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Comparative analysis of base nutrient composition by NMR spectroscopy

© Aleksandr S. Ostyak*, Igor A. Ushakov, Natal'ya M. Khaptanova*, Natal'ya G. Gefan*, Vladimir I. Kuznetsov*, Elizaveta N. Oborina***, Sergei N. Adamovich***, Elena I. Ivanova****, Igor B. Rozentsveig*****

* Irkutsk Antiplague Scientific Research Institute for Siberia and Far East, Irkutsk, Russian Federation
** Irkutsk National Research Technical University, Irkutsk, Russian Federation
*** A.E. Favorsky Irkutsk Institute of Chemistry SB RAS, Irkutsk, Russian Federation
**** Irkutsk scientific center, Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russian Federation

Abstract: *The traditional approach to assessing the quality of nutrient bases involves a determination of amino nitrogen and acidity. The disadvantage of this approach consists in a lack of information, i.e. an inability to detect antibiotics, growth inhibitors and other undesirable compounds. In this regard, more modern and informative methods are required to control the technological process of preparing the nutritional basis and therefore the quality of the products obtained. The aim of this work was to study the physicochemical properties of nutrient bases made from sea and river fish and squid using new approaches (NMR spectroscopy). The following raw materials were used: herring (1), roach (2), pollock (3), squid (4). The raw materials were subjected to enzymatic hydrolysis by the pancreas (according to Hottinger). The qualitative composition of the organic component of hydrolysates (1–4) was determined by ^{1}H, ^{13}C and ^{15}N NMR spectroscopy. All of the ^{1}H NMR spectra had the same appearance, typical of mixtures of amino acids or amino acid sequences. In the high-field part (0.9–2.5 ppm), a set of multiplets was observed, characteristic of aliphatic fragments of molecules. Since most of the signals in the ^{1}H NMR spectra partially overlap, a quantitative assessment of the composition of the organic component appears impossible. All four samples can be confirmed as being qualitatively similar without isolating the dominant compound. Analysis of 2D NMR spectra revealed the presence of the following free amino acids in mixtures of samples (1–4): alanine, valine, threonine, arginine, lysine, leucine, methionine, phenylalanine and glycine. The use of NMR spectroscopy demonstrated that any discrepancies in the component composition of hydrolysates (1–4) were insignificant, allowing manufacturers of nutrient media to choose the most affordable raw materials. The obtained data appear to be applicable for controlling the technological process of preparing the nutrient bases and determining the quality of the resulting products during storage.*

Keywords: *nutrient base, growth medium, NMR spectroscopy, fish hydrolysate, Hottinger hydrolysate*

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Сравнительный анализ состава питательных основ методом спектроскопии ЯМР

© А.С. Остяк*, И.А. Ушаков, Н.М. Хаптанова*, Н.Г. Гефан*, В.И. Кузнецов*, Е.Н. Оборина***, С.Н. Адамович***, Е.И. Иванова****, И.Б. Розенцвейг*****

* Иркутский научно-исследовательский противочумный институт Роспотребнадзора, г. Иркутск, Российская Федерация
** Иркутский национальный исследовательский технический университет, г. Иркутск, Российская Федерация
*** Иркутский институт химии им. А. Е. Фаворского СО РАН, г. Иркутск, Российская Федерация
**** Иркутский научный центр СО РАН, г. Иркутск, Российская Федерация

Резюме: *Традиционным подходом к оценке качества питательных основ является определение аминного азота, кислотности. Недостатком данного подхода является его неинформативность – неспособность выявить антибиотики, ингибиторы роста и другие нежелательные соединения. В этой связи существует необходимость использования более современных и информативных методов для контроля технологического процесса приготовления питательных основ, а следовательно, и качества получаемой продукции. Целью данной работы являлось исследование физико-химических свойств питательных основ, изготовленных из морской, речной рыбы и кальмара, используя новые подходы (ЯМР-спектроскопия). Было использовано следующее сырье: сельдь (1), сорога (2), минтай (3), кальмар (4). Сырье подвергали ферментативному гидролизу с помощью поджелудочной железы (по Хоттингеру). Определяли качественный состав органической составляющей гидролизатов (1–4) с помощью метода ЯМР ^{1}H, ^{13}C и ^{15}N. Спектры ЯМР ^{1}H имели одинаковый вид, типичный для смесей аминокислот или аминокислотных последовательностей. В сильнопольной части (0,9–2,5 м.д.) наблюдался набор мультиплетов, характерный для алифатических фрагментов молекул. Так как большинство сигналов в спектрах ЯМР ^{1}H частично перекрывается, количественную оценку состава органической компоненты сделать нельзя. Можно судить о качественно схожем составе всех четырех образцов без выделения доминирующего соединения. Анализ 2М спектров ЯМР позволил установить присутствие в смесях образцов (1–4) свободных аминокислот: аланин, валин, треонин, аргинин, лизин, лейцин, метионин, фенилаланин, глицин. Применение ЯМР-спектроскопии показало незначительные расхождения в компонентном составе гидролизатов (1–4), что дает возможность изготовителям питательных сред выбирать наиболее доступное сырье. Полученные данные могут быть использованы для контроля технологического процесса приготовления питательных основ и определения качества полученной продукции в процессе ее хранения.*

Ключевые слова: *питательная основа, питательная среда, ЯМР-спектроскопия, рыбный гидролизат, гидролизат Хоттингера*

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INTRODUCTION

Nutrient media are an integral component of microbiological research. Their quality and properties determine the accuracy and informativity of bacteriological analysis [1, 2].

To ensure satisfactory growth properties in the finished nutrient medium, the quality of all components included in its composition, in particular, components of complex and indefinite composition, such as nutrient bases, must be controlled [3].

The most common raw materials used as nutrient bases in the production of microbiological media are of animal origin[1] [4]. In the manufacture of nutrient bases for microbiological media, other protein sources are applicable as meat substitutes [5, 6]. To date, meat bases, where possible, have been replaced with fish and casein, as well as their processed products. Other raw materials in the production of nutrient bases include fish industry waste, yeast autolysates, blood clots, chicken embryos, as well as plant materials, such as chlorella algae, peas, soybeans, tung tree fruits, milk whey, etc. [7–10].

The selection of one or another raw material in the production of nutrient bases is determined by the specifics of their application, as well as the

cost of the raw material itself. In most cases, nutrient bases made from meat hydrolysates can be replaced with fish meal hydrolysates (FMH) without changing the growth properties of the medium [11]. Many manufacturers of domestic dry culture media have taken this path.

In the manufacture of nutrient bases, quality control is carried out at all stages of production. The quality of the finished product is also assessed. The traditional approaches to assessing the quality of the nutrient base include physicochemical studies, such as determination of amine nitrogen and acidity. The main disadvantage of this approach lies in incomplete information or, in other words, the inability to detect antibiotics, growth inhibitors and other undesirable compounds. During quality control monitoring, deficiencies in nutrient media prepared using such nutrient bases can appear.

As a consequence of the foregoing, it becomes necessary to introduce additional methods and criteria for assessing the quality of nutrient bases. This will allow the detection of deviations from the standard during nutrient base production. Consequently, the parameters of hydrolysis can be corrected or the product series can be rejected.

NMR-spectroscopy manifests itself as one of the promising methods for investigating the nutrient bases [12]. The method is widely used in chemistry: by studying the peaks of the NMR spectra, the structure of many compounds can be determined [13, 14]. The method is applicable to uniquely identifying known and new compounds [15]. In the future, the method could be used for qualitative and quantitative determination of the composition of nutrient bases, for the detection of foreign substances, as well as for quality assessment.

The aim of this work is to study the component composition of nutrient bases made from sea and river fish, as well as squid, using new approaches, namely, NMR spectroscopy.

MATERIALS AND METHODS

The following raw materials were used in the work: herring, roach, pollock and squid. Without cleaning the entrails or removing scales, the fish and squid were cut into large pieces (including head), weighed and washed with running water. The chopped material was placed in an enamelled or stainless-steel container and distilled water added at a ratio of 1.5 l per 1 kg of raw material. The raw material was then brought to a boil and cooked for 20–30 minutes. Cooked raw material was removed and crushed together with any bones and the broth was cooled to 50(±5) °C.

3.5 kg of cooked and crushed raw material together with 7l of broth was deposited in 20l bottles. Using a 40% sodium hydroxide solution, the pH was adjusted to a value of 8.0–8.2. Minced pancreas (1%) and chloroform (1–1.5%) were added to each bottle. After mixing the contents, the bottles were closed with rubber stoppers and placed in an incubator at a temperature of 45(±2) °C for 10 days. The contents were mixed every 30 minutes on the first day and 3–5 times a day on the following days. During the first two days, the pH was adjusted to 7.8–8.0 using a 40% sodium hydroxide solution. Amine nitrogen was measured and recorded daily, and the concentration increased to 0.4–0.5 %. After the end of the increase in the concentration of amine nitrogen, the hydrolysate was filtered. With the addition of chloroform (1.5–2.0 %), the finished hydrolysate was capable of storage in a cool place for 6 months.

The qualitative composition of the organic component of hydrolysates was determined using the ^{1}H, ^{13}C and ^{15}N NMR spectroscopy. Native concentrated samples and samples diluted 10 times were studied as follows:

^{1}H NMR spectroscopy. For diluted samples, ^{1}H NMR spectra were recorded. The observed intense H_2O solvent signal was suppressed using standard techniques [16]. A general view of the spectra is shown in Figs. 1 and 2.

^{13}C NMR spectroscopy. For concentrated samples (1–4), the ^{13}C NMR spectra were recorded under the same conditions, allowing their comparison. For the purpose of ensuring the quantitative integrated signal intensity, a pulse sequence was applied yielding no signal amplification due to the Overhauser effect [17].

2D NMR spectroscopy. In order to determine the amino acid composition, two-dimensional (2D) correlation NMR experiments were carried out using standard pulse methods for assigning signals: COSY and TOCSY – for establishing spin coupling in proton spectra, ^{1}H–^{13}C HMBC and ^{1}H–^{13}C HSQC – for assigning signals in the ^{13}C NMR spectra. ^{1}H-^{15}N HMBC 2D experiments made it possible to determine the position of the resonances of free NH_2 groups (from -350 to -335 ppm), which agrees with published data [18].

2D NMR spectra were recorded by DPX400 Bruker pulse spectrometer (^{1}H – 400.1 MHz; ^{13}C – 100.6 MHz; ^{15}N – 40.5 MHz, respectively). 1D NMR spectra were recorded by DPX250 Bruker pulse spectrometer (^{1}H – 250.1 MHz; ^{13}C – 62.9 MHz). Samples with a volume of 0.6 cm^3 were transferred into 5 mm NMR tubes from the provided solutions without the use of deuterated solvents.

The conditions for recording the ^{13}C NMR spectra were selected taking an additional comparison of the integrated signal intensities into account: the signal accumulation time comprised 2 s, the relaxation delay was 10 s and the pulse power and duration corresponded to a 90-degree pulse. In order to increase the integration accuracy, 6000 scans were performed; the recording time for one spectrum was 14 hours.

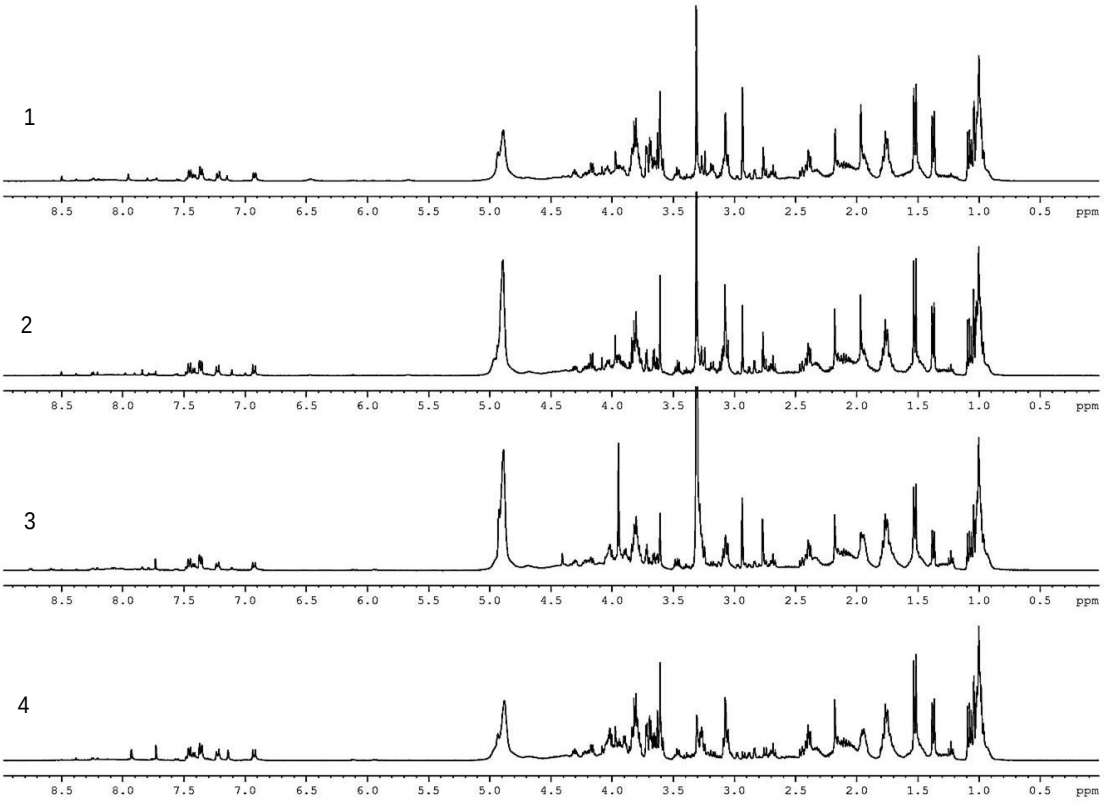

Fig.1. ^{1}H NMR spectra of diluted samples 1–4 (from top to bottom) in the mode of suppressing the H_2O signal (broadened signal in the region of 4.8 ppm is composed by the signal of residual protons H_2O)

Рис. 1. Спектры ЯМР ^{1}H разбавленных образцов 1–4 (сверху вниз) в режиме подавления сигнала H_2O (уширенный сигнал в области 4,8 м.д. – сигнал остаточных протонов H_2O)

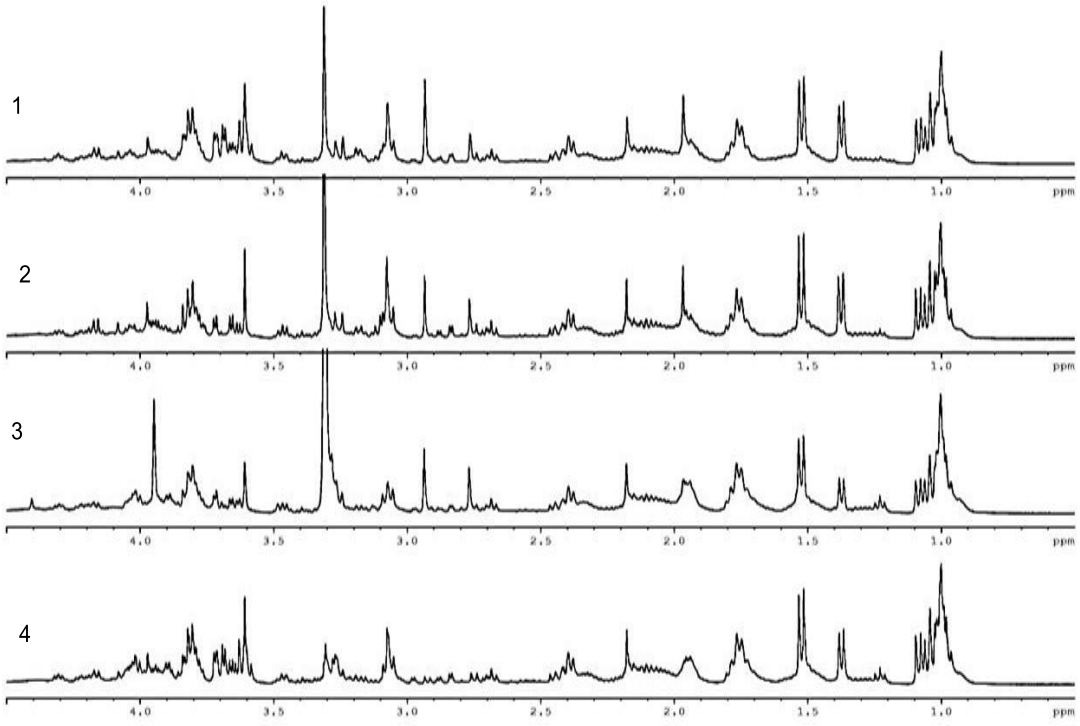

Fig.2. Fragments of the ^{1}H NMR spectra (high-field part) of diluted samples 1–4

Рис. 2. Фрагменты спектров ЯМР ^{1}H (сильнопольная часть) разбавленных образцов 1–4

RESULTS AND DISCUSSION

The ^{1}H NMR spectra had the same appearance, typical of mixtures of amino acids or amino acid sequences. In the high-field part (0.9–2.5 ppm), a set of multiplets was observed, characteristic of aliphatic fragments of molecules. Here, sets of doublets characteristic of isopropyl fragments, as well as complex multiplets of methylene groups, were clearly revealed. In the region of 2.7–3.5 ppm, singlets are presented, characteristic of OMe and NMe resonances; the ratio of their integrated intensities varies from sample to sample. In the spectral range of 3.5–4.5 ppm, sets of overlapping multiplets characteristic of methine protons at NH_2 are apparent. In the region of 4.5–6.7 ppm, resonant signals of significant intensity are not detected. In the part of the spectrum corresponding to the positions of aromatic resonances, i.e. 6.7–7.6 ppm, in all cases, similar sets of multiplets are observed.

Since most of the signals in the ^{1}H NMR spectra partially overlap, a quantitative assessment of the composition of the organic component appears impossible. The qualitatively similar composition of all four samples can be accounted without isolating the dominant compound.

^{13}C NMR spectra of concentrated samples are shown in Figs. 3 and 4. The signals of the same type of carbon atoms have integrated intensities comparable with the content of the component mixture present.

The main sets of signals in the spectra are presented in the range of 10–80 ppm. Here, 50–60 resonances of significant intensity are observed demonstrating the complex nature of the mixture composition. The specified spectral range includes resonances of aliphatic amino acid fragments. Based on the literature and the spectral base of organic compounds of the National Institute of Advanced Industrial Science and Technology (AIST) (www.aist.go.jp; www.acdlabs.com), the areas responsible for $\underline{C}HNH_2$ and $\underline{C}H_2CHNH_2$ resonances and other structural fragments of amino acids can be determined [19]. Due to the strong dependence of the resonance position of the carbon atom (chemical shift (CS)) on the acidity of the solution [20], an unambiguous determination of specific amino acids according to CS ^{13}C NMR appears to be impossible.

In the spectral region of 125 ppm, for all four samples, identical sets of signals characteristic of a para-substituted phenyl ring are observed.

In the range of 175–185 ppm, the ^{13}C NMR spectrum presents carboxyl group signal sets (10–12 signals of different intensities), indicating the presence of COOH quaternary carbon atoms belonging to several amino acids in the mixtures. The determination of CH, CH_2CH_3 and quaternary carbon atom signals was carried out using the method of ^{13}C J-modulation.

Analysis of 2D NMR spectra revealed the presence of the following free amino acids in mixtures of samples (1–4): alanine, valine, threonine, arginine, lysine, leucine, methionine, phenylalanine and glycine. The form (*D, L*) of these representatives according to the available data appears to be impossible to establish. The total proportion of acids such as histidine, tyrosine and tryptophan does not exceed 5%.

According to the NMR spectra of ^{1}H and ^{13}C, the differences in the amino acid composition of mixtures (1–4) were determined to be insignificant. Glycerin is presented as impurities in sample 1 (herring) (73.1 and 63.5 ppm signals in the ^{13}C NMR spectrum) and, in samples (2) and (3), the content of the component is conservative with CS (60 and 157 ppm ^{13}C) and CS 3.3 ppm in ^{1}H NMR. Based on the data of 2D spectra, this component is assigned to N,N-dimethylcarbamide.

The main results were obtained using equipment from the Baikal Analytical Centre for Collective Use of the SB RAS.

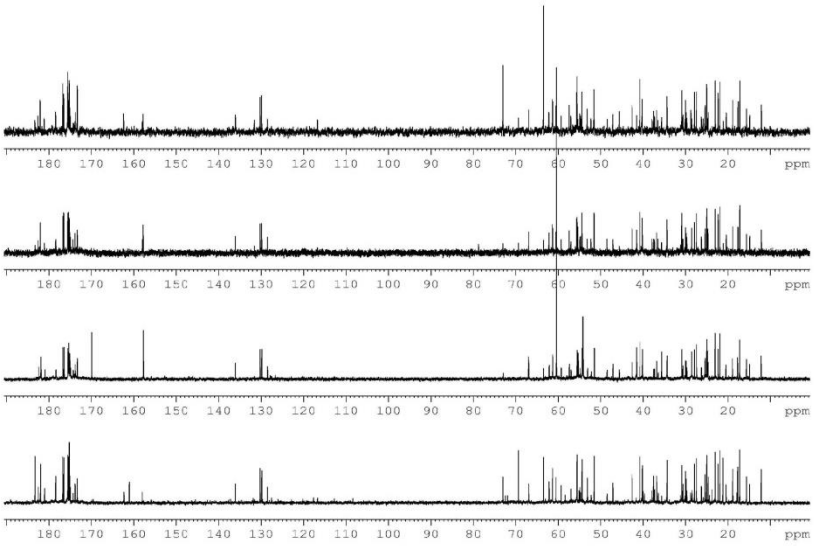

Fig.3. ^{13}C NMR spectra of concentrated samples 1–4 (from top to bottom)

Рис. 3. Спектры ЯМР ^{13}С концентрированных образцов 1–4

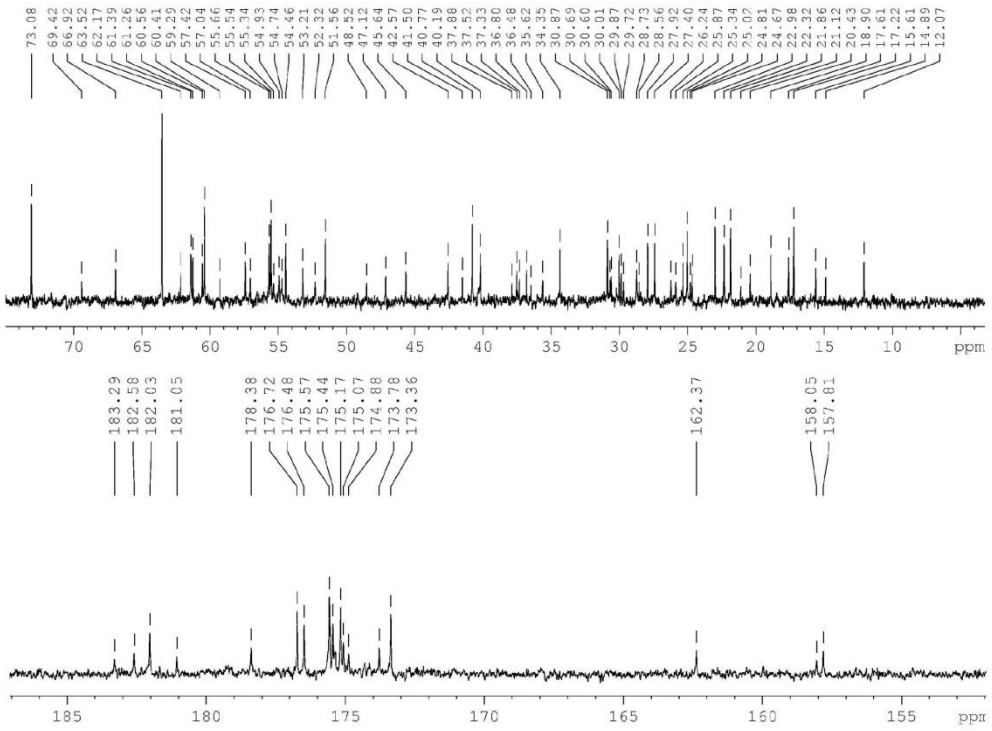

Fig. 4. ^{13}C NMR spectrum of the concentrated sample 1 (top is the aliphatic region, bottom presents resonances of carboxyl groups)

Рис. 4. Спектр ЯМР ^{13}C образца 1 (вверху – алифатическая область, внизу – резонансы карбоксильных групп)

CONCLUSIONS

The use of NMR spectroscopy demonstrated that any discrepancies in the component composition of hydrolysates prepared from different raw materials (herring, roach, pollock and squid) were insignificant, allowing manufacturers of nutrient media to choose the most affordable raw materials.

The obtained data appear to be applicable for controlling the technological process of preparing the nutrient bases and determining the quality of the resulting products during storage.

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Contribution

Aleksandr S. Ostyak, Igor A. Ushakov, Natal'ya M. Khaptanova, Natal'ya G. Gefan, Vladimir I. Kuznetsov, Elizaveta N. Oborina, Sergei N. Adamovich, Igor B. Rozentsveig carried out the experimental work, on the basis of the results summarized the material and wrote the manuscript. Aleksandr S. Ostyak, Igor A. Ushakov, Natal'ya M. Khaptanova, Natal'ya G. Gefan, Vladimir I. Kuznetsov, Elizaveta N. Oborina, Sergei N. Adamovich, Igor B. Rozentsveig have equal author's rights and bear equal responsibility for plagiarism.

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Остяк А.С., Ушаков И.А., Хаптанова Н.М., Гефан Н.Г., Кузнецов В.И., Оборина Е.Н., Адамович С.Н., Иванова Е.И., Розенцвейг И.Б. выполнили экспериментальную работу, на основании полученных результатов провели обобщение и написали рукопись. Остяк А.С., Ушаков И.А., Хаптанова Н.М., Гефан Н.Г., Кузнецов В.И., Оборина Е.Н., Адамович С.Н., Иванова Е.И., Розенцвейг И.Б. имеют на статью равные авторские права и несут равную ответственность за плагиат.

Conflict of interests

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AUTHORS' INDEX

Aleksandr S. Ostyak,
Researcher,
Irkutsk Antiplague Research Institute
of Siberia and Far East,
✉ e-mail: ostykalex@mail.ru

Igor A. Ushakov,
Ph.D. (Chemistry), Assosiat Professor,
Irkutsk National Research Technical University,
e-mail: nmr@istu.edu

Natal'ya M. Khaptanova,
Junior researcher,
Irkutsk Antiplague Research Institute
of Siberia and Far East,
e-mail: khaptnat@mail.ru

СВЕДЕНИЯ ОБ АВТОРАХ

Остяк Александр Сергеевич,
научный сотрудник,
Иркутский научно-исследовательский
противочумный институт Сибири
и Дальнего Востока,
✉ e-mail: ostykalex@mail.ru

Ушаков Игорь Алексеевич,
к.х.н., доцент,
Иркутский национальный исследовательский
технический университет,
e-mail: nmr@istu.edu

Хаптанова Наталья Маркеловна,
младший научный сотрудник,
Иркутский научно-исследовательский
противочумный институт Сибири
и Дальнего Востока,
e-mail: khaptnat@mail.ru

Natal'ya G. Gefan,
Ph.D. (Biology), Head of the Biological and Technological Controls Department,
Irkutsk Antiplague Research Institute of Siberia and Far East,
e-mail: adm@chumin.irkutsk.ru

Vladimir I. Kuznetsov,
Ph.D. (Biology), Head of the Cultural Media Department,
Irkutsk Antiplague Research Institute of Siberia and Far East,
e-mail: adm@chumin.irkutsk.ru

Elizaveta N. Oborina,
Ph.D. (Chemistry), Researcher,
A.E. Favorsky Irkutsk Institute of Chemistry,
e-mail: mir@irioch.irk.ru

Sergei N. Adamovich,
Dr. Sci. (Chemistry), Senior Researcher,
A.E. Favorsky Irkutsk Institute of Chemistry,
e-mail: mir@irioch.irk.ru

Elena I. Ivanova,
Ph.D. (Chemistry), Senior Researcher,
Irkutsk Scientific Center,
e-mail: ivanova.iem@gmail.com

Igor B. Rozentsveig,
Dr. Sci. (Chemistry), Head of the Department,
A.E. Favorsky Irkutsk Institute of Chemistry,
e-mail: i_roz @irioch.irk.ru

Гефан Наталья Геннадьевна,
к.м.н., заведующая отделом,
Иркутский научно-исследовательский противочумный институт Сибири и Дальнего Востока,
e-mail: adm@chumin.irkutsk.ru

Кузнецов Владимир Ильич,
к.м.н., заведующий лабораторией,
Иркутский научно-исследовательский противочумный институт Сибири и Дальнего Востока,
e-mail: adm@chumin.irkutsk.ru

Оборина Елизавета Николаевна,
к.х.н., научный сотрудник,
Иркутский институт химии им. А.Е. Фаворского СО РАН,
e-mail: mir@irioch.irk.ru

Адамович Сергей Николаевич,
д.х.н., ведущий научный сотрудник,
Иркутский институт химии им. А.Е. Фаворского СО РАН
e-mail: mir@irioch.irk.ru

Иванова Елена Иннокентьевна,
к.х.н., ведущий научный сотрудник,
Иркутский научный центр СО РАН,
e-mail: ivanova.iem@gmail.com

Розенцвейг Игорь Борисович,
д.х.н., заведующий лабораторией,
Иркутский институт химии им. А.Е. Фаворского СО РАН,
e-mail: i_roz@irioch.irk.ru