VERIFYING THE INFLUENCE OF THE HPLC METHOD ON CARBON ISOTOPIC FRACTIONATION OF AMINO ACIDS STANDARDS FOR RADIOCARBON DATING

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An experimental investigation is presented concerning the influence of the high-performance liquid chromatography (HPLC) method on the determination of the radiocarbon age of some amino acids standards. The HPLC analysis was performed by use of a Shimadzu system. Amino acids were analyzed in a Primesep A column. It was found that although the HPLC method introduces a fractionation of the carbon isotopic ratio, the age of the amino acid standards was insignificantly influenced.

Keywords: amino acids, HPLC chromatography, graphitisation, accelerator mass spectrometry (AMS), radiocarbon dating

1. Introduction

Carbon dating with the isotope ^{14}C is based on the fact that this radiocarbon is constantly produced in the atmosphere by the interaction of cosmic rays with atmospheric nitrogen. The resulting radiocarbon combines with oxygen to form carbon dioxide, which is incorporated into plants by photosynthesis and then the animals acquire carbon by eating plants. When the animal or plant dies, it stops exchanging carbon with its environment, and the amount of ^{14}C it contains begins to decrease as the ^{14}C undergoes radioactive decay. Measuring the amount of ^{14}C in a sample from a dead plant or animal such as a piece of wood or a fragment of bone provides information that can be used to calculate when the animal or plant died.

In the photosynthetic pathways the carbon is not absorbed in the same isotopic ratios as from the atmospheric CO_2. In the photosynthesis ^{12}C is absorbed more easily than ^{13}C, which is more easily absorbed than ^{14}C. The differential uptake of the three carbon isotopes leads to $^{14}C/^{13}C$, $^{13}C/^{12}C$ and $^{14}C/^{12}C$ ratios in plants that differ from the ratios in the atmosphere. This effect is known as

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isotopic fractionation and is the result of a natural biochemical processes dependent on their atomic mass [1].

To determine the degree of fractionation in a living thing, the isotopic ratio $^{13}C/^{12}C$ is measured and expressed as a difference, in parts per thousand, from a standard. The depletion of ^{13}C relative to ^{12}C is proportional to the difference in the atomic masses of the two isotopes, so that the depletion for ^{14}C is twice the depletion of ^{13}C [2].

The fractionation of ^{13}C of a sample, known as $\delta^{13}C$, is calculated as follows [3]:

$$\delta^{13}C(‰) = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{PDB}} - 1 \right) x1000 \quad (1)$$

where: PDB is international PDB (abbreviation from Pee Dee Belemnite) standard for carbon fractionation (derived from the rostrum of the calcareous fossil Belemnitella americana from the Pee Dee Formation of South Carolina).

Many studies on isotopic fractionation due to photosynthesis on different plants have been carried out [4-8]. It has been noticed that fractionation does not mainly occur as an effect of geographic position but rather depend on the environment, CO_2 diffusion, CO_2 absorption and respiration. According to the photosynthetic mechanism, there are 3 types of plants: C3 plants, C4 plants and CAM plants (with crassulacean acid metabolism). The differences between C3, C4, and CAM photosynthesis mainly consists in the way carbon dioxide is absorbed using the sun energy.

Approximately 85% of the plants on earth have the C3 photosynthesis. In C4, the photosynthesis produces a high concentration of carbon, making the organisms more adapted to survive in habitats with low light and water. Isotope studies demonstrated that C4 plants show less negative $\delta^{13}C$ values than C3 plants. The value of $\delta^{13}C$ for C4 plants is between -15 ‰ and -9 ‰ and for C3 plants between -30 ‰ to -22 ‰ [9]. The CAM plants present a wide variety of carbon isotopic ratios [10]. This difference in isotopic composition has become one of the standard methods to distinguish C4 plants from C3 plants. The $\delta^{13}C$ value provides an indicator for investigating the differences between marine and terrestrial nature of animal and human diet.

One of the usual assumptions in carbon-14 dating is that the sample being analysed has undergone only radioactive decay and has remained unaltered by any other process over the years since it ceased interaction with the biosphere. Remains of bone from archaeological sites depend on their conservation and suffer from various chemical or biological processes. By their degradation, the molecular structure of bones can incorporate exogenous molecules, consisting of organic compounds in soil and sediment, in particular, metabolic products such as

amino acids and lipids from microorganism degradation [11]. Elimination of these contaminants was one of the most discussed issues related to the accuracy of dating. Dating of the individual amino acids is a solution however, only if no other external sources such as bacteria or micro-organisms are present. Hydroxyproline responds to this requirement because it is an amino acid that is not found in significant amounts in most of other animal proteins. It is present in collagen of mammalian bones in a high percentage (10%). Therefore, extraction and dating of hydroxyproline essentially provides a non-contaminated biomarker, and therefore it represents a 'gold-standard' for ^{14}C dating [11].

The $\delta^{13}C$ value is a signature for a sample from the environment, for the origin of the sample originates or for the mixtures of materials used to produce it. Fractionation describes variations in the isotopic ratios of carbon brought by non-natural causes in the laboratory, through a variety of processes such as the lack of attention to detail and incomplete conversion of the sample from one stage to another (e.g. solid to gas or graphitization). In dating of single amino acids extracted from various archaeological samples the HPLC analyze is calibrated according to the corresponding amino acids standards. Unfortunately, such standards are not fully characterized by the providers. Their properties in terms of purity, $\delta^{13}C$, carbon and nitrogen content vary from lot to lot.

The aim of this paper was to investigate the four most commonly used amino acid standards used in ^{14}C dating: glycine (Gly), alanine (Ala), proline (Pro) and hydroxyproline (Hyp). The influence of using the high-performance liquid chromatography (HPLC) method on isotope fractionation of carbon was checked. The dating of the four amino acid standards was carry out using 1 MV Tandetron Accelerator AMS (Accelerator Mass Spectrometry) from IFIN-HH. The $\delta^{13}C$ values and the age value obtained for each standard were correlated with each individual way of manufacturing.

2. Experimental part

The data were recorded in two series of experiments. One included the graphitization followed by AMS measurements and the other included graphitization followed by HPLC analyses and AMS measurements. All aqueous solutions were prepared using ultrapure water from Millipore Milli-Q (Direct 8). Glassware was pre-heated to 500°C for 3 hours before being used to remove organic contaminations. The references standards of amino acids were purchased from Sigma Aldrich.

2.1. Sample preparation

Four types of amino acid standards samples were used in this work: glycine (Gly), alanine (Ala), proline (Pro) and hydroxyproline (Hyp). The product specifications of each standard are presented in Table 1.

Table 1

Theoretical and product data, including nitrogen and carbon content, of amino acid standards

No.	Amino acid	Formula	Molecular mass (g/mol)	N (%)	C (%)	C/N
1	Gly	$C_2H_5NO_2$	75.07	18.6*	32	1.7204
2	Ala	$C_3H_7NO_2$	89.09	15.2 - 16.2	40.0 – 41.0	2.469 - 2.697
3	Pro	$C_5H_9NO_2$	115.13	11.9 - 12.5	51.1 - 53.2	4.088 - 4.470
4	Hyp	$C_5H_9NO_3$	131.13	10.68*	45.80	4.2883

* Theoretical value (is not in Specification sheet)

Each amino acid standard solution was made with ultrapure water in the concentration of 10 mg/mL. Two sets of blank samples were produced. Blank samples were run through the entire laboratory protocol. The first set was produced without HPLC step. Up to 15 mL of ultrapure water had to be reduced to a final volume of 2 mL and the resulting carbon concentration in ultrapure water was enough high to be measured.

2.2. Preparative High-Performance Liquid Chromatography (HPLC)

Chromatography analysis was performed with HPLC Shimadzu system equipped with two delivery pump units (LC-20AD), an auto-sampler (SIL-20A), a UV/VIS photodiode array detector (SPD-M20A), and a fraction collector (FRC-10A). The analyzing column for amino acids was a Primesep A column 22 x 250 mm, with the particle size 5 μm (SIELC Technologies, Prospect, Height, Illinois, SUA). This type of HPLC column is producing the separation of ions combining the reverse-phase with the embedded strong acidic ion-pairing groups in a mix mode.

The sample injection was performed by an auto sampler for a volume of 1000 μL of liquid at a flow rate of 6 mL/min and a total run time of 150 min. The temperature of a column was kept at room temperature. The UV/VIS photodiode of the detector was working on a 205 nm wavelength. The entire analyzing system is computer controlled. To obtain enough material from each amino acid for AMS dating, three successive injections for each sample were necessary. Every fraction of amino acid was collected from the beginning to the end of the peak with a fraction collector. The excess water was removed by vacuum evaporation (Genevac EZ-2).

2.3. Elemental analysis

After water evaporation, the dried samples of amino acids were weighed into a tin capsule and burn in an Elemental Analyzer (EA) to determine the percentage of C and N and the C/N atomic ratio. The CO_2 from sample combustion is then adsorbed on the zeolite trap of the Automated Graphitization Equipment (AGE). Finally, the pure CO_2 is thermally released into a selected

reactor, with iron catalyst on bottom, of the AGE, where it is converted with excess hydrogen into graphite. The final sample is a homogeneous mixture of carbon and iron. The all process is controlled by PC software Lab VIEW [12, 13].

In order to check the contamination with ^{14}C, all HPLC standards of amino acids were directly graphitized in the same way described above.

2.4. Stable carbon isotope and radiocarbon analysis

The stable carbon isotope and radiocarbon analysis were carried out at RoAMS Radiocarbon Dating Laboratory (IFIN-HH) using AMS facility based on a Cockcroft-Walton type 1 MV tandetron accelerator, designed and manufactured by HVEE (High Voltage Engineering Europe, Netherlands). The AMS facility is dedicated for ultra-sensitivity AMS analyses using C, Be, Al, I and Pu elements [14-19].

For a complete age reporting we used Oxa II (SRM 4990C) as a primary standard while for the blank correction graphite powder from Merck was used. After the measurement of all samples and Oxa II, we obtained the ratios of the stable isotopes and the radioactive one: $^{13}C/^{12}C$, $^{14}C/^{12}C$, and after that, the radiocarbon ages with the help of special software BATS developed at ETH Zurich [20] were calculated.

3. Results and discussion

To verify the level of isotopic fractionation of carbon introduced by the HPLC, four standards were analyzed both directly and after using HPLC. It is also important that no significant contamination occurs through the pretreatment procedures. Two sources of extraneous carbon contamination are suspected in the use of the HPLC: one is ^{14}C death due to the column bleed and the other is modern carbon contained in the Milli-Q water or/and dissolved atmospheric CO_2 in the mobile phase [21]. We analyzed the ^{14}C content of various blank samples to determine background value, while the isotopic composition was of secondary interest. For this reason, carbon isotopic ratio was measured by use of AMS instead CF-IRMS (Continuous-Flow Isotope Ratio Mass Spectrometry).

3.1. Determination of C/N ratio

Percentage of C, N and C/N atomic ratio obtained for the four amino acid standards, using Elemental Analyzer (EA), is presented in Table 2 and Table 3. Two replicates were performed for each measurement with different amounts of amino acid. However, the separation from collagen results in different quantities amino acids. The question arises if such mass differences have an influence on C/N ratio, which determines the suitability for radiocarbon dating. The measured data showed that the C/N ratio is not dependent of the amount of used amino acid.

Since the quantity of the amino acid cannot be precisely determined such of the results indicated this dependence is negligible.

Table 2

Experimental data for amino acids standards using direct analysis

No.	Amino acid	Mass (mg)	N (%)	C (%)	C/N
1	Gly	3.67	21.30	36.33	1.7056
		3.28	20.67	35.78	1.7311
2	Ala	2.30	17.93	45.68	2.6466
		4.05	17.51	45.58	2.6035
3	Pro	3.57	11.70	49.97	4.2703
		4.22	11.46	50.16	4.3763
4	Hyp	2.87	12.04	51.28	4.2595
		4.10	12.10	51.51	4.2571

Table 3

Experimental data for amino acids standards using HPLC

No.	Amino acid	Mass (mg)	N (%)	C (%)	C/N
1	Gly	1.11	21.26	38.36	1.8039
		3.56	18.91	34.37	1.8174
2	Ala	2.67	16.01	42.74	2.6694
		3.71	13.84	36.43	2.6322
3	Pro	2.66	10.85	48.04	4.4269
		3.92	9.61	42.45	4.4176
4	Hyp	2.90	11.57	49.87	4.3110
		3.70	11.53	50.31	4.3649

The experimental C/N ratios obtained for each sample are very close to the theoretical C/N values and from Specification Data of each amino acid (see Table 1 and Table 4).

Table 4

Comparative table of experimental and theoretical data for amino acids standards

No.	Amino acid	C/N (direct)	C/N (HPLC)	C/N (theoretical)
1	Gly	1.718±0.018	1.8112±0.009	1.715
2	Ala	2.625±0.030	2.651±0.026	2.572
3	Hyp	4.323±0.075	4.423±0.007	4.287
4	Pro	4.258±0.002	4.338±0.039	4.287

From calculated values of C/N ratios for each collected fraction, it was checked if the amino acid standards contain carbon or nitrogen as contaminants after HPLC step.

Fig.1 presents the plot of the experimental data versus the theoretical data. The intercept of curves with the axis Oy indicates an addition of carbon introduced through the graphitization and HPLC processes.

These increases of C/N value 0.045 and 0.068 for graphitization process and respectively HPLC process are in the range of measurement errors, so that the amount of extraneous carbon can be considered negligible. From the plot in Fig.1,

it can be seen that an amount of extraneous carbon is added using chromatographic technique (slope value is over 1).

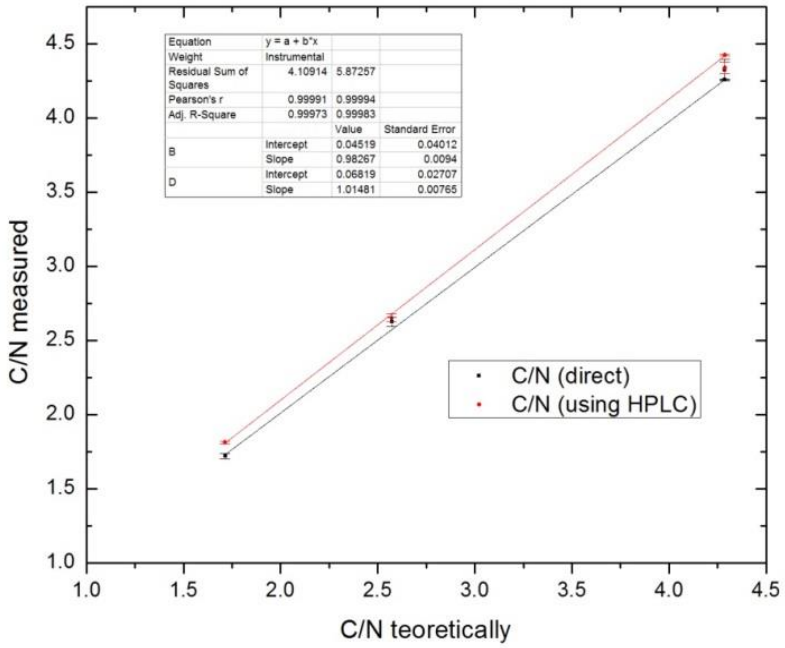

Fig. 1: Study of the deviation of experimental data from the theoretical data

The average carbon concentration for blank samples that passed graphitization and HPLC was 0.28±0.15 μg/mL, insignificant relative to the carbon content of the samples to be analyzed. The manufacturer declared purity was better than 89% and was confirmed by the experimental data obtained (see Fig. 1).

3.2. Isotopic fractionation

In order to provide accurate and precise radiocarbon determinations, it is necessary to make the corrections for "isotopic fractionation" using the stable isotopes ^{13}C and ^{12}C. Regarding the studied amino acid, only partial C fractionation information is presented in literature: glycine suffers a greater $\delta^{13}C$ fractionation (is depleted by -7.3 ‰ in case of derivatized amino acid and enriched by +1 ‰ for underivatized amino acid). This effect on the $\delta^{13}C$ of proline and alanine is negligible [22-25]. In this paper we determine the level of fractionation of each amino acid standard using HPLC relative to $\delta^{13}C$ value of raw amino acid standards. The difference between the two values is given in Table 5 as Δ(HPLC-raw). In Table 5 it can be seen that the magnitude of carbon isotope fractionation tends to increase with the decrease of the mass of molecule, the result obtained also by Bouchard and al. [26].

Table 5

$\delta^{13}C$ experimental data of amino acids measured at 1 MV AMS facility

		Amino acids standards (raw)		Amino acids using HPLC		Δ(HPLC-raw) (‰)
No.	Amino acid	$\delta^{13}C$ (‰)	Relative errors (%)	$\delta^{13}C$ (‰)	Relative errors (%)	
1	Gly	-45.7±0.38	0.83	-18.0±0.39	2.17	+27.7
2	Ala	-24.4±0.33	1.35	-11.1±0.38	3.42	+13.3
3	Pro	-29.0±0.39	1.34	-17.3±0.39	2.25	+11.7
4	Hyp	-20.8±0.31	1.49	-12.4±0.30	2.42	+8.4

3.3. Radiocarbon analysis

Radiocarbon analysis was carried out at RoAMS Radiocarbon Dating Laboratory using AMS facility from IFIN-HH. The results obtained are presented in Table 6.

Table 6

Comparative experimental age of amino acids measured at 1 MV AMS facility

No.	Amino acid	Amino acids without HPLC		Amino acids using HPLC	
		^{14}C age (years)	Percent of modern carbon (pMC, %)	^{14}C age (years)	Percent of modern carbon (pMC, %)
1	Gly	25,370 ± 347	4.25	24,395 ± 292	4.80
2	Ala	31,930 ± 139	1.88	24,077 ± 77	4.99
3	Pro	-505 ± 30	106.49	-531 ± 29	106.83
4	Hyp	-419 ± 44	105.35	-357 ± 44	104.54

The radiocarbon ages of the amino acids standards were calculated with the help of special software BATS developed at ETH Zurich. This software uses the $\delta^{13}C$ correction, but a correction of extraneous dead and modern carbon was not applied.

It was concluded that the use of HPLC insignificantly influences the age of three amino acid standards. Only in the case of alanine it was obtained smaller age than the direct dating, without HPLC. This can be explained by the non-uniformity of the lot from which the samples were collected or by adding a modern carbon contamination due to atmospheric CO_2 dissolved in mobile phase. Measurements on a larger number of samples are to be carried out in the future and the extraneous carbon corrections will be applied. A correlation between the age and the process of manufacturing the four amino acids can be made. In the literature, there are three general procedures for obtaining amino acids: direct chemical synthesis, fermentation and bioconversion using enzymes. Depending on the using of glycine and alanine, the direct synthesis or fermentation procedures are used more or less [27].

Proline and hydroxyproline are obtained by the fermentation method using culture media of microorganisms in atmospheric CO_2 [27]. From experimental data, it can be said that glycine and alanine (for which very old ages was obtained) are produced by synthesis method. On the other hand, the modern ages obtained for proline and hydroxyproline confirm that they were produced by the fermentation method.

4. Conclusions

High performance liquid chromatography of the amino acids standards was performed and AMS measurements showing that no significant contamination throughout the chain of the analysis occurred. The average carbon concentration for blank samples that passed graphitization and HPLC was 0.28±0.15 μg/mL that is insignificant relative to the carbon content in the samples to be analyzed. By the use of HPLC it was found that glycine is enriched in $\delta^{13}C$ relative to the other amino acids. However, it has insignificant influences in the radiocarbon age determination. The same weak influence was found for three of the four amino acid standards that have been studied.

A difference of about 25% in the age determination of alanine was measured when applying HPLC relative to the direct radiocarbon dating. It can be explained by the non-uniformity of the lot from where the samples were collected. The conclusion of this study is that the HPLC method is useful for dating amino acids from bone collagen because it can drastically reduce the contamination with extraneous carbon, leading to more precise results. Improvement of radiocarbon dating is essential since such a procedure is valuable both for the forensic science, environmental pollution, history and for quality control procedures.

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