ŻYWNOŚĆ. NAUKA. TECHNOLOGIA. JAKOŚĆ, 2024, 31, 1 (138), 66 – 77

DOI: 10.15193/zntj/2024/138/487

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THE DERIVATIVES OF 7-AMINO-4-TRIFLUOROMETHYLCOUMARINE AS POTENTIAL FLUORESCENT FOOD DYES – PRELIMINARY RESULTS

S u m m a r y

Background. Unique food products such as black hamburgers, pink bagels and Jelly Beans have been more and more desired, especially among young adults. Following this trend, we focused our interest on a fluorescence phenomenon which has not been considered yet by the food industry to be interesting. Contrary to fluorescent dyes, natural and synthetic ones are widely used to give food attractive colors. The fluorescent properties of coumarin derivatives could be of interest to various industry branches, including the food industry. In this paper, we have tested the water-soluble derivatives of coumarin as a fluorescent dye for alcoholic drinks and obtained promising results. The aim of the research was to obtain through chemical synthesis the derivatives of 7-amino-4-trifluoromethylcoumarine and analyze them in terms of the range of their application and impact on the selected microorganisms.

Results and Conclusions. The derivatives of 7-amino-4-trifluoromethylcoumarine can be considered potential fluorescent dyes for alcoholic drinks. They absorb visible light, may be excited in the region of electromagnetic radiation emitted by incandescent lamps characteristic of nightclubs, the so-called black bulbs. The dye fluorescence is only moderately affected by water present in drinks . The optimal concentration of the dye is low and would probably be safe for human health. Additionally, the dye examined is capable of reducing the growth of selected molds.

Keywords: fluorescent dye; coumarin; fun food, unusual food, alcoholic drinks

Introduction

The use of coloring substances has been known for thousands of years. It was as early as the period of ancient Egypt when various compounds were used, for example, to correct the color of wine or medicines. At that time, however, the technique of obtaining natural or nature-derived dyes was extremely complicated and labor-intensive, resulting in very high prices for many dyes. With the development of civilization, there was a need to look for new sources and methods of dyeing, due to growing consumer

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demands and an increasing range of products. The real revolution took place in the 19th century, when there was a rapid development of the chemistry of organic compounds and a strong increase in the production of synthetic coloring substances [7]. Currently, the choice of dyes is large and it is possible to impart color to a wide range of consumer products, including food. Dyeing food products makes it possible to recover the color of the product that was lost due to a technological process used and to enhance its intensity. It also makes it possible to give a new color to foodstuffs that lack it, making them more attractive and recognizable [15, 24]. As the appearance is the first property of food recognized by the consumer, the color of food is a very important factor [23].

The term *fun food* could be used for products that, through their unusual organoleptic characteristics, stand out from other foodstuffs. With their unusual taste, appearance or smell, they capture the attention of consumers [1, 22, 28]. In 2014, a chain of popular American restaurants Burger King introduced in Japan a black hamburger under the name KURO Pearl [13]. The unprecedented color of the bun, cheese and sauce received a very positive reception and became a popular dish also in European countries. The characteristic color of the burger's ingredients is the result of using sepia, a blackish-brown substance produced by cuttlefish. The process of coloring food with sepia is expensive and time-consuming, hence only a few restaurants serve this type of food. A cheaper equivalent to sepia is coconut carbon (E153), which, in addition to its obvious black color, has also health-promoting properties.

Unusual food colors meet with approval from consumers, which is why more and more *fun food* outlets are springing up these days. In Cracow and Warsaw, the Mr. Pancake restaurants [17], which offer, among other things, soap bubble cocktails, pink pancakes and burgers, and chicken strips in pink coating, enjoy particular popularity. Synthetic and natural food dyes are used to color products. For example, the pink color of buns is achieved by using beet puree as one of the ingredients. This type of food looks very attractive and is highly appreciated, especially among the younger part of the population. The consumption of unusual food is an intensely growing trend in the food industry [26, 29]. Such products have also attracted interest of adult consumers, as neophobia is giving way to curiosity. Unusual foodstuffs could be also prepared using the so-called molecular cuisine methods. Up to date, we have not found any examples of fluorescent foodstuffs, hence a few years ago we started some research into that subject. We have tested some potential candidates for fluorescent dyes for food, but only coumarin derivatives have appeared to be promising so far.

Coumarins are naturally occurring compounds that could be used in pharmacotherapy of cancer [10], have antimicrobial activity [27] and could be also used as fluorescent probes in a biological environment [25]. Coumarins have been found in different kinds of food [14]. Due to the fluorescent ability of 7-amino-4-trifluoro-

methylcoumarine and its derivatives, observed also in highly polar solvents, it has seemed to be a good choice for our research [3]. The presence of trifluoromethyl group not only increases the fluorescence intensity, but also enhances the metabolic stability [18], which is important, if the compound is considered a potential food dye.

Materials and methods

Materials: all the chemicals and solvents used in the research were purchased from Aldrich or Chempur (Polish supplier) and were of analytical quality. The solvents used for spectrophotometric and electrochemical measurements were of HPLC grade.

Spectrophotometric measurements: the absorption spectra were recorded using a Shimadzu UV-2101 PC spectrometer and the fluorescence spectra (with the correction for spectral sensitivity) were measured with a Hitachi F7000 fluorometer. The fluorescence quantum yield measurements were carried out with coumarin 153 in ethanol (Φ_{fl} = 0.38) as an actinometer [6]. For the fluorescence quantum yield, the solution of the dye was not deaerated, to maintain comparability with the real samples. The sample concentration of the dye was ca. $1.5 \cdot 10^{-5}$ M (this corresponds to the absorbance of 0.21 at the excitation wavelength of 400 nm, used for the fluorescence investigation).

Cyclic voltammetry measurements were performed on a potentiostat PalmSens4. The platinum disc working electrode was used, with a diameter of ø = 3 mm. For auxiliary electrode, a platinum wire (ø = 1 mm) was used, whereas a silver chloride electrode Ag/AgCl (RL-100 model from Hydromet – a Polish producer) was used as a reference (E = +0.197±0.003 *vs. SHE*). 0.1 M solution of $NaClO_4$ in water/acetonitrile (1:1) was used as an electrolyte. Prior to measurements, the solutions were purged with argon to remove residual oxygen.

Microbiological assay: DRBC Agar was used as a medium for analyzed molds (*Aspergillus niger, Penicilium expansum, Rhizopus* sp.). The prepared medium was sterilized (Ultrasonic Sterilizer Microjet ML2-0212). A solution of dye in DMSO ($2 \cdot 10^{-4}$ M) was added to the prepared medium solutions at a concentration of 10 % according to the medium. The cultures were performed on solidified, previously prepared culture media using the surface method. The prepared cultures were incubated at 28 °C for 3 days in a laboratory incubator (Laboratory incubator BMT MMM Group Incucell).

Synthesis: 2-[4-(trifluoromethyl)coumarine-7-amino]-ethanosulphonic acid sodium salt and 2,2'-[4-(trifluoromethyl)coumarine-7-amino]-diethanosulphonic acid disodium salt were obtained in the reaction between 7-amino-4-trifluoromethylcoumarine with vinylsulphonic acid sodium salt in hexafluoroizopropanol as a solvent (Fig. 1), following a procedure by De *et al.* [8].

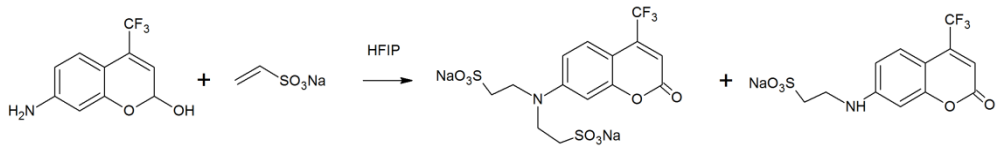

Fig. 1. Reaction scheme for the synthesis of 7-amino-4-(trifluoromethyl) coumarin with vinylsulfonic acid sodium salt in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)

Ryc. 1. Schemat reakcji syntezy 7-amino-4-(trifluorometylo)kumaryny z solą sodową kwasu winylosulfonowego w 1,1,1,3,3,3-heksafluoro-2-propanolu (HFIP)

A 0.229 g (1 mmol) portion of 7-amino-4-trifluoromethylcoumarine was added to a round bottom flask (25 ml), and subsequently, 3 ml of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 1.10 ml (10 mmol) of vinylsulfonic acid sodium salt solution. The mixture was stirred at 60 °C for 24 h. After that time, the reaction mixture was cooled to room temperature, 3 ml of diethyl ether was added and the precipitate which was formed was filtered off, washed with few milliliters of diethyl ether and dried in ambient conditions. The product was used in the crude form. The synthesis and purification procedures must be improved to obtain the pure product.

Results and discussion

In order to determine the potential suitability of the synthesized derivative as a fluorescence dye for alcoholic beverages, the following parameters were determined: the influence of ethanol concentration and dye concentration on the fluorescence intensity, dye oxidation resistance and its influence on the growth of selected microorganisms. Fluorescence intensity was also measured for selected real alcoholic beverages.

Absorption and emission of the dye

The most important, long wave absorption band of synthesized dye is observed at λ_{max} = 383 nm and ε = 17800 (Fig. 2).

That absorption corresponds to $S_0 \leftrightarrow S_1$ transition, which is of $\pi \leftrightarrow \pi^*$ type, as it is observed for coumarin 153 and other related coumarins [12], and as for them is also structureless in a polar solvent. As the band covers visible spectrum, fluorescence could be excited by so called black light bulbs (often used in nightclubs). The fluorescence spectrum (Fig. 2) has a maximum emission at λ_{max}= 480 nm, excited with 400 nm line. The quantum yield Φ of the dye in 96 % ethanol is equal to 0.77, as calculated from equation (1) [6].

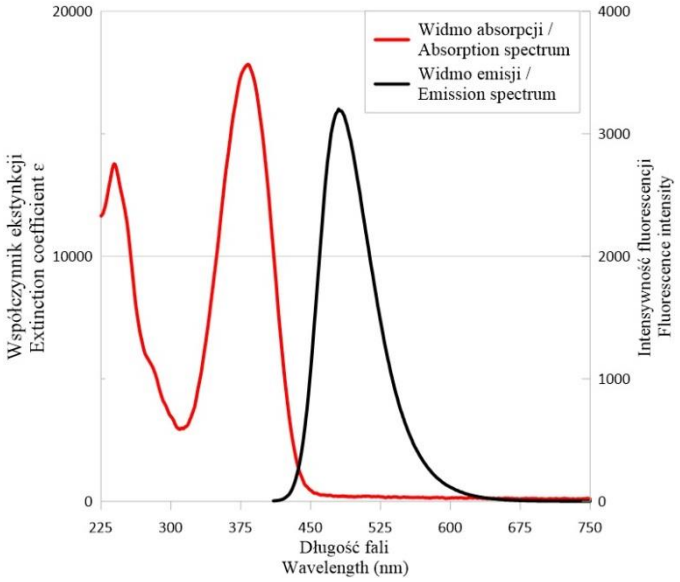

Fig. 2. Absorption and emission spectra of the studied dye in a 96 % ethanol solution.
Ryc. 2. Widma absorpcji i emisji analizowanego barwnika w 96 % roztworze etanolu.

$$\Phi = \frac{F}{F_s} \frac{1-10^{-A_S}}{1-10^{-A}} \frac{n^2}{n_S^2} \Phi_S \quad (1)$$

where: F and F_S – integrated intensities of the sample and standard spectra, respectively, A and A_s – absorbances of the sample and standard spectra, measured at excitation wavelength, n and n_S – the refractive indexes of the sample and reference solutions, and Φ_S is quantum yield of standard.

The influence of dye concentration on emission intensity

The relationship between the concentration of synthesized dye and the intensity of fluorescence was measured, as it is important to obtain the highest intensity with the lowest concentration. The maximum allowable concentration of synthetic food dyes are usually of 10^{-5} M order [21]. A wide group of artificial dyes is approved for use in the food industry, but their potential health risk is still discussed [16]. Coumarins are the group of compounds considered to be only mildly toxic [4, 11], nevertheless, the potential daily intake should be as low as possible [20]. Based on the results obtained, the maximum fluorescence occurs at a concentration of 4 to $5 \cdot 10^{-5}$ M (Fig. 3), which corresponds to the absorbance of ca. 0.65 at the excitation wavelength.

The fluorescence intensity at that concentration corresponds to the maximum of the curve (Fig. 3), but as many different factors affect the fluorescence intensity, the

concentration-fluorescence intensity relation should be measured also for real samples in the ongoing investigations.

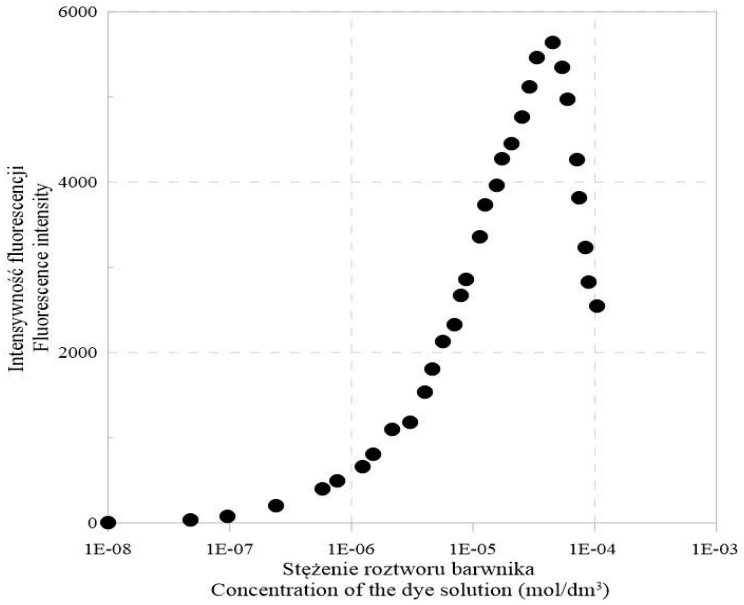

Fig. 3. The dependence of fluorescence intensity on the concentration of dye in the solution (the concentration was presented on a logarithmic scale)

Ryc. 3. Zależność intensywności fluorescencji od stężenia roztworu barwnika (stężenie zostało zaprezentowane w skali logarytmicznej)

The influence of ethanol concentration on the emission intensity

As 96 % ethanol is rarely used as a drink, it is important to measure the fluorescence intensity in the ethanol-water mixtures of various concentrations. The concentration usually observed in alcoholic beverages is 40 vol. % or lower. Our results showed that the fluorescence intensity drops for the concentration lower than 60 vol. %, but even at 10 %, it is still clearly visible (about 40 % of maximum intensity) (Fig. 4).

The quenching of fluorescence caused by the addition of water cannot be easily explained without the detailed photophysical measurements. What is important from the practical point of view is that fluorescence intensity for low ethanol concentrations (found in various types of beers and wines) would be relatively weak, and the best results would be obtained for distilled beverages having a higher ethanol concentration.

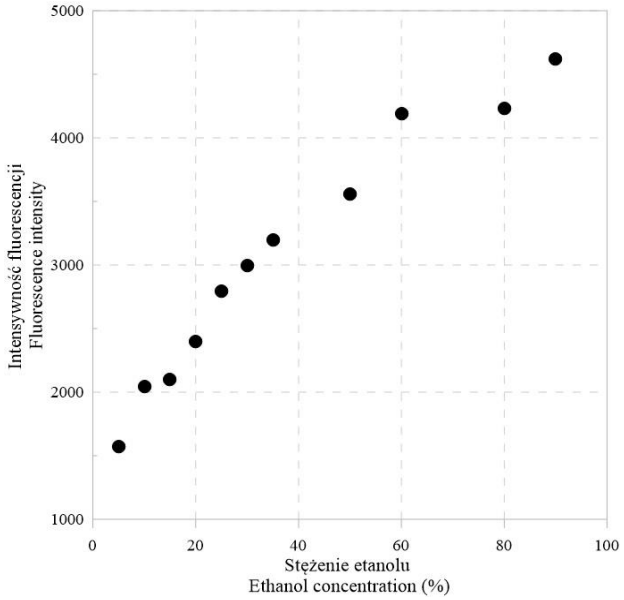

Fig. 4. The dependence of the fluorescence intensity of the studied dye on the concentration of ethanol.
Ryc. 4. Zależność intensywności fluorescencji analizowanego barwnika od stężenia alkoholu etylowego.
Explanatory notes: / Objaśnienia:
emission intensity was read at a wavelength of 480 nm, which is the maximum of fluorescence / intensywność emisji została odczytana dla długości fali 480 nm, dla której fluorescencja barwnika jest najwyższa.

Electrochemical measurement results

To check the resistance to oxygen autoxidation of the studied dye, the cyclic voltammetry measurements were performed. The results (Fig. 5) showed quasi-reversible oxidation peak at +1.11 V.

This value indicates that in the neutral environment the dye should not be oxidized by oxygen, as oxygen potential in neutral condition is equal to +0.81 V [30]. In an acidic environment (often observed in alcoholic beverages, due to the addition of usually acidic fruit juices), the potential of oxygen rises to +1.23 V [5]. Thus, in the acidic environment, the oxidation of the dye is possible, but due to a small difference between the dye and oxygen potentials, the process should be rather slow.

Microbiological assay

To analyze the influence of the dye on the selected microorganisms, they were grown at a medium with the addition of a synthesized dye solution (these samples were made in triplicate). The growth was compared with the reference samples: medium

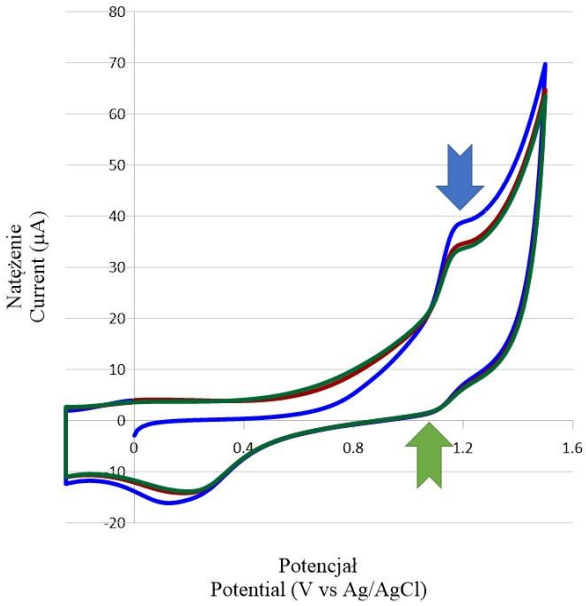

Fig. 5. Voltammograms of the dye solution (at a concentration of 5 mM) in a mixture of acetonitrile and water (1:1) with the addition of an electrolyte, 0.1 M sodium perchlorate.
Ryc. 5. Woltamogramy roztworu barwnika (o stężeniu 5 mM) w mieszaninie acetonitrylu i wody (1:1) z dodatkiem elektrolitu, 0,1 M chloranu(VII) sodu.

Explanatory notes: / Objaśnienia:
The arrows indicate oxidation and reduction processes / Strzałki wskazują procesy utleniania i redukcji.
The potential was measured *vs.* Ag/AgCl reference electrode / Potencjał był mierzony względem elektrody chlorosrebrowej.

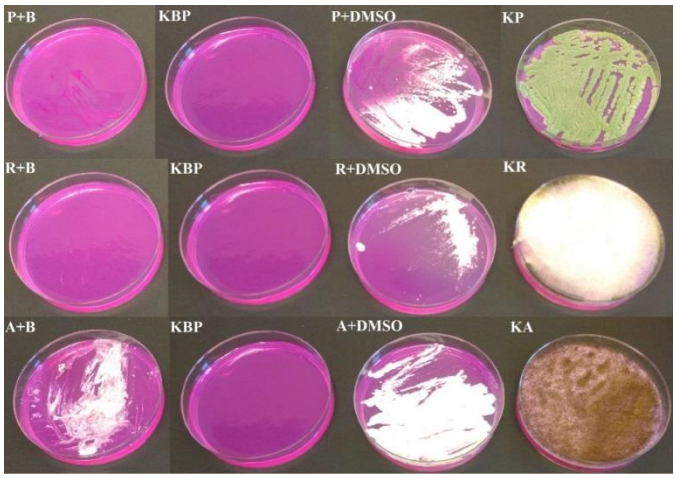

Fig. 6. Grown mold colonies: *P. expansum*, *Rhizopus* sp., *A. niger* on solid media.
Ryc. 6. Wyrosłe kolonie pleśni: *P. expansum*, *Rhizopus sp.*, *A. niger* na podłożach stałych.

Explanatory notes: / Objaśnienia:
First column: P+B (R+B, A+B) – *P. expansum* (*Rhizopus* sp., *A. niger*) seeded on a medium with DRBC and the addition of a dye solution in DMSO; second column: KBP – control sample, DRBC medium with the addition of a dye solution in DMSO without microbial culture; third column: P+DMSO (R+DMSO, A+DMSO) – *P. expansum* (*Rhizopus* sp., *A. niger*) seeded on a medium with DRBC and the addition of pure DMSO; fourth column: KP (KR, KA) – control samples, *P. expansum* (*Rhizopus* sp., *A. niger*) seeded on medium with DRBC.
Pierwsza kolumna: P+B (R+B, A+B) – *P. expansum* (*Rhizopus sp., A. niger*) posiany na podłożu z DRBC i dodatkiem roztworu barwnika w DMSO; druga kolumna: KBP – próbka kontrolna, podłoże z DRBC z dodatkiem roztworu barwnika w DMSO bez hodowli drobnoustrojów; trzecia kolumna: P+DMSO (R+DMSO, A+DMSO) – *P. expansum* (*Rhizopus sp., A. niger*) posiany na podłożu z DRBC i z dodatkiem czystego DMSO; kolumna czwarta: KP (KR, KA) – próby kontrolne, *P. expansum* (*Rhizopus sp., A. niger*) posiany na podłożu z DRBC.

with the addition of the dye solution in DMSO without inoculation of the microorganism and mediums: without any additives and with pure DMSO, inoculated with molds. The results obtained, showed in Fig. 6, indicate that the dye has significant influence on the growth of all studied molds.

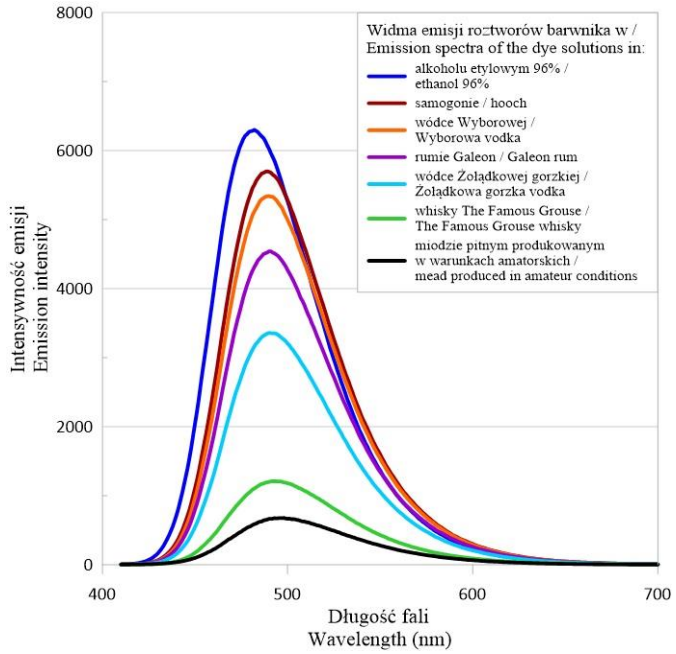

Fig. 7. Emission spectra of solutions of selected alcoholic beverages with the addition of the dye.
Ryc. 7. Widma emisji roztworów wybranych napojów alkoholowych z dodatkiem barwnika.

The ability of the studied dye to inhibit the growth of molds (which are one of the most important microbiological contaminants in beverages [2]) could be the additional benefit. During the investigation, we tested also other microorganisms (selected bacteria and yeasts), but due to the strong influence of DMSO on their growth, the results are ambiguous and are not presented here.

Real samples tests

For a test of the behavior of the studied coumarin derivative in the real alcoholic drinks which could be served by a bartender, we prepared the solutions of the dye in: vodka Wyborowa, vodka Żołądkowa Gorzka, Galeon rum (alcohols of Polish producers), The Famous Grouse Blended Scotch Whisky, homemade mead and hooch, and all were compared with 96 % ethanol (Fig. 7).

The results showed that colorless and almost colorless products (hooch, vodka Wyborowa and rum) decreased fluorescence intensity only slightly, probably mostly due to a decreasing concentration of alcohol in the solution (compare Fig. 4). In the case of colored alcohols, the fluorescence intensity decreased strongly with the growing saturation of the hue (from vodka Żołądkowa to mead). The absorption of the colored ingredients of the alcohols studied partly covers the emission band of *black bulbs* (around 400 nm), which results in the lower excitation of the dye. These yellow-colored components are usually various polyphenols and/or anthocyanines [9, 19].

Conclusions

1. All the results obtained show clearly that the derivatives of 7-amino-4-trifluoromethylcoumarine could be potentially considered fluorescent dyes for selected alcoholic beverages.
2. The absorption spectrum of such dyes covers the visible region, and the fluorescence could be excited with black light bulbs used in many nightclubs. Fluorescence intensity is not affected strongly by the presence of water in the beverages. Only in some cases (e.g. mead), the ingredients of the studied alcohols decreased fluorescence intensity significantly, but even in those cases it was still observable.
3. The optimal concentration of the dye (the maximum fluorescence intensity) is comparable to the allowable concentration of synthetic dyes in foodstuffs. The dye is also capable of decreasing the growth of molds, which are ones of the most important microbiological contaminants in food.
4. Additionally, the coumarin derivatives are not considered toxic, with LD_{50} levels few orders of magnitude higher than the maximum amount possibly taken with beverages consumed.

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POCHODNE 7-AMINO-4-(TRIFLUOROMETYLO)KUMARYNY JAKO POTENCJALNE BARWNIKI FLUORESCENCYJNE DO ŻYWNOŚCI - WYNIKI WSTĘPNE

S t r e s z c z e n i e

Wprowadzenie. Unikalne produkty spożywcze, takie jak czarne hamburgery, różowe bajgle oraz żelki Jelly beans są modne, szczególnie wśród młodej, dorosłej części społeczeństwa. Zbadaliśmy potencjalne zastosowanie fluorescencji w przemyśle spożywczym. Naturalne i syntetyczne barwniki są powszechne i ogólnie stosowane, w przeciwieństwie do barwników fluorescencyjnych. Pochodne kumaryny, wykazujące intensywną fluorescencję, mogłyby okazać się interesujące dla różnych gałęzi przemysłu, w tym przemysłu spożywczego. W naszych badaniach przetestowaliśmy rozpuszczalne w wodzie pochodne kumaryny jako barwnik fluorescencyjny do napojów alkoholowych i uzyskaliśmy obiecujące wyniki.

Celem badań było otrzymanie na drodze syntezy chemicznej pochodnych 7-amino-4-(trifluorometylo)kumaryny oraz analiza otrzymanych pochodnych pod kątem zakresu ich stosowalności i wpływu na wybrane drobnoustroje.

Wyniki i wnioski. Pochodne 7-amino-4-(trifluorometylo)kumaryny można uznać za potencjalne barwniki fluorescencyjne do napojów alkoholowych. Absorbują one światło widzialne, mogą być wzbudzane za pomocą czarnych świetlówek tzw. black bulb, a na fluorescencję słabo wpływa woda obecna w napojach. Optymalne stężenie barwnika jest niskie i prawdopodobnie byłoby bezpieczne dla zdrowia ludzkiego. Badane barwniki hamują również wzrost wybranych pleśni.

Słowa kluczowe: barwnik fluorescencyjny, kumaryna, ciekawe jedzenie, niezwykłe jedzenie, napoje alkoholowe