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[^{13}C] GC–C-IRMS analysis of methylboronic acid derivatives of glucose from liver glycogen after the ingestion of [^{13}C] labeled tracers in rats

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ABSTRACT

We developed a complete method to measure low [^{13}C] enrichments in glycogen. Fourteen rats were fed a control diet. Six of them also ingested either [U-^{13}C] glucose (n = 2) or a mixture of 20 [U-^{13}C] amino acids (n = 4). Hepatic glycogen was extracted, digested to glucose and purified on anion–cation exchange resins. After the optimization of methylboronic acid derivatization using GC–MS, [^{13}C] enrichment of extracted glucose was measured by GC–C-IRMS. The accuracy was addressed by measuring the enrichment excess of a calibration curve, which observed values were in good agreement with the expected values (R = 0.9979). Corrected delta values were −15.6 ± 1.6 δ^{13}C (‰) for control rats (n = 8) and increased to −5 to 8 δ^{13}C (‰) ‰ and 12–14 δ^{13}C (‰) ‰ after the ingestion of [U-^{13}C] amino acids or [U-^{13}C] glucose as oral tracers, respectively. The method enabled the determination of dietary substrate transfer into glycogen. The sequestration of dietary glucose in liver glycogen 4 h after the meal was 35% of the ingested dose whereas the transfer of carbon skeletons from amino acids was only 0.25 to 1%.

1. Introduction

It is difficult to measure low levels of [^{13}C] enrichment (APE < 0.1%) of stored or circulating glucose in biological pools after an oral tracer [^{13}C] administration because purification procedures involve several complex steps which can generate important sample losses. Unlike plants, in which starch stores are abundant, in animals glucose is limited to small amount in either the circulating or glycogen pools. Although of small size (50–500 mg for liver glycogen and 5–20 mg for circulating glucose in rats, depending on their nutritional and physiological state), those pools play a fundamental role in various physiological and metabolic events, and the disruption of glucose homeostasis can result in pathological situations such as diabetes.

During the postprandial phase, the synthesis of glycogen through the indirect pathway, i.e. from gluconeogenic precursors (amino acids, lactate and glycerol), has been suspected on the basis of metabolic or tracer studies [1,2]. We recently hypothesized that dietary amino acid carbon skeletons could be used to synthesize glycogen during the postprandial phase [3]. To quantify the contribution of dietary precursors to the direct and indirect pathways, we have monitored incorporation into liver glycogen of carbons from dietary glucose or amino acids after the ingestion of [U-^{13}C] oral tracers. To measure the incorporation of [^{13}C] from oral tracers in liver glycogen, the sample preparation and more specifically the purification step is critical. Several methodological problems were encountered because of the lack of a full description of the different steps in the literature.

The first step consisted in the precipitation and hydrolysis of glycogen. Two different methods are described in the literature: enzymatic digestion [4] and acid hydrolysis [5]. We chose an enzymatic method to hydrolyze hepatic glycogen because it is more specific and less denaturing. Second, the purification of glucose must be performed following an anion and cation exchange procedure. However, different adaptations of the initial method [6] exist in the literature and may not be suitable with respect to subsequent derivations. Third, it was necessary to choose a derivation that would add the fewest carbons possible in order to avoid excessive [^{13}C] dilution with derivative reagents.

Several techniques can be used to derivatize monosaccharides, involving reagents such as trimethylsilyl (TMS), methylboronic acid (MBA), butylboronic acid (BBA) or alditol acetate [7]. Of these different techniques for carbohydrate derivatization, the methylboronic method, introduced by van Dongen et al. [8,9] and described by

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Gross and Glaser [10] as well as Jackson et al. [7], was used because it only supplies five carbons. Furthermore, MBA monosaccharide derivatives are stable at room temperature.

Lastly, it was necessary to determine both the chromatographic conditions and mass spectrometry parameters. We used GC–MS to identify MBA-glucose and GC–C-IRMS to measure [^{13}C]-MBA glucose.

This paper describes the entire procedure in detail, including liver sampling in rats, the measurement of [^{13}C] enrichment in glucose produced by glycogen hydrolysis and quantification of [^{13}C] oral tracers transferred to glycogen during the postprandial phase. This methodology is accurate in determining low levels of [^{13}C] enrichment in various glucose pools in animals, such as blood or muscle glucose.

2. Experimental

2.1. Chemicals and animals samples

2.1.1. Chemicals

[U-^{13}C] glucose and [U-^{13}C] amino acids (98 at%) were purchased from CortecNet (Paris, France). [U-^{13}C] amino acids were a mixture of 20 amino acids. Amyloglucosidase from *Aspergillus niger* was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France), absolute ethanol, perchloric acid and D-glucose from Prolabo (Fontenay sous bois, France), sodium acetate anhydrous from Promega (Charbonnière, France), acetic acid from VWR (Fontenay sous bois, France), potassium carbonate and pyridine from Acros Organics (Noisy Le Grand, France), Dowex AG-50W X8 hydrogen form and Dowex AG1-X8 formate form from Biorad (Marne-La-Coquette, France), methylboronic acid (MBA) from Fluka (Saint-Quentin Fallavier, France) and *N*,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) from Supelco (Saint-Quentin Fallavier, France).

2.1.2. Rat liver samples

Fourteen Wistar male rats (Harlan-France, Gannat, France) were studied in accordance with the guidelines of the French National Animal Care Committee. The rats were placed under a reversed light rhythm (lights on from 8:00 pm to 8:00 am) and adapted to the experimental conditions for one week. The rats were trained to rapidly consume a 4 g meal (dry matter) in the morning and then had free access to food between 14:00 pm and 18:00 pm. They had free access to water. Their diet was constituted of 14% of protein, 76% of carbohydrate (14% sucrose, 86% starch) and 10% of fat as energy. On the day of the experiment, after an overnight fasting, the rats received a calibrated meal of 4 g dry matter. Among the 14 rats, four rats received in their diet 5 mg of a mixture of 20 [U-^{13}C] amino acids and two received 0.75 mg of [U-^{13}C] glucose, in order to measure the ability of the method to detect the transfer of an oral tracer into the glycogen. The other eight rats (rat 1 to 8) were used as controls to measure the basal abundance of [^{13}C] in liver glycogen. The rats were injected intraperitoneally with pentobarbital 4 h after the meal and the liver was harvested rapidly and stored at −20 °C until the analytical studies.

2.2. Samples preparation

2.2.1. Glycogen extraction

Rat liver samples (about 250 mg) were homogenized for 20 s in 1 mL of UHQ water using an Ultra-Turrax (Fisher Scientific Bioblock, Illkirch, France) on ice. The homogenate was kept on ice for 30 min to initiate glycogen release. Glycogen was then precipitated with 1 mL of absolute ethanol and centrifuged at 2500 *g* at 4 °C for 15 min. After removing the supernatant, the pellet was rinsed with 1 mL 80% ethanol and centrifuged at 2500 *g* at 4 °C for 15 min. The pellet containing the glycogen fraction was then dried and kept for hydrolysis.

2.2.2. Glycogen hydrolysis

Enzymatic glycogen hydrolysis was performed according to the method described by Keppler and Decker [4], with a few modifications. The pellet was resuspended in 900 μL sodium acetate–acetic acid buffer (0.2 mol/L, pH 4.6) supplemented with amyloglucosidase from *Aspergillus niger* (188 units per 200 mg of liver) and incubated in a shaking water bath at 60 °C for 90 min. After incubation, 50 μL of the homogenate were collected and neutralized for the determination of glucose concentrations using the glucose-oxydase method adapted for a microplate spectrophotometer. To assess the losses due to the precipitation step, intact liver homogenates were also digested and glycogen content was determined as the difference between glucose content before and after the hydrolysis.

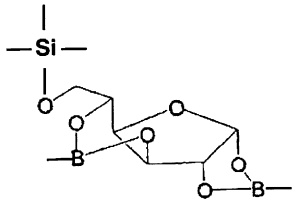
1.2 M of perchloric acid was added to the digested homogenate to stop the reaction and precipitate the proteins. After centrifugation at 9000 *g* at 4 °C for 10 min, the supernatant was collected and the pH was adjusted to between 5.5 and 6.2 with 100 μL potassium carbonate (3.2 mol/L) according to the method described by Lemosquet et al. [11]. The CO_2 produced by the reaction was released at 4 °C overnight. The next day, the samples were spun (at 2500 *g* for 10 min) and the pH of the supernatant was again neutralized with potassium carbonate (3.2 mol/L) or perchloric acid (1.2 mol/L), after which it was filtered through a 0.22 μm filter adapted on a 1 mL syringe (Fisher Bioblock Scientific, Illkirch, France).

2.2.3. Glucose purification

The supernatant was successively passed through an anion (AG1-X8: formate form) and a cation (AG50-X8: hydrogen form) exchange column to successively trap organic acids and amino acids, as described by Lemosquet et al. [11]. Free glucose was eluted with 6 mL UQH Water in a 10 mL glass tube. The glucose solution was dried overnight in a speed vacuum concentrator (Fisher Scientific Bioblock, Illkirch, France) and then dissolved in 300 μL UHQ water. 20 μL of the solution was collected to measure the glucose concentration. The solution was then dried again under a speed vacuum prior to derivatization.

2.2.4. Glucose derivatization

500 μL of a solution of 3 mg MBA in 0.5 mL of pyridine was added to 0.1–5.3 mg of purified glucose. The solution was mixed, kept for 2 h at room temperature and then heated at 60 °C for 60 min. After cooling, 30 μL BSTFA was added and the sample was heated at 60 °C for 30 min. The structure of glucose MBA derivative is presented below:



In order to optimize the glucose derivatization reaction, several different quantities of glucose (1, 3, 6, 12 and 18 mg), MBA (3, 10 and 20 mg) and BSTFA (30, 60, 100 and 150 μL) were used. The derivatives were prepared in duplicate and were analyzed twice using gas chromatography/mass spectrometry (GC–MS) in electron impact ionization mode (GC 6890 N chromatograph coupled to a 5973 INERT quadrupole mass spectrometer, Agilent Technologies, Massy, France). A capillary column (HP 5-MS, 30 m long, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies, Massy, France)

with helium as the carrier gas (1 mL/min) was used for this study. The derivative sample (1 μL) was injected in the splitless mode. The GC injector was set at 200 °C and the transfer line was held at 280 °C. The chosen temperature was programmed from 70 °C (1 min) to 180 °C at 4 °C min^{-1}, with an isothermal of 4 min, then to 280 °C at 10 °C min^{-1} with an isothermal of 1 min. Electron ionization (EI) at 70 eV was used with an ion source temperature of 230 °C and quadruprole temperature at 150 °C. Full mass spectra were obtained by scanning the mass range m/z 50–550 in approximately 0.5 s.

2.3. Determination of ^{13}C enrichment of the glucose derivative by GC/C/IRMS

Analysis of the ^{13}C enrichment of the glucose MBA derivative was performed on an Isoprime in continuous flow (Micromass, Villeurbanne, France) coupled online with an HP 5890 series II gas chromatograph (Fisons Instruments, Arcueil, France) via a combustion interface (Fisons Instruments, Arcueil, France).

Glucose derivative that was introduced into the column could be directed to a flame ionization detector (FID) or a mass spectrometer via a pressure-back-pressure system controlling a heart split valve (HS). The FID was used to determine the optimum chromatographic conditions and to program opening and closing of the HS valve. During the analysis, solvent and derivative glucose were respectively directed towards the FID and the mass spectrometer *via* a combustion interface. The sample was then combusted to CO_2 that was introduced into the mass spectrometer while H_2O was removed through a cryogenic trap before the introduction of the sample in the IRMS. Three pulses of reference CO_2 gas (99.995% purity, Air Liquide, France), calibrated on an international standard (Pee Dee Belemnite, isotopic ratio $[^{13}C]/[^{12}C]$: 0.011237), were injected into the source before detection of the sample signal in order to determine the $[C^{13}]/[C^{12}]$ isotopic ratio of the sample. Calibration of CO_2 reference gas was performed using urea standard (Fisons, France, −36.4 $\delta^{13}C$ (‰)).

The chromatographic conditions were optimized using a capillary column (HP 5-MS, 30 m long, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies, Massy, France) with helium as the carrier gas (1 mL/min). The derivative sample (1 μL) was injected in splitless mode. The GC injector was set at 200 °C and the oven temperature was programmed from 130 °C (1 min) to 220 °C at 4 °C min^{-1} (isothermal for 1 min), then to 320 °C at 30 °C min^{-1} (isothermal for 2 min).

CO_2 enrichment, expressed as a $\delta^{13}C$ value (‰) with reference to Pee Dee Belemnite, was calculated using the Craig corrections [12]. $\delta^{13}C$ values were corrected to take account of the dilution of glucose carbons by the derivative reagent. They were expressed according to the following equation:

$$\delta^{13}C_{glucose}(‰) = \frac{(N_{der}\delta^{13}C_{der} + F - (N_{MBA}\delta^{13}C_{MBA} + N_{BSTFA}\delta^{13}C_{BSTFA}))}{N_{glucose}}$$

where N_{der}, N_{MBA}, N_{BSTFA} and $N_{glucose}$ are the number of carbon atoms of the derivative, the derivatization reagents and the glucose molecule, respectively. $\delta^{13}C_{der}$ is the values of glucose derivative and was obtained by GC–C-IRMS. $\delta^{13}C_{MBA}$ (‰) (−43.32 ± 0.15) and $\delta^{13}C_{BSTFA}$ (‰) (−38 ± 0.25) are the values of MBA and BSTFA, respectively, as measured by EA-IRMS (elementary analyzer/isotopic ratio mass spectrometer, Euro EA 3000, Eurovector, Italy) using the same value for the reference gas as that used for GC–C-IRMS measurements. F is the correcting factor accounting for the kinetic isotope fractionation.

2.4. Isotopic accuracy and isotopic fractionation

The accuracy of $[^{13}C]$ determination in glucose MBA derivatives by GC–C-IRMS was checked using a calibration curve (from 0 to 0.75 APE). The fractionation effect of derivatization was assessed by measuring the $[^{13}C]$ enrichment of the basal point by EA-IRMS before derivatization. Urea standard (Fisons Instruments, France) was used to determined the correction factor of CO_2 reference gas ($-34.71\delta^{13}C$ (‰)) for both GC–C-IRMS and EA-IRMS.

In tracer studies, it is necessary to express the isotopic enrichment as abundance, which represents the percentage of $[^{13}C]$ atoms relative to the total carbon atoms for CO_2.

$$\text{Atom\%(AP)} = \left[\frac{R_{ref}(1 + (\delta^{13}C\,‰/1000))}{1 + [R_{ref}(1 + (\delta^{13}C\,‰/1000))]}\right] \times 100$$

AP values are then transformed to APE (atom% excess), which is the difference between the value obtained after tracer administration and the value obtained in control animals.

The percentage of the ingested tracer recovered in the glycogen pool was determined as follows:

% tracer = glycogen C (mmol) × 100 × (APE sample/APE tracer)/ingested C tracer (mmol); where glycogen C is the amount of carbon in liver glycogen (total liver glycogen content × 6/180), APE sample is the excess of enrichment in the liver sample above the basal value and APE tracer is the excess of enrichment of the tracer.

Table 1
Rat liver glycogen concentrations and amounts of purified glucose released from glycogen used for the derivatization reaction.

Rats	Size of liver sample (mg)	Total glycogen concentration[a] (mg of glucose/g of liver)	Glycogen concentration after precipitation[b] (mg of glucose/g of liver)	Quantity of glucose after purification (mg of glucose/g of liver)	Quantity of glucose purified (mg)
1	269.6	7.06	5.73	2.63	0.7
2	270.3	9.76	7.52	4.03	1.1
3	263.2	39.43	30.09	9.82	2.6
4	247.1	31.47	20.96	7.60	1.9
5	237.3	ND	26.14	10.22	2.4
6	231.9	9.13	6.89	4.94	1.1
7	234.0	ND	17.1	10.4	2.4
8	229.0	ND	19.4	10.7	2.4

ND: not determined.

[a] The total concentration of glycogen in the liver was measured without any glycogen precipitation and was calculated as the difference of glucose concentration measured before and after the enzymatic digestion.

[b] The concentration of glycogen after precipitation was determined from the glucose concentration measured after glycogen enzymatic digestion.

Table 2
Optimization of glucose MBA derivatization using GC–MS.

	Test									
	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	No 10
Glucose (mg)	0.1	1	3	6	6	12	12	18	18	18
MBA[a] (mg)	3	3	3	3	10	3	10	3	10	20
Pyridine (μL)	500	500	500	500	500	500	500	500	500	500
BSTFA[b] (μL)	30	30	30	60	60	100	150	100	150	150
Number of peaks	1	1	1	>1	1	>1	>1	>1	>1	1

[a] MBA: methylboronic acid.
[b] BSTFA: *N*,O-bis(trimethylsilyl) trifluoroacetamide.

3. Results and discussion

3.1. Extraction and purification of glucose

Four hours after the meal, the liver glycogen content ranged from 6 mg to 30 mg/g of liver in the eight control rats (Table 1). This was consistent with findings described in the literature. At this postprandial time point (4 h), Gannon and Nuttal found liver glycogen levels of 6–17 mg/g in rats receiving either casein or glucose by gavage [13]. The glycogen content measured without any precipitation step was found to be 18–35% higher, showing that the losses induced by the ethanol precipitation were variable. In consequence, for the calculation of tracer sequestration in liver glycogen, total liver glycogen must be determined without glycogen precipitation on a separate procedure from that used for glucose purification and derivatization. After purification on anion (AG1-X8: formate form) and cation (AG50-X8: hydrogen form) exchange columns, 0.7–2.4 mg of glucose was recovered (Table 1), accounting for 22–60% of the glucose precipitated. These results suggest that neutralization and purification of the hydrolyzed homogenate constituted a critical step. A higher volume of water for elution might have improved the recovery ratio but would have led to a dramatic increase in drying time. However, this quantity of glucose was sufficient for [^{13}C] determination using mass spectrometry. In addition, to perform the derivatization reaction, strict control of the quality of the purification was essential, as purified samples needed to be colorless after drying. Thus if the samples presented a brown coloration, they were purified again.

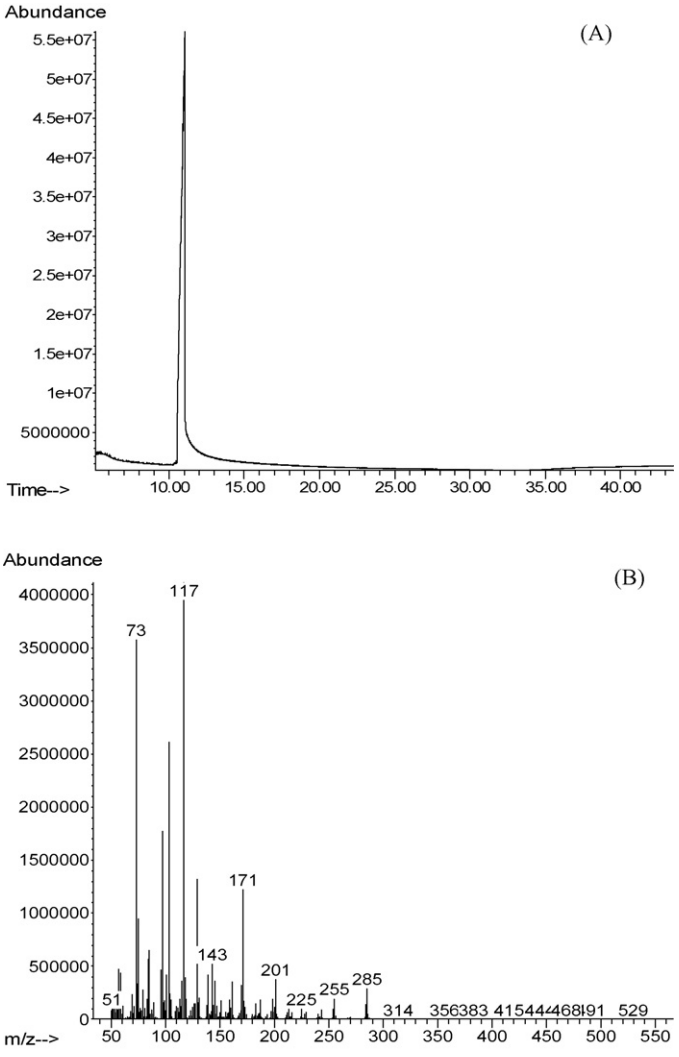


Fig. 1. Chromatogram (A) and mass spectra (B) obtained by GC–MS after the derivatization of 3 mg glucose with 3 mg MBA in 500 μL pyridine and 30 μL BSTFA.(test No. 1 in Table 1).

3.2. Optimization of glucose derivatization

The results in Table 2 show that for quantities of glucose ranging from 0.1 to 3 mg, it was necessary to use 3 mg MBA and then 30 μL BSTFA. For 6 and 18 mg of glucose, 10 and 20 mg MBA were required, together with 60 and 150 μL BSTFA. Under these conditions, a single and narrow peak for glucose with a retention time of about 12 min was detected using GC–MS, with reproducible mass spectra (Fig. 1). The major fragment ions and their relative abundance are given in Table 3 and are fully consistent with those reported by Jackson et al. [7]. The ratio 103/104, 129/130 and 171/172 were reproducible whatever the amount of glucose derivatized (1, 3, 6 and 12 mg): their respective values were 0.093 ± 0.33, 0.121 ± 0.62 and 0.138 ± 0.66 (Table 4).

Under the other conditions of BSTFA and MBA, we obtained several peaks that were indicative of incomplete derivatization (Fig. 2). Gross and Glaser [10] had also observed several peaks using larger quantities of BSTFA (more than 50 μL BSTFA for 4.5 mg MBA and 100 μg monosaccharide), that they explained by replacing MBA with trimethylsilyl (TMS) bonds if the reaction times were longer than 5 min. We observed complete derivatization when the MBA concentrations increased in line with those of BSTFA

Table 3
Rationalization of the fragmentation patterns of [^{12}C] and [U^{13}C]-glucose-MBA in GC–MS.

Fragment ion	Mass	Abundance (%)
$(CH_3)_3Si^+$	73	81
$CH_3BO_2C_3H_3^+$	97	43
$(CH_3)_3SiOCH_2^+$	103	63
$(CH_3)_3SiOCH_2CH_2^+$	117	100
$(CH_3)_3SiOCH_2CHCH^+$	129	37
$(CH_3)_3SiOCH_2CHCHCH_2^+(CH_3)_3SiOCH_2COC^+$	143	13
$(CH_3)_3SiOCH_2CH(CH)OCH_3^+$	171	34

Table 4
Isotopic ratio of the three specific major glucose-MBA fragments in GC–MS at different glucose concentrations.

	Ratio 103/104	Ratio 129/130	Ratio 171/172
1 mg	0.09337	0.1213	0.1369
3 mg	0.0935	0.1206	0.1386
6 mg	0.0933	0.1218	0.1381
12 mg	0.0940	0.1202	0.1390
Mean	0.0935	0.1209	0.1381
Standard deviation	0.0003	0.0007	0.0009
Coefficient of variation (%)	0.3264	0.6163	0.6613

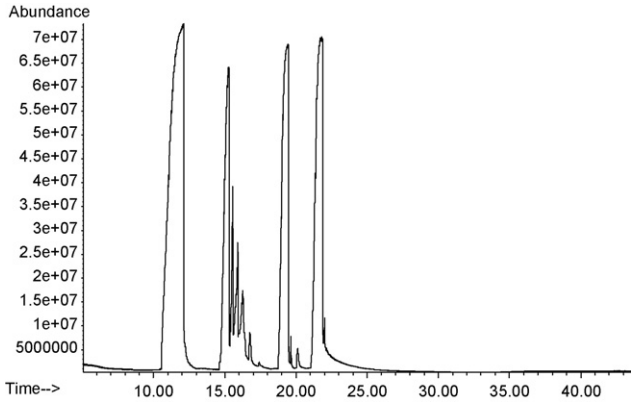


Fig. 2. Chromatogram obtained by GC–MS after the derivatization of 6 mg glucose with 3 mg MBA in 500 μL pyridine and 60 μL BSTFA (test No. 4 in Table 1).

with an incubation period of 30 min. In conclusion, depending on the concentrations of glucose recovered after hepatic glycogen hydrolysis and purification (2–5 mg dried glucose used for derivatization), we used 3 mg MBA dissolved in 500 μL pyridine and 30 μL BSTFA.

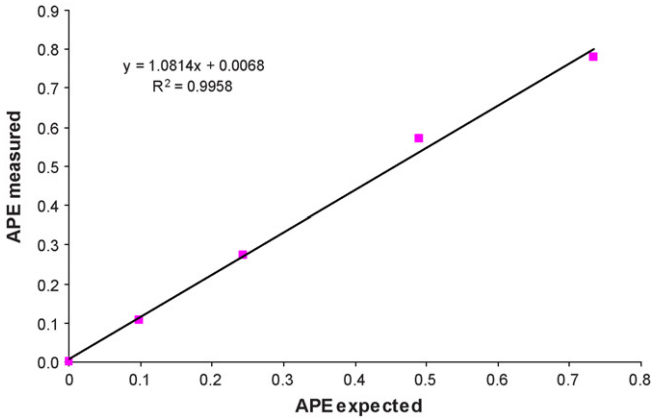


Fig. 4. [^{13}C] enrichment (APE) measured with GC–C-IRMS in standard solutions.

3.3. Measurement of ^{13}C-glucose by GC/C/IRMS

After the derivatization of approximately 2–5 mg glucose, we obtained a single and narrow glucose peak by GC–C-IRMS with a retention time of about 18 min and an intensity of 5–9 nA (Fig. 3). It was possible to analyze glucose quantities smaller than 1 mg with the same sensitivity, by using smaller quantities of derivatization reagents and by concentrating the derivative sample under nitrogen. The measurement of standard solutions ranging from 0% to 0.75% APE showed the linearity of the response ($R^2 > 0.99$) and the good agreement between expected and measured enrichment excess ($R = 0.9979$) (Fig. 4).

In order to assess the fractionation occurring during derivatization, we also measured simultaneously the enrichment of glucose by EA-IRMS (without derivatization) and GC–C-IRMS. Values obtained by EA-IRMS and GC–C-IRMS showed a difference resulting in a correcting factor of −40. Information concerning this factor is scarce since Gross and Glaser [10] determined it but did not

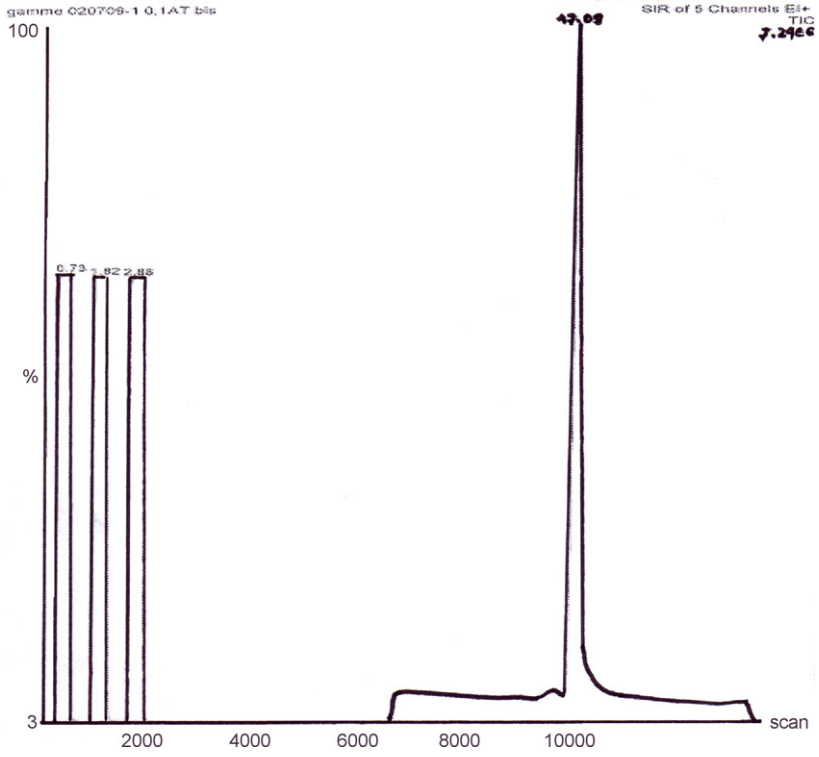


Fig. 3. Chromatogram of MBA glucose from hepatic glycogen obtained using GC–C-IRMS.

Table 5
[^{13}C] glucose enrichment measured by GC–C-IRMS in the liver glycogen of control rats and after the administration of [^{13}C] oral tracers.

Group	Rat	Measured δ^{13}C(‰)	Corrected δ^{13}C(‰)	Corrected atom % (AP)
Control[a]	1–8	−23.0 ± 0.9	−15.6 ± 1.6	1.0941 ± 0.002
[^{13}C] glucose[b]	9	−7.8	12.3	1.1248
	10	−6.9	13.9	1.1265
[^{13}C] AA[c]	11	−16.7	−4.0	1.1068
	12	−16.3	−3.2	1.1078
	13	−17.2	−4.9	1.1059
	14	−10	7.4	1.1194

[a] Control rats did not receive any oral [^{13}C] tracer.
[b] [^{13}C] glucose rats received a single dose (0.75 mg) of [U-^{13}C] glucose with the meal.
[c] [^{13}C] AA rats received a single dose (5 mg) of [U-^{13}C] amino acids with the meal.

report the values. In contrast, van Dongen et al. [8] found a negligible fractionation. Using an alditol acetate derivatization of glucose, Docherty et al. [14] reported a correcting factor of −60, which is close to our but hardly comparable with the fractionation that can occur with MBA derivatization. However, an uncertainty on the correcting factor does not impair our final result (as described below), since the calculation of the enrichment above the basal value (APE) leads to the neutralization of the correcting factor in the equation.

The [^{13}C] enrichment was measured in rats that received an oral tracer (either [U-^{13}C] glucose or [U-^{13}C] amino acids) with the experimental meal (Table 5). In the [U-^{13}] glucose group, one of the three rats was not included because it spilled its meal. Compared to the value obtained in control rats (−15.6 ± 1.6 δ^{13}C (‰)), the [^{13}C] enrichment in glycogen was slightly increased when [U-^{13}C] amino acids were added to the meal (rat 12 to 14) and to a higher extent with [U-^{13}C] glucose.

The same sample (from animal 15) was analyzed five times in order to calculate the reproducibility of [^{13}C] enrichment measurements using GC–C-IRMS. Using this technique, the coefficient of variation was less than 5% for crude delta but was of 8% for corrected delta (Table 6). In this last case, the coefficient of variation varied with the correcting factor value. In the absence of any correcting factor, the coefficient of variation was only 3.2%. Nevertheless, the coefficient of variation for the AP value was very low, indicating that the measurement of [^{13}C] enrichment in liver glycogen was reproducible, using this complete procedure.

The enrichment excess reached in rats with [U-^{13}C] amino acids (0.012 to 0.025 APE) indicated that some glycogen was produced from dietary amino acid carbon skeletons during the postprandial phase. Although the indirect pathway has already been evidenced during tracer studies [2,15,16] we are the first group to have demonstrated *in vivo* the existence of this pathway from a dietary amino acid source. However, the quantity of tracer sequestered at that time-point in the glycogen store was very low since it represented only 0.25 % to 1 % of the tracer ingested. This low sequestration was expected given the high carbohydrate content of the meal (76 % of energy), but could be higher after the ingestion of a low carbohydrate meal. In rats that ingested [U-^{13}C] glucose (rats 9 and 10), enrichment excess was 0.03 APE, resulting in a 31–37% tracer recovery in hepatic glycogen. It thus indicates that about one third of dietary carbohydrates was postprandially stored in hepatic glycogen. The other two thirds can thus be either oxidized or directed toward muscle glycogen, a proportion that agrees with data obtained by indirect calorimetry in our laboratory with the same meal composition and feeding design (unpublished data) as well as in postprandial studies performed in humans [17–19].

Table 6
Reproducibility of [^{13}C] glucose enrichment measured using GC–C-IRMS.

	Measured δ^{13}C (‰)	Corrected δ^{13}C (‰)	Corrected AP (%)
RUN 1	−10.5	7.4	1.1194
RUN 2	−9.9	8.5	1.1206
RUN 3	−10.0	8.3	1.1204
RUN 4	−9.8	8.7	1.1208
RUN 5	−9.2	9.8	1.1220
Mean	−9.9	8.6	1.1206
Standard deviation	0.47	0.76	0.0008
Coefficient of variation (%)	4.8	8.9	0.07

The test for reproducibility was performed on rat 14, which ingested the meal + [U-^{13}C] amino acids.

The precision of the measurement, as estimated by the standard deviation obtained in control rats (1.6 δ^{13}C (‰)), accounts not only for measurement uncertainty but also for the interindividual variability and the uncertainty generated by the purification procedures. In rats that ingested [^{13}C] glucose, a variation of 1.6 δ^{13}C (‰) generated a variation of 0.0018 AP and 0.001 APE, finally resulting in a variation of glucose sequestration in glycogen of 2%.

Our method can be interestingly applied to several nutritional conditions to assess the transfer of dietary substrates to glycogen pool. However, it requires several steps that are time consuming, including the time required for GC–C-IRMS measurements. A method based on the *in vitro* catabolism of glucose by yeast has recently been developed, allowing the use of EA-IRMS for [^{13}C] glucose enrichment in plasma [20]. It could be of interest to test whether this method could be accurate for our purpose in order to reduce the time required for measurements.

4. Conclusion

We have developed a complete biochemical and analytical procedure during which the main critical step concerns the precipitation of glycogen and purification of glucose on ion exchange columns, leading to a cumulated loss of 45–75% of the initial liver glycogen. However, amounts of 250 mg of liver were sufficient to recover enough glucose for derivatization. We could evidenced and quantify the transfer of small amounts of [^{13}C] oral tracers into glycogen. Under our conditions, about 35% of dietary carbohydrates were sequestered into glycogen 4 h after the meal whereas the contribution of amino acid carbon skeleton was negligible. This method for the measurement of low levels of [^{13}C] enrichment in small samples could be adapted to muscle glycogen or blood glucose during metabolic studies. It use could also be extended to the measurement of low or natural [^{13}C] enrichment in all types of carbohydrates, including in plants.

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