pasture. As previously mentioned, the oven-drying could have affected the concentrations of C_{27}, C_{29}, C_{31}, C_{33} and C_{35}, reducing the capacity to discriminate among treatments. However, the results indicate that C_{33} and C_{35} using the oven-dry method could correctly discriminate changes in diet. This result confirms the Sanchez Chopa *et al.* (2012) finding that suggested that the use of C_{33} could provide reliable intake estimates from feces samples oven-dried at 60 ºC.

A lag of two to three d was observed in detected changes in *n*-alkanes after a change in diet, which partly concurs with the result reported by Benvenutti *et al.* (2014). In their study, the time lag existing between a decrease in forage intake and the subsequent change (statistical detection) in the concentration of some fecal markers was 3–5 d.

This study concludes that changes in the diet of animals, from 100% pasture-fed to 100% silage:concentrate diet (60:40), were rapidly reflected in changes in fecal concentrations of *n*-alkanes. Significant differences were evident on d 2 of the study, with the altered concentrations becoming stable by d 9 and remaining at this level until the end of the experiment. In the case of animals that had a gradual change in diet, from pasture to a silage:concentrate diet (60:40), the C_{27}, C_{29} and C_{31} fecal *n*-alkanes allow one to discriminate diets consisting of 4 kg of DM d^{-1} of 60:40 ratio of silage:concentrate and 2 kg of DM d^{-1} of pasture from animals that are fed 100% on pasture, whereas C_{33} and C_{35} concentrations are

more sensitive and could differentiate animals that are fed 100% on pasture from those fed 4 kg of DM d^{-1} of pasture and 2 kg of DM d^{-1} of the silage:concentrate (60:40).

Under the conditions of the present study, the results indicate that fecal *n*-alkanes can differentiate pasture-fed animals from animals fed in feedlots or with moderate concentrations of concentrates (30% of DMI), including short-term changes in diet.

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Resumen

M.E. Martínez, A. Benavente, Y R. Morales. 2017. Potencial de los *n*-alcanos como biomarcadores de novillos alimentados a pradera. Cien. Inv. Agr. 44(3): 239-251. El objetivo del presente estudio fue evaluar el efecto del tipo de dieta (pastoreo vs. alimentación tipo feedlot) y de los cambios en la dieta en la concentración de n-alcanos en las heces de vacunos alimentados con estas dietas. El ensayo duró 35 días (15 de adaptación y 20 de toma de muestras). Se dividieron treinta novillos de raza Holstein Friesian en tres grupos de diez, los cuales fueron asignados a uno de los tres tratamientos: GZ: dieta consistente en 100% pastoreo; SC: dieta consistente en ensilaje de pradera: concentrado en proporción 60:40; y MX: dieta con disminución gradual del pastoreo e inclusión de cantidades crecientes de ensilaje y concentrado cada cinco días hasta alcanzar la misma dieta del tratamiento SC en los últimos 5 días del estudio. Se tomaron diariamente muestras fecales de cada animal, que fueron analizadas mediante cromatografía gaseosa para determinar su concentración de n-alcanos. Bajo las condiciones del presente estudio, los datos obtenidos mostraron cambios evidentes en la concentración de n-alcanos en las heces de los novillos de dos a tres días después de un cambio de dieta, mientras que las concentraciones permanecieron estables en las heces de los animales que no sufrieron cambios en la dieta. Los resultados obtenidos pueden utilizarse como base para desarrollar una herramienta que determine la dieta recibida por los animales los días previos al sacrificio.

Palabras clave: Autenticación, carne, engorda con grano, pastoreo, trazabilidad.

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