

# Determination of the $^{13}\text{C}$ Content of Glycerol Samples of Different Origin

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The average carbon isotope value ( $\delta^{13}\text{C}$ ) of 63 samples of glycerol from over 30 different sources has been determined. The results indicate that it is possible to distinguish the glycerol obtained from the glycerides produced in plants following C-3 and C-4 carbon fixation pathways. The samples obtained from animal sources seem to reflect the composition of the material consumed, as well as that produced by sugar fermentation.

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**Keywords:** *Authenticity proof; carbon-13; glycerol; animal, vegetal, and fermentative sources*

## INTRODUCTION

Glycerol (**1**) is a primary metabolite of living organisms, occurring naturally mainly in esterified form as fats and oils (Bauman et al., 1988). Its derivation from carbohydrates in alcoholic fermentation was recognized in a pioneering work by Pasteur (1858), and subsequently, the enzymic nature of the biogenesis process was established (Buchner and Rapp, 1901). The discrete steps of the enzymic machinery responsible for the conversion of C-6 fructose 1,6-diphosphate into C-3 glycerol have been identified (Belitz and Grosch, 1987). Glycerol is used in fairly large quantities in industrial food preparations such as chewing gum products, the main supplier being the fats and oils industry (Young et al., 1986). However, consumer consciousness has grown, and today people are very selective about food products as well as the ingredients they contain. Moreover, it is considered inappropriate for ethnic and religious reasons to use natural components extracted from certain animals in food manufacture. These circumstances have stimulated the search for analytical methods enabling an exact definition of the origin of food ingredients. To this end, the measurement of the stable isotope composition, i.e.  $^2\text{H}$  and, in particular,  $^{13}\text{C}$  (Martin and Martin, 1995; Schmidt, 1986; Doner, 1991; Guillou et al., 1991), seems highly promising.

The recent appearance on the retail market of foods labeled "nonanimal" with regard to their glycerol content raises the problem of the analytical method used

clearly defining the precise origin of the material. We envisaged that one way to recognize the glycerol origin could be the determination of the global  $^{13}\text{C}$  content. Such a measurement ensures a fast and reliable result thanks to the use of isotope ratio mass spectroscopy interfaced with an elemental analyzer (EA-IRMS). To this end, we carried out this kind of analysis on 63 samples of glycerol derived from over 30 different sources to verify whether the isotopic composition can be attributed to certain origins. While the study was being completed, a paper appeared on the determination of the  $^{13}\text{C}$  labeling pattern of glycerol through the same technique (Weber et al., 1997). This highly sophisticated study concentrated mainly on the explanation of the remarkable depletion of  $^{13}\text{C}$  in natural glycerols compared to the sugars from which they are synthesized. As a consequence, an authentication of glycerol present in wines becomes possible. This work includes, besides the determination of the total content of  $^{13}\text{C}$ , the definition of the isotopic composition of the single carbon atoms of the molecule, achieved through chemoenzymatic selective degradation of the C-3 carbon framework of glycerol. However, the investigation was applied to a limited number of samples only. We therefore consider it instructive to now present our results, performed on a wide set of samples, integrating the above-mentioned results and showing the potentials and the limitations of the technique in the determination of the origin of glycerol.

## MATERIALS AND METHODS

**Glycerol Samples.** The examined glycerol samples belong to four sets. (i) Glycerol of animal origin was obtained from cattle fat (samples 1 and 2 from breeding cattle in the countryside around Brescia, Italy; samples 3 and 4 from a butcher shop in Milan), butter (samples 5–8 from dairy farming in Italy: sample 5 from Cremona, samples 6 and 7 from the countryside around Milan, sample 8 from Varese, and sample 9 from Ireland), lard (sample 10 from a butcher shop in Milan), pig fat (samples 11–13 from a livestock breeder in the Brescia area, Italy), goat fat (sample 14 from a butcher shop in Milan), and fish oil (samples 15–18 from herrings from Portugal). (ii) Glycerol of vegetal origin was obtained from olive oil (samples 19–21), grape kernel oil (samples 22–24), carduus oil (sample 25), peanut oil (sample 26), walnut oil (samples 27 and 28), sunflower oil (samples 29–31), soya oil (sample 32), cotton oil (sample 33), castor oil (samples 34 and 35), sesame oil (sample 36), wheat oil (sample 37), coconut oil (sample 38), almond oil (sample 39), and maize (samples 40–42). (iii) Glycerol of sugar fermentation was obtained from maize (sample 43), beet (sample 44), and wines (samples 45, 46, and 47 obtained from German EU-DB wines in the years 1993, 1994, and 1995, respectively). (iv) Glycerol was also obtained from commercial sources; the origins were specified by the suppliers as cattle fat (samples 48–49), plant oil (sample 50), chemical synthesis (unspecified method) (samples 51 and 52), and petrochemical (unspecified method) (samples 53–55). The following additional commercial samples were also used: glycerol of unspecified origin (samples 56–60), glycerol from the hydrolysis of a commercial specimen of "monoglyceride" (sample 61), glycerol from the hydrolysis of the glycerides present in a commercial sample of gum base used in chewing gum manufacture (sample 62), and, finally, glycerol labeled "nonanimal glycerol" (sample 63) purchased from a dealer.

**Hydrolysis of the Glycerides.** The glycerides in 10 volumes of methanol were treated with 10% in weight of sodium methoxide. At the end of the reaction, the volume of the mixture was reduced under vacuum to small volume and the residue separated out into hexane and water. The aqueous phase was brought to pH 7 by the addition of 2 N HCl and dried out. Glycerol was recovered from the residue by extraction with ethanol and purified by bulb-to-bulb vacuum distillation.

**Glycerol from Wine and from Sugar Fermentation.** The glycerol from the above-mentioned sources was isolated according to the method described earlier (Weber et al., 1997).

**Carbon Isotope Analysis.** The isotopic discrimination occurring during the photosynthetic CO<sub>2</sub> fixation, conventionally presented as  $\delta^{13}\text{C}$ , indicates the difference per mill in the  $^{13}\text{C}/^{12}\text{C}$  ratio of the sample relative to an international standard, i.e. CO<sub>2</sub> obtained from Pee Dee Belemnite (PDB) limestone of South Carolina. The  $\delta^{13}\text{C}$  value is calculated according to the equation  $\delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3$ , where  $R = ^{13}\text{C}/^{12}\text{C}$ . The carbon isotope ratios were determined following the experimental methodology previously described (Angerosa et al., 1997).

## RESULTS AND DISCUSSION

The study involved the determination of the  $^{13}\text{C}$  content of 63 different samples of glycerol divided in 4 sets. These include (i) glycerol obtained by hydrolysis of animal fats (18 samples), (ii) glycerol similarly produced from vegetal oils (24 samples), (iii) glycerol extracted from wine and produced by fermentation of sugars of different origin (5 samples), and (iv) commercial glycerol available either in the free form or obtained from glycerides by hydrolysis (16 samples).

Examination of the results (Tables 1–4) indicates that the distribution of the isotopic content among the various sets is not random but conceivably determined by natural processes involved in the generation of

**Table 1. Total  $\delta^{13}\text{C}$  Values of Glycerol Samples of Animal Origin**

entry	origin	$\delta^{13}\text{C}$ (‰)
1	cattle fat	−20.5
2		−22.3
3		−22.6
4		−20.3
5	butter	−23.5
6		−23.1
7		−23.0
8		−22.9
9	Irish butter	−32.7
10	lard	−20.2
11	pig fat	−26.0
12		−26.4
13		−25.2
14	goat fat	−26.2
15	fish oil	−21.4
16		−21.0
17		−21.4
18		−21.3

**Table 2. Total  $\delta^{13}\text{C}$  Values of Glycerol Samples of Vegetal Origin**

entry	origin	$\delta^{13}\text{C}$ (‰)
	C-3 Plant	
19	olive	−31.2
20		−31.3
21		−31.6
22	grape kernel	−28.2
23		−28.3
24		−29.0
25	carduus	−31.6
26	peanut	−29.1
27	walnut	−28.8
28		−30.0
29	sunflower	−30.5
30		−30.7
31		−30.0
32	soya	−30.4
33	cotton	−29.3
34	castor-oil	−31.4
35		−30.4
36	sesame	−29.5
37	wheat	−30.2
38	coconut	−27.6
39	almond	−30.8
	C-4 Plant	
40	maize	−15.6
41		−14.6
42		−15.3

glycerol. In particular, samples of vegetal origin (Table 2) show carbon isotopic ratios falling around −30‰ (entries 19–39) except for maize (entries 40–42), which shows the mean value of −15‰. These results are in agreement with the operation of C-3 and C-4 carbon dioxide fixation processes, respectively (Park and Epstein, 1960; Bender, 1971; Galimov, 1985).

More intriguing are the values relative to the glycerol samples of animal origin (Table 1). The animal body is in equilibrium with the food consumed (DeNiro and Epstein, 1978; Metges et al., 1990). Consequently, the composition of animal glycerol is linked to the composition of diet. Animals fed predominantly on C-3 plants, i.e., grass and alfalfa, will accumulate glycerol in their body lipids reflecting the isotopic composition of that kind of feedstock, and the same is true for those fed on C-4 plants. Animals supplemented with mixtures of C-3 and C-4 plants will produce glycerol with  $^{13}\text{C}$  composition reflecting the proportion of the two materials, i.e., grass, alfalfa, and maize silage, respectively. Indeed, glycerol from cattle fat (entries 1–4) shows fewer

**Table 3. Total  $\delta^{13}\text{C}$  Values of Glycerols from Sugar Fermentation**

entry	origin	$\delta^{13}\text{C}$ (‰) glycerols	$\delta^{13}\text{C}$ (‰) must sugars <sup>a</sup>	$\delta^{13}\text{C}$ (‰) ethanol <sup>b</sup>	no. of samples
43	maize	-16.5			
44	beet	-30.6			
45	D wine 1993	-27.97 $\pm$ 1.48 <sup>c</sup>	-23.4	-24.92 $\pm$ 1.33 <sup>c</sup>	15
46	D wine 1994	-30.92 $\pm$ 0.66 <sup>c</sup>	-26.96	-28.46 $\pm$ 0.54 <sup>c</sup>	14
47	D wine 1995	-31.63 $\pm$ 0.99 <sup>c</sup>	-27.5	-29.0 $\pm$ 0.72 <sup>c</sup>	29

<sup>a</sup>  $\delta^{13}\text{C}$  values of the carbohydrates from which the wine glycerol and ethanol are derived; they are calculated from  $\delta^{13}\text{C}_{\text{ethanol}} + 1.5$  (Rossmann et al., 1996). <sup>b</sup>  $\delta^{13}\text{C}$  values of the wine ethanol. <sup>c</sup> Mean value and standard deviation for the given number of glycerols.

**Table 4. Total  $\delta^{13}\text{C}$  Values of Glycerols of Commercial Origin**

entry	origin	$\delta^{13}\text{C}$ (‰)
48	cattle fat	-29.4
49		-22.6
50	plant oil	-27.5
51	synthesis	-28.6
52		-34.3
53	petrochemicals	-22.7
54		-24.2
55		-27.1
56	unknown	-23.8
57		-25.6
58		-22.2
59		-28.5
60		-27.6
61	monoglyceride	-18.8
62	gum base	-27.4
63	nonanimal	-22.1

negative values than those from pig and goat (entries 11–14): this is probably due to the fact that the diet of the former is based more on maize silage than that of the latter animals. Similarly, the sample of lard examined (entry 9) provides glycerol with an isotopic composition quite similar to that of cattle fat. The same reasonings hold for butter (entries 5–8). However, Irish butter (entry 9), with the value of -32.66‰, indicates a derivation from C-3 diet, attributed to cows fed on pure grass (e.g. in mountain pasture).

The glycerol from fish oil (entries 15–18) shows a composition similar to that of cattle fat. An explanation for this result might be that the photosynthesis in the marine environment occurs via the C-3 pathway. However, in this instance the carbon source in the photosynthesis is bicarbonate, which is enriched in  $^{13}\text{C}$  vs air  $\text{CO}_2$  by ~8‰ (Hoefs, 1973; Galimov, 1985).

The  $^{13}\text{C}$  content of wine glycerol isolated from German EU-DB wines of three seasons first shows how  $\delta^{13}\text{C}$  is influenced by the season. This had already been stated for other wine ingredients [e.g. ethanol (Rossmann et al., 1996) and organic acids (Weber et al., 1997)]. This effect caused by the climate in a specific year results in a depletion of  $^{13}\text{C}$  in must sugars and subsequently in other products from wine in wet and cold years (i.e. 1994 and especially 1995 in Germany). An enrichment of  $^{13}\text{C}$  occurs in warm and hot years (i.e. 1993 in Germany). The same factors are responsible for higher  $\delta^{13}\text{C}$  in ingredients from wines from wet and/or colder climatic zones [regional variation of  $\delta^{13}\text{C}$  (Rossmann et al., 1996)]. In addition to the seasonal and regional variation of the  $\delta^{13}\text{C}$  in wine glycerol, there is a specific depletion of all natural glycerol related to the carbohydrates (sugars) from which they are synthesized during biosynthesis (Weber et al., 1997). The practical consequence is a depletion of  $^{13}\text{C}$  in wine glycerol by 2.5–3.5‰ compared to ethanol from the same wine and by 4–5.5‰ compared to the must sugars. Approximately the same depletion can be found in the comparison

between other natural glycerol samples and the sugars from which they biochemically originate (Table 3).

Finally, as far as the isotopic composition of commercial samples of glycerol is concerned (Table 4), the values relating to entries 48–50 (cattle fat and plant oil) are in agreement with those of Tables 1 and 2. In particular, entry 50 (plant oil) shows the value of -27.5‰, similar to that of entry 38, -27.6‰ (coconut oil). A range of values from -22.2 to -34.31‰ is observed for samples 51–60 (samples of declared miscellaneous origin). More interestingly, a commercial sample of monoglyceride (entry 61) sold for food applications shows the value of -18.8‰, which suggests a derivation from oils predominantly extracted from C-4 plants. The opposite is true for the glycerol from the hydrolysis of gum base components (entry 62), which shows a value of -27.7‰, thus excluding an animal derivation [apart from Irish butter (entry 9) of Table 1]. The latter value is in fact similar to that of entries 38 (coconut oil) and 50.

Finally, a commercial sample of glycerol sold as nonanimal (entry 63) shows the value of -22.1‰, which suggests, in the hypothesis that only vegetal sources have been used in its production, a derivation from a 1:1 mixture of C-3 and C-4 derived natural extractives.

Seen together, these results show that there is a significant difference in the total  $^{13}\text{C}$  content between glycerol samples of animal and vegetal origin. In addition, in the latter case it is possible to distinguish C-3 derived products from those using the C-4 pathway. However, the method is not suitable for the characterization of mixtures of glycerol samples derived from the two pathways or of synthetic glycerol. In the latter case it can be useful to apply positional  $^{13}\text{C}$  measurement (Weber et al., 1997). Supplementary information may also be derived from natural abundance deuterium NMR studies or from the determination of the  $\delta^{18}\text{O}$  of glycerol by IRMS.

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