Stable Isotope Characterization of Milk Components and Whey Ethanol

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A multi-isotopic study of several components of milk has been carried out on commercial samples and on milk produced in feeding experiments involving different kinds of diets originating from C_3 or C_4 photosynthetic metabolisms and exhibiting a relatively wide range of isotope ratios. The dispersion of the carbon, hydrogen, and nitrogen isotope parameters of dried matter and of the lactose, protein, and lipid components has been estimated. In addition, the carbohydrates were represented by the site specific isotope ratios (SNIF-NMR) of ethanol resulting from standardized fermentation of lactose. The rates of response of the isotopic parameters to changes in the feeding materials is slower for the minor components, proteins, and lipids than for lactose and ethanol. For similar diets, the nonexchangeable sites of lactose and the methyl site of ethanol, in particular, are relatively enriched in deuterium in the case of polygastric animals, cow, goat, and ewe, as compared to the monogastric species, sow and mare, and woman. From an analytical point of view, the carbon and hydrogen parameters of ethanol provide efficient criteria for identifying a whey origin with respect to other agricultural and fossil sources.

Keywords: *Milk; isotope ratios; authentication; whey ethanol; isotope ratio mass spectrometry; SNIF-NMR*

INTRODUCTION

The production of lactoserum in Europe is on the order of several millions tons and, considering that 2 kg of lactose yield 1 kg of ethanol, the amount of whey wine potentially available is considerable and could compete with the more conventional sources of agricultural ethanol. Indeed, several species of *Kluyveromyces* yeast have been used in the industry to ferment large quantities of lactose after hydrolysis into glucose and galactose by β-galactosidase. Consequently, there is a need for analytical methods capable of characterizing the lactose source of ethanol. The investigation of site-specific natural isotope fractionation by nuclear magnetic resonance (SNIF-NMR) (Martin and Martin, 1981), which provides powerful authentication criteria (Martin and Martin, 1990), has been applied to the case of milk (Vallet et al., 1998). The lactose component extracted from milk can be isotopically characterized by resorting to the ethanol probe obtained in a fermentation by *Kluyveromyces fragilis* conducted under standardized conditions. However, ethanol derived from milk is a heterotrophic product that cannot be directly compared to other ethanols fermented either from C_3 (grape, sugar beet, wheat, potato, ...) or C_4 (sugar cane, maize, ...) plants. Whereas isotopic contents of plant constituents are governed by the biosynthetic mechanism and the environmental conditions of the photosynthesis, the isotopic ratios of animal products reflect those of the feeding materials (Minson et al., 1975; De Niro and Epstein, 1978; Tieszen et al., 1983). In addition, significant fractionation effects may occur in the course of the trophic chain (Peterson and Fry, 1987; Kennedy and Krouse, 1990; Wada et al., 1991) and the isotopic distribution secondarily depends, to a limited extent, on the physiological characteristics of the animal. It has been shown in particular that the overall carbon-13 content of milk, measured by isotope ratio mass spectrometry (IRMS), closely approaches that of the cow diet (Tyrrell et al., 1984; Boutton et al., 1988; Metges et al., 1990; Kornexl et al., 1997). In this context, the deuterium content of milk lactose is also expected to vary with the diet and to respond specifically to the metabolic pathway undergone in different mammals. Then in the present work, an analytical strategy is developed in order to study the influence on the isotopic distributions—of the nature of the animal which produced the milk, of the nature of the feeding materials for cows grown in intensive conditions (closed stalling), and of the geographical origin of milk obtained from cows grown in the extensive conditions of field stalling where grass is the main source of food. The isotopic data are then analyzed to estimate to which extent a whey origin of ethanol can be differentiated from other natural or fossil sources.

MATERIALS AND METHODS

Lactose Samples: Milk Products. Most of the lactoses studied were extracted from milk according to conventional procedures. The proteins contained in milk were precipitated by addition of 10% trichloroacetic acid at a low temperature, and lipids were extracted by solvent extraction ($CHCl_3$/CH_3-OH 2/1 v/v). The remaining aqueous phase was evaporated under a vacuum until a syrup was obtained and lactose was recrystallized from acetone, washed with 25% ethanol, and

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Table 1. Isotopic Properties of Lactose and Its Fermentation Products for Commercial α-Lactose (a) and Lactoserum (b)[a]

	raw materials			fermentation products				
	lactose	water	biomass	ethanol			water	biomass
case no.	$\delta^{13}C$ (‰)	$(D/H)_W^S$	$\delta^{13}C$ (‰)	$(D/H)_I$	$(D/H)_{II}$	$\delta^{13}C$ (‰)	$(D/H)_W^Q$	$\delta^{13}C$ (‰)
1a	−26.6	150.1	−24.1	113.4	128.9	−25.2	150.6	−24.7
2a	−18.7	150.1	−17.6	110.8	126.0	−17.2	153.2	−17.8
3a	−18.7	150.1	−18.0	111.3	128.1	−17.0	151.2	−18.0
4a	−28.3	150.1	−24.6	113.6	129.7	−26.6	150.4	−25.6
5a	−27.6	149.6	−24.3	114.2	129.7	−26.2	150.4	−25.5
6a	−26.7	149.6		113.8	126.5	−26.1	150.6	
7a	−28.1	149.6		113.2	123.8	−27.2	150.6	−25.9
8a	−26.7	149.6		115.3	130.0	−24.9	150.6	−24.6
9b	−23.5	149.6	−24.0	113.4	126.9	−22.2	150.6	−22.4
10b	−23.1	149.6	−24.0	114.6	128.8	−22.0	150.6	−22.4

[a] The overall carbon isotope deviations, $\delta^{13}C$ (‰) (eq 1), have been measured by IRMS, on lactose, on ethanol resulting from its fermentation by *Kluyveromyces fragilis*, and on the biomass before and after fermentation (Vallet et al., 1998); $(D/H)_W^S$ is the hydrogen isotope ratio of the starting water and $(D/H)_W^Q$ that of the aqueous medium at the end of the fermentation; $(D/H)_I$ and $(D/H)_{II}$ are the hydrogen isotope ratios of the methyl and methylene sites of ethanol. Samples 1−10 were obtained from different commercial sources.

dried. Commercial lactoses were purchased from different sources (Table 1). A sample of woman milk was kindly made available in the frame of a research program on nutrition (INRA, Rennes).

Fermentation of Lactose into Ethanol. The procedure used has been described previously (Vallet et al., 1998). The strain of *K. fragilis*, supplied by the mycotheque of the Catholic University in Louvain (Belgium), was grown at pH 6.2 on agar slants and stored at 4 °C. The yeast was kept in good fermenting conditions for several weeks and used for fermentation after reactivation.

The concentration of lactose was about 50 g/L since higher contents in lactose decrease both the conversion rate and the yield in ethanol. This concentration is similar to that in the permeate. The hydrogen isotope ratio of water used in the experiments was 149.8 ppm. The composition of the fermentation medium was as follows: $(NH_4)_2SO_4$ 4.0 g/L, KH_2PO_4 3.0 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, peptone 2.0 g/L. This medium is convenient for both pure lactose and whey permeates but the procedure of lactose extraction and purification described above must be strictly obeyed in the case of permeates. The whey wines obtained have an alcoholic grade on the order of 2.5% v/v, and the distillation carried out to extract quantitatively ethanol from wine had to be conducted with great care. Yields higher than 95% in ethanol having a grade higher than 91% w/w were usually obtained. Under these conditions, glucose and galactose were quantitatively converted (>99%) into ethanol (>91%) and biomass. Micrometabolites were negligible.

Stable Isotope Determinations. The carbon-13 contents of the starting lactose and those of the biomass and ethanol produced were determined by isotope ratio mass spectrometry (IRMS). They are reported in ‰ with respect to the international reference, V.PDB (for Vienna. Pee Dee Belemnite),

$$\delta‰ = 1000\left[\frac{(H/L)_{sample} - (H/L)_{ref}}{(H/L)_{ref}}\right] \quad (1)$$

where H and L denote the numbers of heavy and light isotopes, respectively.

The ^{15}N contents are also expressed as δ deviations and reported in ‰ with respect to atmospheric nitrogen.

The overall hydrogen isotope ratios, D/H in ppm, of water, of lactose, and in some cases of biomass, were measured by IRMS on the V.SMOW (for Vienna. Standard Mean Ocean Water) scale. The site-specific isotope ratios of the methyl, $(D/H)_I$, and of the methylene, $(D/H)_{II}$, sites of ethanol, reported in ppm, were determined by the SNIF-NMR method (site-specific natural isotope fractionation studied by nuclear magnetic resonance) (Martin and Martin, 1981, 1990).

Precision of the Isotopic Measurements. The repeatability (r) and reproducibility (R) of the different isotopic methodologies used have been determined in interlaboratory studies and have the following values (r, R): $(D/H)_I$ 0.7 and 1.0 ppm (Martin et al., 1996), $\delta^{13}C$ 0.3 and 0.7‰ (Martin, 1997), $\delta^{18}O$ 0.24 and 0.50‰, and $(D/H)_{water}$ 0.6 and 1.2 ppm (Koziet et al., 1995).

Feeding Experiments. A cow from the Frisonne breed, 8 years old and having calved four times, was fed successively with different regimes. First it was fed in the stable with 35 kg/day of ray grass (*Lolium italicum*) during 1 week. The milk production was on the order of 12.8 kg/day. Two liters of milk were collected the last day of the week for analysis. After 1 week of the conventional diet, the same feeding cycle was repeated with maize silage (*Zea mays* 50 kg/day). The milk production was 10.6 kg/day. A third feeding cycle was administrated to the cow with a 50/50 mixture of wheat and barley (*Triticum sativum* and *Hordeum vulgaris*) (5 kg/day) and dry hay (5 kg/day). The milk production was then equal to 9.8 kg/day. Finally, the cow was given 40 kg/day of sugar beet (*Beta vulgaris*) for 1 week. During the whole period of feeding, the cow drank water from a well having a deuterium content of 149.8 ppm.

In another series of experiments, the same cow was fed with maize from the same pool during 8 days but it was given, twice every day, 15 L of water enriched in deuterium at a level of 450 ppm.

The samples of milk collected at the end of the different feeding cycles were analyzed to determine their protein and carbohydrate composition, and isotopic analyses were carried out on the purified components.

RESULTS AND DISCUSSION

The carbon and hydrogen isotope parameters of the raw materials and fermentation products corresponding to eight samples of pure commercial lactose and two samples of lactoserum have been determined in order to estimate the isotopic dispersion of lactose (Table 1). With a view to determine the influence of the feeding materials on the isotope contents of milk products, feeding experiments involving different types of C_3 and C_4 plants have been conducted on a given cow, as described in Materials and Methods. The isotopic results are given in Table 2. The data obtained for several types of living organisms, animal and human, are compared in Table 3. In addition, Table 4 describes the influence of the deuterium content of the drinking water on the isotopic parameters of milk produced by a given cow.

Variability of the Isotopic Parameters of Commercial Lactose and Lactoserum. The results collected in Table 1 illustrate a relatively large dispersion

Table 2. Influence of the Nature of Feeding on the Isotopic Contents of Milk Products[a]

diet			milk components								fermentation products				
			dry extract		proteins		lipids	lactose		water	ethanol			water	biomass
nature	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{13}C$	$(D/H)_{NEX}$	$(D/H)_W^M$	$(D/H)_I$	$(D/H)_{II}$	$\delta^{13}C$	$(D/H)_W^Q$	$\delta^{13}C$
ray grass	−29.1		−28.5	5.8	−25.6	5.9	−21.5	−28.7	156.1	153.1	113.2	128.2	−27.5	153.5	−25.9
maize (silage)	−12.6	3.6	−15.6	5.6	−16.1	6.2	−20.5	−13.5	158.0	152.4	115.1	130.7	−11.7	154.3	−18.0
wheat−barley (50/50)	−26.3	2.3	−25.3	4.8	−23.2	5.5	−23.1	−24.7	154.9	152.2	112.1	128.9	−23.5	153.2	−24.2
sugar beet	−23.3	6.3	−23.9	6.2	−21.5	6.0	−21.7	−25.1	150.5	149.6	105.2	117.4	−24.1	150.6	−23.6
C3 (100%)			−25.7	5.6	−23.4	5.8	−22.1	−26.2	153.8	152.9	110.2	124.8	−25.0	152.4	
C3−C4 (50/50)			−20.7	5.6	−19.8	6.0	−21.3	−19.8	155.9	153.2	112.6	127.8	−18.4	153.4	

[a] The same cow was given different foods harvested in the same geographical area (Nantes region). The isotopic parameters are defined in the legend to Table 1. $(D/H)_{NEX}$ is the hydrogen isotope ratio of the nonexchangeable sites of lactose, and $(D/H)_W^M$ is the isotope ratio of milk water. The isotopic deviations $\delta^{13}C$ and $\delta^{15}N$ (eq 1) are expressed in ‰ with respect to V.PDB and atmospheric nitrogen, respectively, and the hydrogen isotope ratios are in ppm. The carbon-13 content of the starting biomass was $\delta^{13}C = -25.1$‰, and the isotope ratio of the drinking water was 149.8 ppm. The hydrogen isotope ratios of ethanol resulting from the fermentation by *Saccharomyces cerevisiae* of three kinds of feeding raw materials are the following (in ppm):

	$(D/H)_I$	$(D/H)_{II}$
beet sucrose	93.0	129.4
wheat starch	103.5	128.3
corn starch	111.6	123.9

Table 3. Influence of the Nature of the Living Organism on the Isotopic Properties of Milk Derivatives[a]

living organism[b]	diet[c]	raw materials			fermentation products					
		lactose		water	ethanol			water	biomass	
		$\delta^{13}C$ (‰)	$(D/H)_{NEX}$	$(D/H)_W^S$	$(D/H)_I$	$(D/H)_{II}$	$\delta^{13}C$ (‰)	$(D/H)_W^Q$	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
cow (a)	c	−20.7	156.7	151.2	116.4	130.6	−19.2	151.9	−21.2	1.3
cow (a)	d	−26.8	156.1	151.0	114.8	127.2	−25.4	*151.5*	−24.7	
goat (a)	c	−21.5	154.0	152.2	114.6	127.2	−20.2	152.4	−20.9	2.4
ewe (a)	d	−26.9	153.9	151.8	114.5	128.0	−26.3	152.8	−24.0	2.3
sow (b)	c	−22.1	150.3	150.2	108.0	130.6	−21.7	*150.9*	−21.4	2.2
mare (b)	d	−27.5	*151.5*	*151.0*	108.6	128.6	−26.5	*152.0*	−25.2	2.0
woman (b)	C3	−25.3	150.7	150.5	105.6	128.1	−24.1	151.9	−24.2	5.8

[a] The isotopic parameters are defined in the legends to Tables 1 and 2. The isotopic deviations of the starting biomass used in the fermentation experiments were $\delta^{13}C = -25.2$‰ and $\delta^{15}N = 2.6$‰. [b] (a) polygastric organism; (b) monogastric organism. [c] (c) intensive mixed diet; 50% maize, 50% C_3 plants; (d) extensive C_3 meadow breeding.

Table 4. Influence of the Deuterium Content of Drinking Water on the Isotopic Properties of Milk Derivatives[a]

		milk components								fermentation products			
	water	dry extract		proteins		lipids	lactose		water	ethanol			water
t(days)	$(D/H)_W^O$	$\delta^{13}C$ (‰)	$\delta^{15}N$	$\delta^{13}C$ (‰)	$\delta^{15}N$	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$(D/H)_{NEX}$	$(D/H)_W^M$	$(D/H)_I$	$(D/H)_{II}$	$\delta^{13}C$ (‰)	$(D/H)_W^Q$
0	150.8	−15.6	5.6	−16.1	6.2	−20.5	−13.5	158.0	153.4	115.1	130.7	−11.7	153.5
1	433.0	−16.8	5.5	−16.0	5.6	−18.2	−14.6		163.7	116.2	129.1	−13.7	150.8
2	475.0	−15.7	5.4	−15.8	5.8	−17.1	−14.5	173.8	171.9	122.9	131.4	−13.3	150.5
3	473.0	−15.7	5.4	−15.1	5.5	−15.4	−14.5		187.3	132.1	133.0	−13.3	150.8
4	446.0	−15.4	5.1	−14.9	5.4	−17.5	−14.6	201.1	204.5	138.8	133.0	−13.1	150.6
5	439.0	−14.8	5.4	−14.5	5.4	−16.2	−13.4		214.3	146.7	134.8	−12.2	150.6
6	448.0	−15.3	5.3	−15.2	5.2	−16.3	−14.9	224.7	226.5	153.0	135.3	−13.4	151.4
7	439.0	−15.3	5.3	−14.4	5.3	−14.7	−15.6		238.0	159.0	134.7	−13.3	151.2
8	499.0	−15.7	5.5	−14.8	5.5	−16.4	−15.6	244.0	245.6	164.0	136.7	−14.1	151.6

[a] The isotopic parameters are defined in the legends to Tables 1 and 2, $(D/H)_W^O$ is the isotope ratio of the drinking water. The experiments were conducted with the same cow as that involved in the experiments described in Tables 2 and 3. This cow was fed with a C_4 diet (maize silage). The enriched lactose samples recovered from the milk were fermented in water characterized by the isotopic value $(D/H)_W^S = 149.8$ ppm.

of the isotopic parameters (Vallet et al., 1998). In particular, variations of up to 10‰ in the ^{13}C content of lactose and ethanol are observed (mean = −23.5‰ and standard deviation = 3.8‰). These variations cover a large proportion of the gap that separates C_3 ($\delta^{13}C \simeq -25$‰) and C_4 ($\delta^{13}C \simeq -11$‰) plants. The deuterium contents of the methyl, I, and methylene, II, sites of fermentation ethanol extend over smaller ranges since the means (standard deviations) are, respectively, equal to 113.4(1.4) and 127.8(2.0) for $(D/H)_I$ and $(D/H)_{II}$. This behavior suggests, as will be further discussed in the next section, that the lactose samples investigated were produced by living organisms (probably cows) fed with different (C_3 and C_4) diets that influence the ^{13}C distribution much more than the ^2H distribution. A first group (case nos. 1, 4, 5, 6, 7, and 8), characterized by a mean $\delta^{13}C$ value of −27.3‰, is indicative of a C_3 diet, whereas the $\delta^{13}C$ value of −21‰ determined for the second group (cases 2, 3, 9, and 10) is typical of a mixed C_3/C_4 diet. The reliability of the carbon-13 determina-

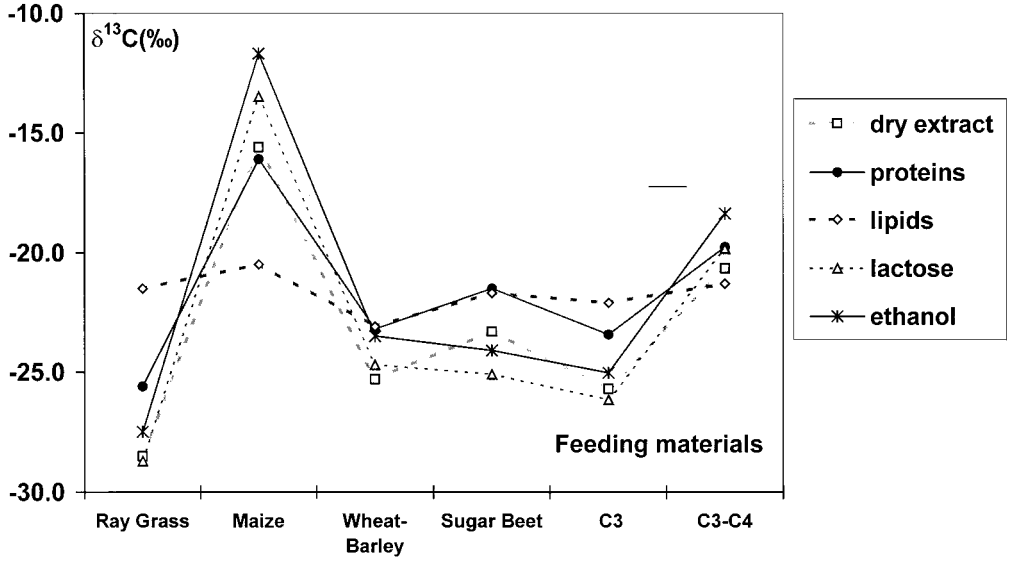

Figure 1. Variation of the $\delta^{13}C$ values of several components of milk produced by the same cow, as a function of the nature of the feeding materials.

tions is supported by the observation of a systematic enrichment of ethanol with respect to the starting lactose. This enrichment, which reaches a mean value of +1.3‰ (Table 1), contrasts with the depletion of the same order of magnitude (−1.4‰) observed in the fermentation of sucrose or plant glucose and fructose by *Saccharomyces* strains. This behavior may be related to differences in the isotopic distribution of sugars associated with the different mechanisms of glucogenesis in animals and in plants.

Influence of the Diet on the Isotopic Parameters of Milk Products. It is now recognized that, in living organisms, the natural abundance quantities of nonexchanging atoms such as ^{13}C and ^{15}N are closely related to those of the corresponding food (Schoeller et al., 1980; Wada et al., 1991). However, in the case of deuterium, isotope fractionation effects due to enzymic hydrogen transfers and exchange reactions with body water may intervene in the course of the metabolic pathway of the living organism. Since such effects are very sensitive to environmental conditions, the hydrogen isotopic connection between the feeding materials and the metabolites is more likely to depend on metabolic peculiarities.

Although the overall ^{13}C content of milk reaches rapidly (4 days) a new isotopic equilibrium after a change in the type of diet (Boutton et al., 1988), the turnover rate may be slower for minor components and complete equilibria are not obtained within the selected interval of 7 days. Nevertheless, the experiments, conducted as described in Materials and Methods, with a cow successively fed with different well-defined and controlled diets illustrate the adaptation of the milk components to the regime (Table 2). In a multi-isotopic approach, the carbon-13 ($\delta^{13}C$) and nitrogen-15 ($\delta^{15}N$) parameters were measured on the dried matter of milk and on the isolated proteins, lipids, and lactose components. In addition, the deuterium contents were determined (1) on the water medium before, $[(D/H)_W^S]$, and after, $[(D/H)_W^Q]$, fermentation, (2) on the nonexchangeable sites of lactose, $[(D/H)_{NEX}]$, and (3) on the ethanol probe derived from lactose $[(D/H)_I$ for the methyl and $(D/H)_{II}$ for the methylene groups]. A systematic increase in the $\delta^{13}C$ value of ethanol with respect to lactose is again observed, confirming that ethanol faithfully translates the isotopic behavior of lactose whatever its origin. Since proteins are the main source of nitrogen in milk, their $\delta^{15}N$ values remain very close to those of the dried matter, which are themselves slightly higher than those of the diet, as already noted (Steele and Daniel, 1978). In contrast to trends usually observed in plants, the lipid components are not depleted in carbon-13 with respect to carbohydrates. The impoverishment usually observed in plant lipids can be explained by cumulated fractionation effects occurring in the biosynthetic chain that largely evolves as an open system. In the present situation, a significant proportion of milk lipids is indirectly derived from feed through a contribution of body tissues (Wilson et al., 1988). Consequently, the longer and more complex metabolic pathway than that of carbohydrates results in a slower response of lipids to changes in the nature of the diet. More generally, such differences in the times necessary to reach steady-state conditions may be responsible for important differences of amplitude and some lack of parallelism between the evolutions of the carbon-13 deviations as a function of the type of diet, in the different components: dry matter, carbohydrates, proteins, lipids, and ethanol (Figure 1). Thus, whereas similar variations in the carbon isotope ratios of individual constituents of lentils grown in different environments have been observed (Zhang et al., 1991), significantly reduced variations are exhibited by proteins and lipids of milk as compared to lactose and ethanol.

The deuterium contents in lactose and its fermentation ethanol also reflect the cow's diet. Thus, when going from sugar beet diets to maize diets, the isotope ratio of the methyl site of milk ethanol exhibits a deuterium enrichment of the order of 10 ppm, which reflects that of the plant sugars themselves (Martin et al., 1991; Zhang et al., 1995). However, higher $(D/H)_I$ values are observed in ethanol from lactose as compared to ethanol resulting from fermentation of the diet.

Influence of the Nature of the Living Organism on the Isotopic Parameters of Milk. Differences between the ^{13}C and ^{2}H isotope ratios corresponding to different animal origins (Table 3) are of the same order of magnitude as those produced, for a given cow, by

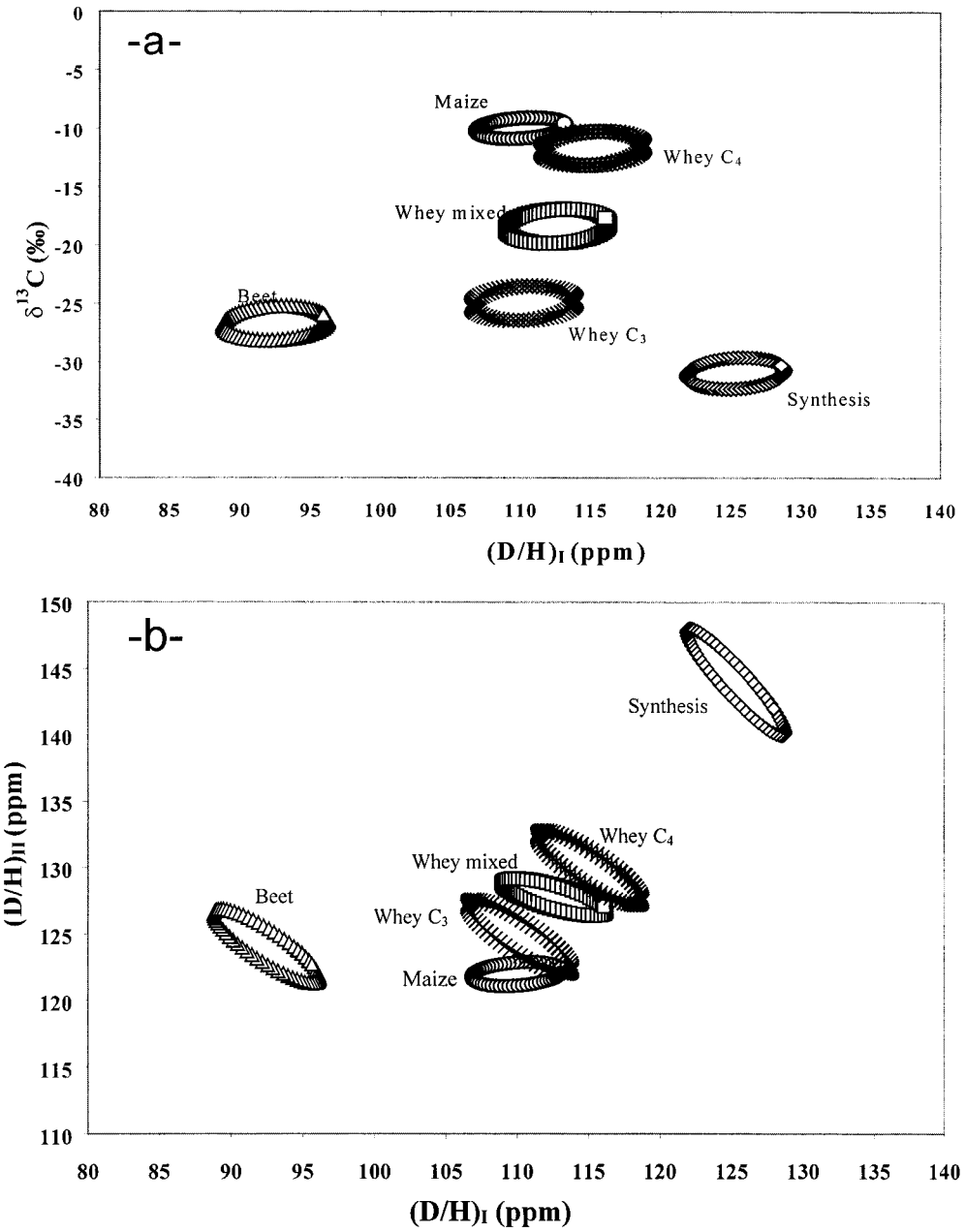

Figure 2. Representation of bivariate existence domains of ethanol from whey, beet, maize, and fossil sources. A 95% confidence level has been adopted. The samples are represented in the plane of the $(D/H)_I$ and $\delta^{13}C$ variables (part a) and in the plane of the $(D/H)_I$ and $(D/H)_{II}$ variables (part b).

changes in the feeding materials (Table 2). Nevertheless, for a similar extensive C_3 breeding, both the nonexchangeable sites of lactose and the methyl site of ethanol are significantly depleted in deuterium in the case of mare milk as compared to cow milk. More generally, Table 3 enables the isotopic results to be compared for three polygastric animals, cow, goat, and ewe, on one hand, and two monogastric animals, sow and mare, and a woman on the other hand. The animals were fed with two different diets according to the type of stalling, pure C_3 from grass in pastures or mixed C_3/C_4 from intensive breeding in the stable. Whereas the $\delta^{13}C$ values of lactose and ethanol mirror those of the diet, whatever the digestive system of the animals, the monogastric species are characterized by smaller deuterium contents in both the nonexchangeable sites of lactose and the methyl site of ethanol. The mean values of $(D/H)_{NEX}$ and $(D/H)_I$ in the polygastric mammals are on the order of 155 and 115 ppm, respectively, whereas they are equal to 151 and 107 ppm for monogastric animals and human. As compared to monogastric species, polygastric animals, such as cow, goat, and ewe, are characterized by higher digestibility coefficients. In particular, different contributions of food cellulose to the pool of deuterium atoms recycled in the glucogenesis pathway are expected as a result of prestomach decomposition specific to the ruminant (Schulze and Giese, 1993).

It is also observed that the biomass is enriched in ^{13}C in the course of the fermentation as a result of some consumption of lactose usually characterized by a higher isotope ratio than the starting cells. In contrast, the biomass is subject to a slight depletion in ^{15}N except in the fermentation of woman milk, which contains a higher proportion of nitrogenated carbohydrates.

Influence of the Deuterium Content of the Drinking Water on the Isotopic Parameters of Milk. To authenticate milk and milk products in terms of their

geographical origin, it may be argued that the deuterium content of the milk components depends on that of the water drunk by the animal. For example, it may be anticipated that cows that graze in elevated extensive pastures will produce milk depleted in deuterium. Indeed, this behavior is corroborated by comparison of pastures situated near the sea level (Vendée) with pastures far away from the cost at an altitude of 800 m (Jura). The deuterium contents measured on the different milk components exhibit the decrease expected when going from sea level to mountain: milk water, 153.1 and 151.0 ppm; lactose, 156.1 and 153.9 ppm; ethanol $(D/H)_I$, 113.2 and 112.6 ppm; $(D/H)_{II}$, 128.7 and 128.2 ppm.

To quantify more precisely the relation between the isotope ratio of the drinking water of the cow and the deuterium content of the milk products, an experiment (Table 4) was conducted with a cow given a C_4 diet and watered with slightly enriched water (≃450 ppm). The cow was milked every day, and the milk was handled in the usual way, but unfortunately, the cow declined significantly after 8 days and the experiment had to be stopped. At this time, the plateau of the curve representing the variation of the deuterium content of the milk water as a function of time was not completely reached. However, the relative increases of $(D/H)_{NEX}$, $[(D/H)_W^M]$, and $(D/H)_I$ with the isotope content of the drinking water, $[(D/H)_W^O]$, were nearly stabilized. Therefore quantitative responses of the deuterium content of the main milk components and derivatives to changes in the isotopic composition of the water drunk by the cow may be estimated (the symbol Δ refers to the variation of the considered parameter with respect to the normal conditions):

$$\frac{\Delta((D/H)_{NEX})}{\Delta((D/H)_W^O)} = 0.25; \frac{\Delta((D/H)_W^M)}{\Delta((D/H)_W^O)} = 0.25; \frac{\Delta((D/H)_I)}{\Delta((D/H)_W^O)} = 0.15 \quad (2)$$

On this basis, the 4 ppm variation in the ^2H content of the rain water associated with the geographical and climatic conditions of the two regions considered above (Vendée and Jura) is expected to induce a 1 ppm variation for lactose and milk water and a 0.6 ppm variation of $(D/H)_I$. In fact, eq 2 underestimates the lactose and milk water changes but fits nicely the observation made on ethanol.

In regard to the carbon-13 parameters, the increase of $\delta^{13}C$ of ethanol as compared to $\delta^{13}C$ of lactose (1.5‰) is again observed. Moreover, the setting up of new deuterium contents introduced by the enrichment of the drinking water seems to be accompanied by regular variations in the ^{13}C contents of the various constituents. Whereas $\delta^{13}C$ of proteins and lipids increases by a few per mil (≈ 2.5‰), $\delta^{13}C$ of lactose decreases by about 2‰. As discussed above, differences in the rates of response of the individual components to changes in the diet used in these experiments must be taken into account.

CONCLUSION

Although they are mainly conditioned by the nature of the feeding materials and the isotopic properties of the drinking water, the isotope ratios of milk components may also be subject to some systematic effects of the animal species. This is the case, in particular, for the nonexchangeable hydrogens of lactose and the methyl hydrogens of its fermentation ethanol. From an analytical point of view, the present results show that whey ethanol can be distinguished, with an acceptable degree of confidence, from ethanol obtained from other agricultural or fossil sources. The 95% confidence domains of ethanol samples from different origins, defined on the basis of the three isotopic parameters, $(D/H)_I$, $(D/H)_{II}$, and $\delta^{13}C$, are represented in Figure 2. Since the raw materials used by the farmers to feed cows do not have a well-defined composition, three existence domains only have been considered for milk: C_3 diet, C_4 diet, and mixed diet. No overlap at the 95% level is observed in Figure 2a between C_4 whey ethanol and the maize raw material. Moreover, the overlap remains very small at the 99% confidence level. When the bivariate domains built on $(D/H)_I$ and $(D/H)_{II}$ are considered, an unambiguous distinction is possible. More generally, in the three-dimensional space defined by $(D/H)_I$, $(D/H)_{II}$, and $\delta^{13}C$, ethanol from whey is easily identified with respect to the other origins, whatever the nature of the feeding stuffs of the animal.

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