Comparative analysis of base nutrient composition by NMR spectroscopy

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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

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430 ФИЗИКО-ХИМИЧЕСКАЯ БИОЛОГИЯ / PHYSICOCHEMICAL BIOLOGY
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), соя (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 1H, 13C и 15N. Спектры ЯМР 1H имели одинаковый вид, типичный для смеси аминокислот или аминокислотных последовательностей. В сильнозольной части (0,9–2,5 м.д.) наблюдался набор мультиплетов, характерный для алифатических фрагментов молекул. Так как большинство сигналов в спектрах ЯМР 1H частично перекрываются, количественную оценку состава органической компоненты сделать нельзя. Можно судить о качественно-количественном составе всех четырех образцов без выделения доминирующего соединения. Анализ 2М спектра ЯМР позволил установить присутствие в смеси образцов (1–4) свободных аминокислот: аланин, валин, треонин, аргинин, лицин, лейцин, метионин, фенилаланин, глицин. Применение ЯМР-спектроскопии показало незначительные расхождения в компонентном составе гидролизатов (1–4), что дает возможность изотермам 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 analysis. 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 [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, nutriti
ent 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 as-
sessed. The traditional approaches to assessing
the quality of the nutrient base include physico-
chemical studies, such as determination of amine
gas 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. Dur-
ing quality control monitoring, deficiencies in nutri-
tent media prepared using such nutrient bases can
appear.

As a consequence of the foregoing, it be-
comes 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 com-
ponents [15]. In the future, the method could be
used for qualitative and quantitative determination
of the composition of nutrient bases, for the de-
tection of foreign substances, as well as for quality
assessment.

The aim of this work is to study the compo-
nent composition of nutrient bases made from sea
and river fish, as well as squid, using new ap-
proaches, 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 enam-
elled or stainless-steel container and distilled wa-
ter added at a ratio of 1.5 l per 1 kg of raw mate-
rial. The raw material was then brought to a boil
and cooked for 20–30 minutes. Cooked raw materi-

al 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 7 l of broth was deposited in 20 l bot-
tles. 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% so-
dium hydroxide solution. Amine nitrogen was
measured and recorded daily, and the concentra-
tion 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 
1H, 13C and 15N NMR spectroscopy. Native
concentrated samples and samples diluted 10 ti-
tes were studied as follows:

1H NMR spectroscopy. For diluted samples,
1H NMR spectra were recorded. The observed
intense H2O solvent signal was suppressed using
standard techniques [16]. A general view of the
spectra is shown in Figs. 1 and 2.

13C NMR spectroscopy. For concentrated sam-

13C NMR spectra. For concentrated sam-
plexes (1–4), the 13C NMR spectra were recorded
under the same conditions, allowing their compar-
ison. 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 sig-
als: COSY and TOCSY – for establishing spin
coupling in proton spectra, 1H–13C HMBC and
1H–13C HSQC – for assigning signals in the
13C NMR spectra. 1H–15N HMBC 2D experiments
made it possible to determine the position of the
resonances of free NH2 groups (from -350 to
-335 ppm), which agrees with published data [18].

2D NMR spectra were recorded by DPX400
Bruker pulse spectrometer (1H – 400.1 MHz; 13C –
100.6 MHz; 15N – 40.5 MHz, respectively). 1D
NMR spectra were recorded by DPX250 Bruker
pulse spectrometer (1H – 250.1 MHz; 13C –
62.9 MHz). Samples with a volume of 0.6 cm3
were transferred into 5 mm NMR tubes from the
provided solutions without the use of deuterated
solvents.

The conditions for recording the 13C NMR
spectra were selected taking an additional compari-
son 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-degrees pulse. In order
to increase the integration accuracy, 6000 scans
were performed; the recording time for one spec-
trum 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$_2$O signal (broadened signal in the region of 4.8 ppm is composed by the signal of residual protons H$_2$O).

Рис. 1. Спектры ЯМР $^1$H разбавленных образцов 1–4 (сверху вниз) в режиме подавления сигнала H$_2$O (уширенный сигнал в области 4,8 м.д. – сигнал остаточных протонов H$_2$O).

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 $\text{CH}_2\text{CHNH}_2$ and $\text{CH}_2\text{CHNH}_2$ 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$_2$CH$_3$ 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.

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

Рис. 3. Спектры ЯМР $^{13}$C 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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