Bioanalytical Technologies for Safety Control of Fish and Seafood by Sensitive Rapid Tests for Phycotoxins

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Abstract. Wide and operate safety monitoring of foodstuffs is highly demanded in modern society. This work aims to develop and characterize new test systems for control of phycotoxins, dangerous contaminants of fish and seafood. For rapid and productive testing, 2 methods have been implemented: immunochromatographic assay and fluorescence polarization immunoassay. Various approaches for reducing the limit of detection of target analytes have been considered, including changes in the use of optical and fluorescent labels and varied order of the detected complexes formation. Antibodies and aptamers have been considered as receptor molecules. The developed techniques provide rapid (20–30 minutes, including sample preparation) and sensitive testing. Their effectiveness has been shown for different kinds of fish and seafood. Portable detectors have been proposed that allow testing directly at the sampling points, without transportation to centralized laboratories.

1. Introduction
The present study is aimed at developing new methods for the rapid and highly sensitive detection of phytotoxins, dangerous contaminants of food products. Phycotoxins are produced by several algae, microalgae, and cyanobacteria, so they are included at the initial points of the marine food chain and transferred to fish and other animals used in the food industry; they are thus becoming a threat to humans [1-3].

The various negative biological effects of phycotoxins have led to the introduction of official standards governing their maximum permissible levels [4, 5]. However, currently the main methods to control phycotoxins are chromatographic [6]. The implementation of chromatography requires the use of sophisticated equipment and the involvement of highly qualified personnel; therefore, it is only possible in a limited number of specialized laboratories. In this regard, the development and implementation in practice of alternative methods, which are simple, cheap, and suitable for mass monitoring, are in demand [7].
A promising way to solve this problem is to use bioreceptor recognition by antibodies, aptamers, or other molecules. Enzyme-linked immunosorbent assay (ELISA) is the most widely applied method from bioreceptor-based analytical techniques. It has been developed for main phycotoxins and provides a sensitive and specific detection with high productivity because of simultaneous testing of dozens of samples [8]. However, ELISA involves multiple time-consuming steps and laborious sample extraction. Therefore, the actual situation necessitates a methodological update of immunological techniques for the detection of phycotoxins so they can be used for point-of-care testing.

Two formats of rapid immunodetection seem to be the most efficient for this purpose and have been chosen for the presented study, namely, fluorescence polarization immunoassay (FPIA) and immunochromatographic analysis (ICA). The advantages of FPIA include the absence of long diffusion-dependent interactions between immobilized and solved reagents and multistage incubations with separation of reacted and unreacted components [9]. The demand for ICA as a means of rapid off-laboratory testing is due to the simplicity of working with immunochromatographic test strips, on which all the necessary reagents are preliminarily applied, and the contact of the strips with the sample initiates all further processes that occur without the participation of operator [10]. As a result, the given approaches provide rapid assays with a minimum of additional reagents and equipment, wherein sample addition immediately initiates specific interactions resulting in signal generation and an “on-the-spot” decision about the presence of food contaminants. Successful developments of such techniques for phycotoxins have been recently described [11], but the tasks of increasing their sensitivity and adaptation to different types of samples still need to be accomplished.

The target analytes of the study were three priority phycotoxins produced by cyanobacteria, namely microcystin, domoic acid, and okadaic acid (see Figure 1).

Figure 1. Structural formulae of target phycotoxins. Active groups of the molecules for their conjugation with carriers and labels are marked with circles.
2. Results and discussion

2.1. Immunochromatographic analysis

Because phycotoxins are low molecular weight compounds, competitive ICA formats were implemented for their analysis, when specific antibodies immobilized on the surface of nanoparticles competitively interact with the antigen in the sample and with the antigen-protein conjugate immobilized in the analytical zone of the test strip. Thus, the nanoparticle is both a carrier for antibodies and a detectable colored label.

The first question that was addressed to increase the sensitivity of the analysis was the choice of optimal nanoparticles. For their preliminary selection, a set of characterization techniques were proposed, including the assessment of nanoparticles' size and surface as well as their minimum number that can be detected from a fixed area of immunochromatographic membranes. The advantages of using gold nanoparticles in ICA because of their plasmon-caused enhanced optical signal, were shown. Two types of nanomarkers that differed in shape and dimensional characteristics were obtained and characterized: spherical gold nanoparticles (AuNPs) and branched nanoparticles, so-called nanoflowers (AuNFs). The AuNPs and AuNFs were synthesized using similar methods based on the reduction of chloroaureic acid with sodium citrate. To obtain AuNPs, a one-stage synthesis was used, while AuNFs were obtained growing spherical nanoparticles. Transmission electron microscopy showed the absence of aggregates and an average diameter of about 30 and 90 nm for AuNPs and AuNFs, respectively. The resulting nanoparticles were conjugated with phycotoxin-specific and anti-species antibodies by physical adsorption. Different loading ratio (antibody : nanoparticle) were compared.

When implementing ICA using the reagents obtained, the main task was to choose the interaction mode that provides the lowest detection limit. For this purpose, direct and indirect schemes differing in the method of introducing a nanodispersed marker were compared. The direct format was based on the use of gold-labeled specific antibodies to the analyte and was performed in one step. Indirect ICA, where gold-labeled anti-species antibodies were used, included 2 stages: preincubation of analyte-containing sample with specific antibodies, incubation of a test strip with this mixture, and revealing of the formed immune complexes by gold conjugate. The advantages of the second scheme were confirmed, as well as it excluded nonproductive analyte binding with multivalent antibody complex that did not cause inhibition of the analytical zone coloration. In addition, different conditions of reactants preincubation were tested with varying duration of this stage. The final optimization was focused on the choice of concentrations and composition of all immunoreagents causing low detection limit and reliable visual distinctions between negative and positive results. Figure 2 demonstrated examples of positive and negative results of testing with the use of AuNPs and AuNFs under the optimized conditions.

![Figure 2. Examples of positive (a, c) and negative (b, d) testing results for ICA of microcystin-LR using gold nanoparticles (a, b) and gold nanoflowers (c, d).](image-url)

On the example of microcystin-LR (MC-LR), metrological parameters of the developed assay were estimated [12]. As a result of the optimization, the instrumental limit of detections of MC-LR were 0.1
and 0.2 ng/mL in the case of AuNFs and AuNPs, respectively. The visual limits of detection were 1 ng/mL in both cases, and the time of analysis was 18 min.

The obtained data allowed approbation of the developed ICAs for real samples. Specific features for testing different fish (cod, trout) and seafood (shrimp, squid, mussels, octopus) preparations were characterized. To unify the assay procedure, a common sample preparation technique was proposed with the fixed composition of extracting buffer and the durations of homogenization, extraction and separation stages. For all tested samples, the possibility of reliable phycotoxins revealing without nonspecific coloration and loss in sensitivity was conformed. The revealing value for the case of MC-LR was no less than 80%.

2.2. Fluorescence polarization immunoassay

The FPIA is based on the competitive interaction of the analyte and its labeled derivative with specific antibodies and the measurement of the fluorescence polarization (mP) value. A small conjugate molecule rotates rapidly and, accordingly, has a low mP. When the tracer binds to the antibody, a high molecular weight complex is formed, that is characterized by slow rotation and high mP. Therefore, the mP reflects the ratio of the bound and free fractions of the tracer and is inversely proportional to the concentration of the analyte in the sample.

For the FPIA, four fluorescent derivatives (see Figure 3) were used as a fluorescent label. Their conjugates with phycotoxins were synthesized by carbodiimide activation, purified by thin layer chromatography, and characterized by mass spectrometry. It was shown by the FPIA that the conjugates retained the ability to interact with both antibodies. Working dilutions of both the conjugate and antibodies were selected for each analytical system.

**Figure 3.** Structural formulae of four fluorophores used for PFIA. Active groups of the molecules for their conjugation with phycotoxins are marked with circles.

Implementation of the PFIA in a competitive format provided the possibility of sensitive and rapid detection of MC-LR with limit of detection of 20 ng/mL and a working range of determined
concentrations of 58–470 ng/mL, within 5 min. The results obtained confirmed the potential of FPIA for the detection of phycotoxins in fish and seafood.

An additional direction of development was the use of aptamers as alternate receptors for phycotoxins in homogeneous assays with fluorescence polarization registration. Aptamers, short oligonucleotides selected for their ability to affine and specific bind target analytes, appear to be a promising alternative to antibodies because of the simplicity and low cost of production and molecular design, stability under a wide range of conditions, and ease of regeneration. The corresponding aptamers for domoic and okadaic acids were synthesized and tested; the optimization of their assays is in progress.

2.3. Instrument base for fluorescence polarization measurements
For out-of-laboratory application of the developed assays, the mobility of their final stage, namely, results registration and processing, should also be ensured. The situation with ICA in this respect is more favorable because of the abundance of several portable optical detectors, including those adapted to work with test strips, as well as programs for smartphones and other commonly used communication devices [13].

FPIA is usually performed using stationary optical technology. In this regard, there is demand for recent development [14], in which a portable detection for fluorescence polarization measurements was proposed and characterized. By this way, FPIA was carried out using a portable FP analyzer (the dimensions of the device are 35 × 15 × 15 cm).

During the following studies, fluorescein isothiocyanate and a fluorescent dye emitting red at a wavelength of 647 nm (HiLyte Fluor™ 647) were used as a fluorescent label. HiLyte Fluor™ 647 has been shown to provide analyte detection at lower concentrations. Approaches to leveling the adsorption of fluorescently labeled analyte and specific antibodies on the surface of the microchannel wall of the device are considered. The microfluidic device for measurement has 9 independent microchannels and can measure 9 samples simultaneously. Thanks to the software, the detector made it possible to carry out automatic measurement of mP and data processing to calculate the analytes content.

3. Conclusions
The features of the presented developments determining their advantages in comparison with the known immunoanalytic systems phycotoxins, include the following:
- Development of signal amplification methods based on the controlled formation of target detectable complexes of functionalized nanoparticles during analysis
- Control of the threshold level for distinguishing positive and negative results by varying the composition of the intermolecular conjugates used in the analysis
- Development of simple quick extraction of target compounds
- The use of developed systems for not only qualitative but also quantitative control and documentation of the analysis results, carried out through the use of portable optical detectors
- Approbation of the developed test systems for a wide panel of various fish and seafood products

4. References
[1] Rasmussen S A, Andersen A J, Andersen N G, Nielsen K F, Hansen P J and Larsen T O 2016 J. Nat. Prod. 79 (3) 662–673
[2] Farabegoli F, Blanco L, Rodríguez L P, Vieites J M and Cabado A G 2018 Mar. Drugs 2018 16 (6) 188
[3] Leal J F and Cristiano M L S 2021 Nat. Prod. Rep. DOI: 10.1039/d1np00009h
[4] Brown A R, Lilley M, Shutler J, Lowe C, Artioli Y, Torres R, Berdalet E and Tyler C R 2020 Reviews in Aquaculture 12 1663–1688
[5] Murk A J, Nicolas J, Smulders F, Bürk C and Gerssen A 2019 Chapter 9 in: Food Safety Assurance and Veterinary Public Health. Wageningen Acad. Publ. 7 207–239
[6] Van Egmond H P 2004 Anal. Bioanal. Chem. 378(5) 1152–1160
[7] Hess P 2010 Anal. Bioanal. Chem. 397 683–694
[8] Dubois M, Demoulin L, Charlier C, Delahaut P, Campbell K, Elliott C, Godefroy S and Singh G 2010 Food Addit. Contam. A 27(6) 859–868
[9] Hendrickson O D, Taranova N A, Zherdev A V, Dzantiev B B and Eremin S A 2020 Sensors 20 (24) 7132
[10] Tripathi P, Upadhyay N and Nara S 2018 Crit. Rev. Food Sci. Nutr. 58(10) 1715–1734
[11] Anfossi L, Baggiani C, Giovannoli C, D’Arco G and Giraudi G 2013 Anal. Bioanal. Chem. 405 467–480
[12] Zvereva E A, Hendrickson O D, Zherdev A V, Dzantiev B B and Eremin S A 2021 Appl. Biochem. Microbiol. 57 (3) 403–409
[13] Urusov A E, Zherdev A V and Dzantiev B B 2019 Biosensors 9(3) 89
[14] Wakao O, Satou K, Nakamura A, Galkina P A, Nishiyama K, Sumiyoshi K, Kurosawa F, Maeki M, Ishida A, Tani H, Proskurnin M A, Shigemura K, Hibara A, and Tokeshi M 2019 Lab on Chip 19 2581–2588

Acknowledgements
This study was financially supported by the Russian Science Foundation (project 20-43-07001, Sections 2.1, 2.2) and the Strategic International Collaborative Research project promoted by the Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan (Section 2.3).