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SAMPLE PREPARATION FOR MASS-SPECTROMETRIC ANALYSIS OF $^{13}C/^{12}C$ ISOTOPE FRACTIONATION FROM ENVIRONMENT TO THE PLANT CARBON POOL

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In order to study the differences in the ^{13}C and ^{12}C isotopes assimilation degree related to the rate of photosynthetic reactions, we have developed a number of procedures of sample gasification and a hardware experimental complex for sample preparation before mass-spectrometric isotope analysis of carbon involved in plant life. A setup for concentrating the carbon dioxide located around the plant was designed and made. The setup makes catalytic afterburning of organic microimpurities available for increasing the carbon content more than a hundred times. A reaction procedure for oxidation of leaf glucose by yeast generating carbon dioxide was suggested, reagent concentrations selected. The collected samples were free from impurities (not exceeding 10^{-5}). The developed sample preparation technique was used to study the effect of the light exposure characteristics on the carbon isotope interchange between atmospheric CO_2 and the plant carbon pool.

Keywords: carbon isotopes, plant, sample preparation, mass-spectrometric analysis, CO_2 concentration

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ПРОБОПОДГОТОВКА ДЛЯ МАСС-СПЕКТРОМЕТРИЧЕСКОГО АНАЛИЗА ФРАКЦИОНИРОВАНИЯ ИЗОТОПОВ $^{13}C/^{12}C$ ИЗ ОКРУЖАЮЩЕЙ СРЕДЫ В УГЛЕРОДНЫЙ ПУЛ РАСТЕНИЙ

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С целью изучения различий в степени ассимиляции изотопов ^{13}C и ^{12}C в ходе жизнедеятельности растений (различия связаны со скоростью протекания фотосинтетических реакций), разработан ряд методик и создан аппаратный комплекс для сбора и подготовки пробы, предшествующих масс-спектрометрическому изотопному анализу углерода. Спроектирована и изготовлена установка для концентрирования углекислого газа, находящегося вокруг растения, с каталитическим дожиганием органических микропримесей, позволяющая повысить его относительное содержание

более чем в 100 раз. Предложена методика проведения реакции окисления глюкозы листьев растений дрожжами с образованием углекислого газа, подобраны концентрации реагентов. Полученные пробы свободны от интерферирующих примесей, доля которых не превышала 10^{-5}. Разработанная методика пробоподготовки использована для изучения влияния спектральных характеристик световой среды на взаимообмен изотопов углерода между атмосферным воздухом и углеродным пулом растений.

Ключевые слова: изотопы углерода, растение, пробоподготовка, масс-спектрометрический анализ, концентрирование углекислоты, окисление дрожжами

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Introduction

Photosynthesis is accompanied by fractionation of isotopes of the elements constituting organic products; in particular, plants selectively absorb stable carbon isotopes ^{12}C and ^{13}C [1–5]. The distribution of isotopes between carbon dioxide in the air and the products of photosynthesis depends on the reactivity of molecules of different isotopic compositions; notably, the isotope whose participation accelerates the reaction is accumulated in the reaction products. Plants rapidly accumulate the ^{12}C isotope: its relative content in plant tissues is 15–25‰ higher than in the atmosphere. Differentiation of isotopes during photosynthesis presumably consists of two stages: the first is preferential absorption of carbon dioxide $^{12}CO_2$ from atmospheric air and its dissolution in the cytoplasm of plants, which is due to the kinetic effect; in turn, the fraction enriched with the ^{12}C isotope is extracted from carbon dioxide CO^2 dissolved in cytoplasm at the second stage, during synthesis of organic compounds [6]. Analysis of isotopic composition is of great interest for studies on distribution of carbon in the plant–soil–atmosphere continuum [7, 8], and on the reactions of plant organisms to changing external conditions [9].

Electrochemical gas sensors are used to monitor gas exchange processes in the plant - root system; analysis of carbon dioxide flows in closed chambers is performed using processing and modeling algorithms [10]; the Warburg apparatus is used to study dark respiration in plants [11]. The radioactive isotope 14C [11, 12] is widely used as an indicator of metabolism, movement of carbon and formation of photosynthesis products. However, these methods are not applicable to analysis of isotopic processes occurring during conversion of carbon dioxide from the air to the carbon pool of a growing plant.

Mass spectrometry is the most common and effective method for measuring the $^{13}C/^{12}C$ isotopic ratio. However, standard methods and hardware systems for collecting and preparing samples for such measurements are not suitable for studies of living plants.

Thus, the goal of of this study was to develop a technique for collecting and preparing samples that is suitable for studying the interchange of carbon isotopes between a plant and the atmosphere by conducting mass-spectrometric analysis of the $^{13}C/^{12}C$ ratio simultaneously in atmospheric carbon dioxide around the plant, and in tissues of the living plant.

Technique and devices used for preparing samples for mass-spectrometric analysis of carbon isotopes participating in plant life cycle

We proposed a technique for studying the isotopic composition of carbon in plant tissues and the degree of its fractionation from the air, and created a hardware system that allows to prepare the samples for mass-spectrometric isotopic analysis of carbon involved in the life cycle of plants.

The measured difference of the isotopic ratio of the sample from the standard is usually expressed as $\delta^{13}C$:

$$\delta^{13}C = [(^{13}C/^{12}C)_{smp}/(^{13}C/^{12}C)_{stnd} - 1]\cdot 10^3‰,$$

where $^{13}C/^{12}C$ is the isotopic ratio of carbon; the subscripts *smp* and *stnd* correspond to the sample and the standard, respectively.

We used the commonly accepted standard Belemnitella Americana (PDB) from the Peedee Formation (South Carolina), dating from the Cretaceous period, with the isotopic ratio

$^{13}C/^{12}C = 1123.72 \cdot 10^{-5}$, to compare data in isotopic analysis of carbon.

The $^{13}C/^{12}C$ ratio is determined in CO_2 whose concentration in the sample should be sufficiently high (more than 2–3%) and constant. We face the following problems in working with plants:

the concentration of carbon dioxide in the air surrounding the plants is low (~$3 \cdot 10^{-4}$% $^{13}CO_2$);

organic matter of plant tissues has to be transformed to gaseous state.

Therefore, the gas mixture should be enriched for mass-spectrometric analysis, which we implemented by freezing in nitrogen vapors (see below). Furthermore, to convert solid matter into gaseous state, we proposed and implemented a method alternative to thermal decomposition, which consists in using yeast as oxidizing agents for carbon-containing compounds.

The analysis of the $^{13}C/^{12}C$ ratio was carried out with Helicomass, a specialized static magnetic mass spectrometer, developed at the Ioffe Insitute (St. Petersburg, Russia) [13]. We used a laboratory standard calibrated against PDB with a Thermo Scientific Delta mass spectrometer (by Thermo Fisher Scientific, USA).

The three-collector detection system operating in spectrograph mode allows to detect molecular ions of carbon dioxide (CO_2^+) with the following mass-to-charge ratios:

$m/z = 44$ is the value corresponding to the main isotopic modification of $^{12}C^{16}O^{16}O$;

$m/z = 45$ to the sum of isotopic modifications of $^{13}C^{16}O^{16}O$ and $^{12}C^{17}O^{16}O$;

$m/z = 46$ to the sum of isotopic modifications of $^{12}C^{18}O^{16}O$ and very insignificant additions of $^{13}C^{17}O^{16}O$.

We only used the m/z values equal to 44 and 45 as carriers of analytical information about the $^{13}C/^{12}C$ isotopic ratio.

Given the isotopic abundance of carbon

$$^{13}C\ /^{12}C = 0.01123 : 1{,}000,$$

oxygen

$$^{18}O : {}^{17}O : {}^{18}O : = 2.004810^{-3} : 3.909310^{-4} : 1{,}000,$$

and the detector characteristics, we calculated $\delta^{13}C$ by the following algorithm:

Step 1. Find signal intensity for ^{13}C and ^{12}C isotopes taking into account the contribution of oxygen isotopes

$$^{13}C = (I_{45}/33) - 2I_{44} \cdot 3.9093 \cdot 10^{-4};$$

$$^{12}C = I_{44} + 2I_{44} \cdot 2.0048 \cdot 10^{-3} + 2I_{44} \cdot 3.9093 \cdot 10^{-4},$$

where I_{45}, I_{44} are the signal intensities for the m/z values of 45 and 44, respectively.

Step 2. Calculate the normalization coefficient k by the formula

$$k = 0.0106956/R_e,$$

where R_e is the average value of $^{13}C/^{12}C$ for laboratory reference gas, normalized by a coefficient of 0.0106956, which is the absolute content of the ^{13}C isotope in laboratory standard and measured against the PDB standard.

Step 3. Calculate $\delta^{13}C$ values by the formula

$$\delta^{13}C = [(kR_s/R_{PDB}) - 1] \cdot 10^3,$$

where R_s is the measured $^{13}C\ /^{12}C$ ratio for the sample; R_{PDB} is the $^{13}C/^{12}C$ ratio for the PDB standard, equal to 0.0112372.

We used a TEKHMAS MS7-100 quadrupole mass spectrometer developed at the Institute for Analytical Instrumentation of RAS (St. Petersburg, Russia) for molecular analysis of the composition of gas mixtures obtained in preparing samples, determining the concentrations of individual components and recording them in dynamic mode. The device allows to determine the composition of the gas mixture in the range of mass numbers from 2 to 100 amu, making it possible to detect substances and fragments of molecules interfering with carbon dioxide.

Setup for enriching carbon dioxide and determining the isotopic ratio of carbon in the air surrounding the plant

The gas mixture is enriched with carbon dioxide for mass-spectrometric analysis of carbon in the atmosphere surrounding the plants. Freezing is one of the methods for concentrating carbon dioxide. Carbon dioxide transforms to solid state at an absolute pressure of 760 mm Hg and a temperature of −78.9 °C. Freezing is carried out in thermal mode, so that carbon dioxide is crystallized on the walls of the collection tank, and in the absence of snow in airflow. In this case, the temperature difference between the air and the walls should not exceed 30 °C, and the gas flow rate should not exceed 3 m/s (to prevent the deposited crystals from getting separated and carried away).

In view of the described conditions, we have developed a technique for enriching the gas mixture with carbon dioxide. The setup for concentrating carbon dioxide is shown schematically in Fig. 1. Small streams of atmospheric air were pumped through test tube *3* placed in heat-insulating vessel *4*, filled approximately one-third with

liquid nitrogen. The tube was placed in nitrogen vapor so that its bottom temperature was about −100 °C. Desiccator *1* was used as a sealed chamber for the test plant.

The freezing system consisted of two circuits, where the temperature just below zero was maintained in loop *2* ensuring deposition of water and dehumidification of the gas mixture, while the temperature below −100 °C was maintained in refrigerating heat exchanger *3*, made in the form of a glass tube with a volume of 235 cm^3, allowing to transform carbon dioxide to solid phase. Flow rate did not exceed 0.5 m^3/ h, so solid carbon dioxide could be desorbed on the walls of the tube. A system of pneumatic tubes connecting all the components of the setup and flow controller *5* allowed the gas mixture to circulate from the plant through a system of containers back to the chamber with the test object.

After a freezing cycle lasting 15 min, concentrated carbon dioxide in tube was transformed to gaseous state by heating at room temperature. The tube was equipped with a 35 × 1 mm platinum catalytic chamber, maintaining a temperature over 900 °C at a heating current of 3.5 A. Catalytic combustion of impurities was performed in the test tube after defrosting for $\tau \approx$ 600 s. We estimate that the total concentration of impurities that can interfere in carbon isotope measurements for target ions with m/z 44 and 45 did not exceed about 10^{-5}.

Technique for determining the isotopic ratio for glucose carbon in plant tissues

Based on the developed technique for studying the fractionation of carbon isotopes by heterotrophic microorganisms [14], we proposed using oxidation by yeast to transform simple plant sugars to gaseous phase with carbon dioxide forming in order to determine the isotopic composition of glucose carbon in plant tissues.

Yeast mainly consume glucose by two pathways:

glycolytic splitting, that is, two pyruvate molecules are formed from a glucose molecule;

partial oxidation of glucose in oxidative pentose phosphate cycle, when three carbon dioxide molecules and pyruvate are formed from a glucose molecule [14].

Pyruvate synthesized through both of these pathways for glucose metabolism can then be oxidized in tricarboxylic acid cycle with the carbon dioxide molecule removed and coenzyme A (CoA) added to form acetyl-CoA:

$$CH_3{-}CO{-}COOH + SH\text{-}CoA + NAD \rightarrow$$
$$\rightarrow CO_2 + CH_3{-}CO{-}S\text{-}CoA + NAD{\cdot}H_2.$$

Alcohol fermentation proceeds in the absence of oxygen; its total equation has the following form:

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_5OH.$$

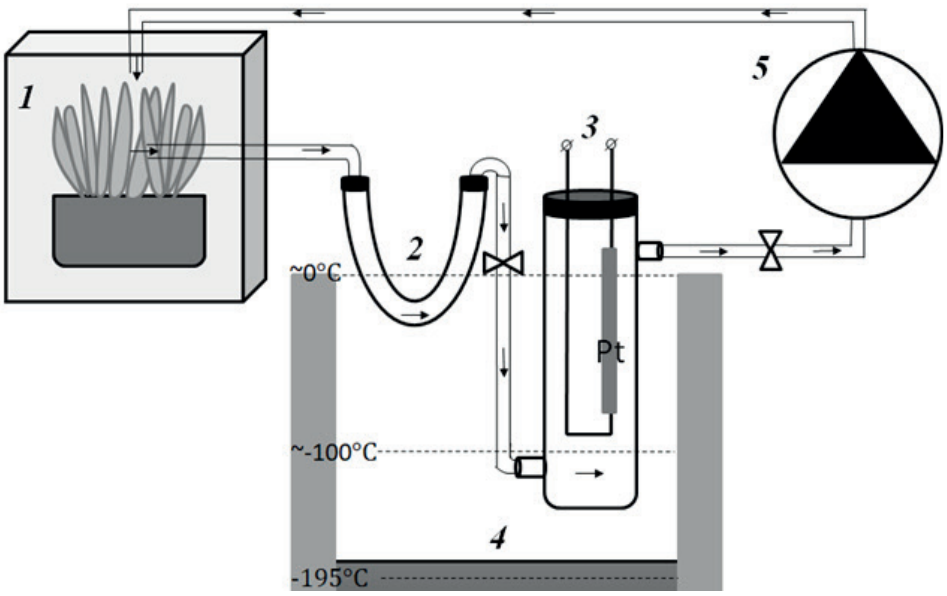

Fig. 1. Setup for enriching carbon dioxide from atmosphere surrounding the plant: sealed chamber *1* with air exhaust and supply pipes, *U*-shaped tube *2* for draining the gas mixture, Wurtz flask *3* for depositing carbon dioxide in nitrogen vapor with integrated catalytic chamber for afterburning organic impurities, heat-insulating vessel *4* with liquid nitrogen, flow controller *5* Temperature is controlled by a thermocouple

The acetaldehyde formed during the reaction has a molar mass of 44 g/mol, which does not allow it to be separated from the target compound, carbon dioxide. However, since glucose is the starting material for the carbon constituting acetaldehyde CH_3COH, this substance does not introduce errors in the isotopic composition of the plant. Ethyl alcohol with a molar mass of 46 g/mol begins to form approximately 24 h after the start of the reaction, after yeast have consumed the nutrients represented in our case by glucose in plant tissues.

The technique was as follows: ground plant tissue, water and dry yeast were placed in a sealed test tube (Fig. 2); after a 30-minute reaction, the synthesized carbon dioxide was taken for mass-spectrometric analysis.

The complete mass spectrum of the gas mixture formed in the reaction tube during oxidation by yeast was recorded with an MS 7-100 quadrupole mass spectrometer (Fig. 3). Gases in atmospheric air exhibited increased peak intensity in the mass spectrum for $m/z = 44$ (CO_2) (by 53 times) and for $m/z = 50–70$ (by 5–10 times) during the reaction (within 15 min).

According to [15], mainly signals from fragment ions lie in the range $m/z = 50–70$, and there are no data on the peaks related to interference of fragments at $m/z = 45$ (this mass corresponds to the CO_2 molecule with the ^{13}C isotope). In addition, the absence of a peak at $m/z = 46$ indicates the absence of ethanol vapor, which is usually the main interfering agent in isotopic measurements of carbon, in the gas mixture. In our estimation, the total concentration of impurities whose molecular or fragment ions are capable of interfering with the target ions used in isotopic measurements at mass numbers 44 and 45 did not exceed the level of 10^{-5}, providing the necessary measurement accuracy of 1‰.

The ratio of reaction components for alcohol fermentation should be as follows: 1 kg of sugar, 4–5 l of water, 100 g of pressed yeast or 20 g of dry yeast. Because the concentration of sugars in the tested plant tissues is not known exactly, we conducted an experiment to select the concentrations of reagents (see Table). We could find no significant differences of in $\delta^{13}C$ values of leaves. The standard deviation for the obtained values was 1.3‰. For example, the $\delta^{13}C$ value for 1 mg of plant tissue per 1 ml of water (second row of Table) was -35.3 ± 0.9‰ 60 min after the start of the reaction, and -33.6 ± 0.9‰ after 90 min.

In addition to studying plant sugars, we obtained and analyzed the isotopic composition of sugars corresponding to different types of photosynthesis and used as substrate for yeast.

The isotopic ratio obtained for yeast oxidation of beet sugar isolated from C3 plants (fixing carbon dioxide by the C3 mechanism of photosynthesis [16]) was

$$\delta^{13}C = -‰1.9 \pm 33.4.$$

The value for cane sugar synthesized from a C4 plant (higher plants with C4 photosynthesis [16]) was

$$\delta^{13}C = -‰1.6 \pm 14.6.$$

The values obtained are in agreement with the data given in literature for these types of photosynthesis, which means that the proposed technique can be applied for a wide range of objects.

Application of developed technique for preparing the samples

The given methods were used to study the effect of the spectral characteristics of lighting on the degree of exchange of carbon isotopes between atmospheric air and plant organs that carry out photosynthesis. Apparently, isotopic composition of the leaves substantially depends on the spectrum of light that the growing plant was exposed to. For example, when the spectrum changed from

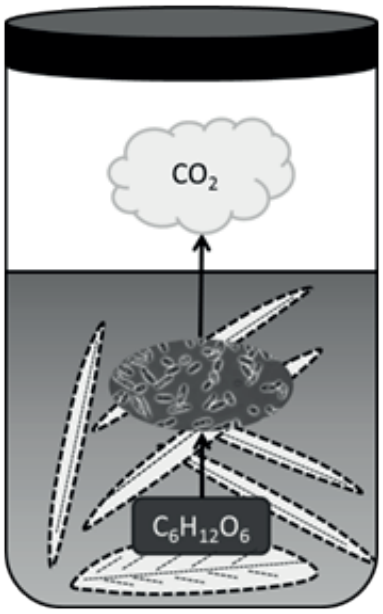

Fig. 2. Glucose from plant leaves oxidated by yeast until carbon dioxide is obtained in reaction tube

Table

Dependence $\delta^{13}C$ for glucose in leaves on reagent concentration in oxidation by yeast

Amount of reagent (mg) per 1 ml of water		$\delta^{13}C$,‰
Plant tissue	Yeast	
0.5	1.05	–33.6 ± 0.9
1.0	1.04	–33.6 ± 0.9
2.0	1.05	–33.6 ± 0.9

red to blue, the $^{13}C/^{12}C$ ratio changed in the range from −35 to −‰23, and the dependence on wavelength was nonmonotonic. The difference between the carbon isotope composition in the air surrounding the plants and in their leaves varies from 7 to 19‰ depending on the spectral composition of light and characterizes the rate of carbon assimilation due to photosynthetic reactions and photorespiration. This difference reflects the degree of isotope fractionation during the life of plants and can be used as a phytomonitoring parameter. We plan to publish more detailed findings later.

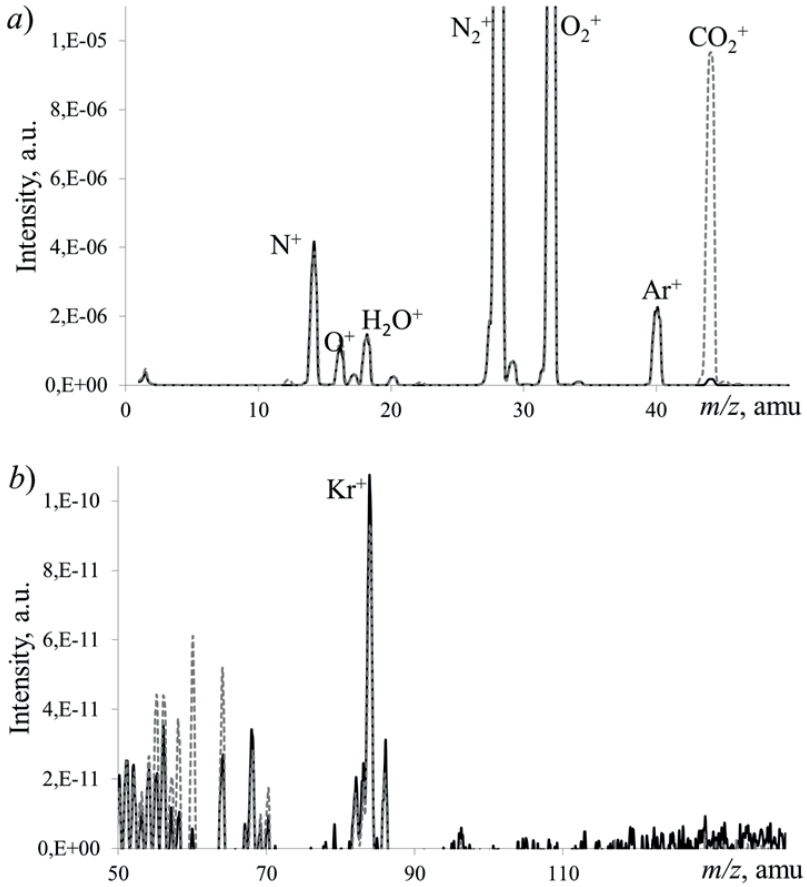

Fig. 3. Mass spectra of air in reaction tube (solid curve) and in gas mixture formed during oxidation of glucose in plant leaves by yeast (dashed curve) in m/z ranges of 0–45 amu (*a*) and 45–95 amu (*b*)

Brief results and conclusions

We have developed a system for collecting and preparing samples for mass-spectrometric analysis of fractionation of $^{13}C/^{12}C$ isotopes from the environment into the carbon pool of plants. Development of the system comprised the following stages:

constructing a setup for collecting and enriching a carbon dioxide sample from the air surrounding the plant *in vivo* by freezing carbon dioxide at a temperature of liquid nitrogen vapor;

developing and applying a technique for obtaining carbon dioxide samples from glucose contained in leaves via biochemical oxidation by yeast.

As a result of experimental studies conducted using this setup by the developed technique, we have found that the ratio of carbon isotopes in carbon dioxide released during oxidation of plant tissue by yeast remained unchanged for three hours.

The isotopic ratios obtained for oxidation by yeast were

$$\delta^{13}C = -33.6 \pm 0.9‰$$

for a leaf of a C3 plant;

$$\delta^{13}C = -‰0.9 \pm 33.6$$

for beet sugar isolated from C3 plants;

$$\delta^{13}C = -‰1.6 \pm 14.6$$

for cane sugar synthesized from a C4 plant.

These values are in agreement with the data given in literature for these types of photosynthesis, which means that the proposed technique can be applied for a wide range of objects.

The developed system for collecting and preparing the samples provided a significant increase in the accuracy of isotope measurements due to concentration of carbon dioxide from the atmosphere surrounding the plant and elimination of interfering organic impurities.

The technique for preparing the samples was successfully used to measure the dependence of isotopic ratio on the spectral composition of the light that the plants were exposed to during growth. We have found significant differences in the isotopic composition of carbon dioxide in the atmosphere and in plant leaves.

Thus, we can recommend to use the $^{13}C/^{12}C$ isotopic ratio as an important indicator of the photosynthesis reaction rate, while the difference between the $\delta^{13}C$ values for the air surrounding the plant and involved in its metabolism and $\delta^{13}C$ values of the carbon pool of plant tissue may reflect the degree of isotope fractionation during plant life cycle.

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