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Chapter 15

DYES AND PIGMENTS WITH ECOLOGICALLY MORE TOLERANT APPLICATION

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ABSTRACT

A review of the basic ecological problems related to the application of dyes and pigments for textile, foods and polymers is presented. A modern approach for solving the problems is shown. Along with a review of the synthesis and application of functional dyes and pigments already reported, in this paper we present a synthesis of 20 new bifunctional azo- and anthraquinone dyes-triazine derivatives. Six of them are metallized (Cu, Cr^{III} and Co^{III}) dyes and two of them contain a tetramethylpiperidine (TMP) stabilizer fragment in their molecule. In a color assessment of the new dyes, the percentage of exhaustion and fixation to cotton was found to be with 10-15 % higher compared to those of the model ones.

The photo degradation of 10 fluorescent naphthalimide dyes, synthesized before, was investigated and the structure-stability dependence was determined.

The copolymers of acryl amide and five fluorescent 9-phenylxanthene dyes derivatives, allylic ether-esters were obtained. The percentage of the chemical bound in the polymer dye was found to be 45-80, providing an intensive, stable to wet treatment and solvent's color and fluorescence, thus making the dyes suitable for ecologically tolerant application in food and cosmetics.

The photo stability of the triazine azodyes with a TMP fragment in the molecule was 25-30 % higher than those of the similar bifunctional azodyes without TMP. Their polymers of acrylonitrile were obtained and it was found that they have a good stabilizing effect on the photo degradation of the copolymer thus making these dyes suitable for "one-step" coloration and stabilization of materials with more tolerant ecological behavior.

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INTRODUCTION

Dyes and pigments are among the most widespread products applied in human life. We can not even imagine our life without colors and colored materials. The important influence of color on humans was determined a long time ago. Nowadays, there are no places in our lives without the application of dyes and pigments. At the same time, their application has caused various ecological problems. In spite of this, no one proposed their prohibition, because the most important principle in modern ecology is the principle of prevention but not of prohibition [1].

In the present paper we will try, while describing the main ecological problems in the application of dyes and pigments, to concentrate on the possibilities for the synthesis of products with more tolerant behavior and application.

Nowadays, depending on their nature and properties, the dyes and pigments are applied in the following areas:

1. Coloration of textile materials;
2. Coloration of polymers;
3. Dyes and pigments for foodstuffs and cosmetics; and
4. Dyes and pigmanets in high technologies (most recent application).

We will focus on these areas of application consecutively to express the kind of ecological problems presented and what approaches to take for their solution.

1. COLORATION OF TEXTILE MATERIALS

1.1. Ecological Problems

Ecological problems resulting from the coloration of textile materials arise at three different times: during the dyeing of the respective material, during the use of the colored material, and at the time of its washing and cleaning.

- *Ecological problems arising during the dyeing process*

  Textile materials of natural origin are colored with different dyes [2, 3]. No matter what the conditions of coloration are, some quantity of the dye remains in the bath. First of all it is an important economical problem, because a part of the dye is lost with the waste water. But also as important, it is an ecological problem. Furthermore, the water undergoes a physical, chemical and biological purification, so the organic products can be destroyed or made harmless.
- *Problems arising at the time of the application*

  Dyes, which are used for coloration of textile materials, are fixed to the material forming hydrogen, ion and/or van der Waals bonds with it. It is known that these bonds are not stable and during the application, due to different factors such as rubbing, light, heat, sweat and others, these bonds can be broken down. This releases the dye and its

molecules migrate and could fall onto human skin. This is a serious ecological problem because dyes, in many cases, could cause allergic or toxic reactions.

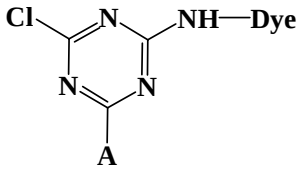
Another problem appeared when, during the application, the material was subjected to wet treatment or cleaning. During this procedure, a part of the dye was released into the wash water or into the solvents.

1.2. Approaches for Solving these Problems

One rather successful decision made to solve the ecological problems mentioned above was the synthesis of the reactive dyes [2, 3]. Nowadays almost all companies, working in organic synthesis, produce different brands of reactive dyes. In contrast to the other classes, the coloration by these dyes is due to the forming of covalent (chemical) bonds with the material, which guarantees durability of the color on physical factors. The most important part of the reactive dyes are the 1, 3, 5-triazine derivatives. In the dichlorotriazine dyes, the two chlorine atoms are the active groups.

The dichlorotriazine dyes showed one very serious disadvantage. During the coloration, because of its high reactivity, a part of the dye is lost in the bath. This additional problem was successfully solved with the synthesis of the monochlorotriazine dyes and especially the bifunctional ones [3]. The last ones contain two types of active groups in their molecule, which are able to react consecutively or simultaneously with the material. With the contemporary brands of bifunctional dyes, it was possible to reach an over 80% degree of exhaustion of the dye from the bath and a more than 90% degree of fixation to the material. The most common combination of active groups is chlorotriazine and vinylsulfone ones.

Keeping in mind the above mentioned, we synthesized a group of bifunctional triazine dyes with a general formula 1, where the Dye is different azo- and anthraquinone chromophors, and A is an unsaturated group like allylamine or allyloxy [4].



Formula 1.

The novelty in these dyes was that the two active groups are chlorine and allylamino- or allyloxy ones. The difference between the traditionally applied active vinylsulfone or bromoacrylic groups is that they can easily be involved in the triazine ring [4]. We synthesized two yellow azotriazine dyes with formula 1. Our investigations showed that these groups had good activity and the dyes had a good ability for coloration of cotton with a high degree of exhaustion (70-79%) and of fixation (98%). On the other hand, they are bifunctional as well, because of their ability to take part in copolymerization with some monomers, thus self-colored polymers can be obtained. The copolymers of these dyes with acryl amide (ACA) and acrylonitrile (AN) were obtained with a color stable when exposed to

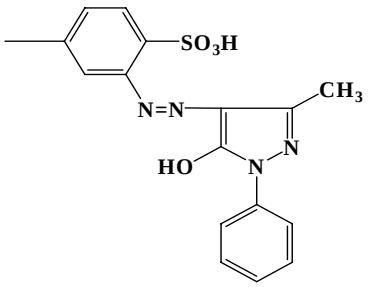
wet treatment and solvents. These results made those types of dyes suitable for coloration of materials of natural and synthetic origin and their blends as well.

The above-mentioned studies encouraged us to continue our investigations into the synthesis of other derivatives of that class.

Synthesis of Bifunctional Reactive Triazine Dyes

The synthesis of 10 new triazine derivatives (yellow, orange, red, and blue in color) took place with formula 1, where the meanings of Dye are as follows:

1.1



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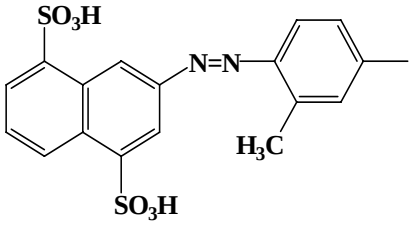

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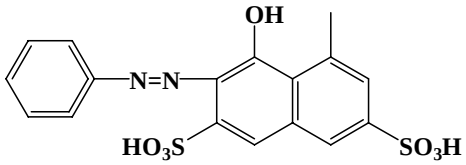

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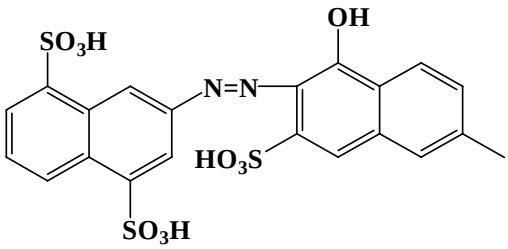
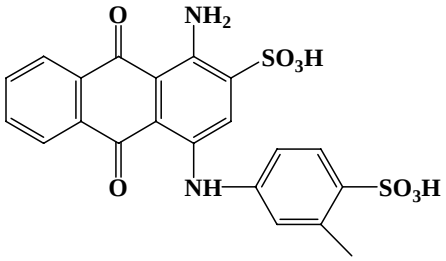
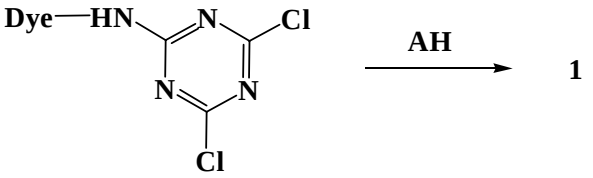


1.5



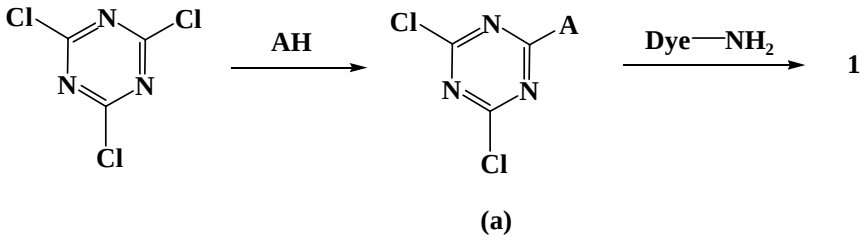
With each chromophore we obtained 3 sub-derivatives where the meanings of A are: 1.1^a A is $–NHCH_2CH=CH_2$, 1^b A is $-OCH_2CH=CH_2$ and 1^c A is $–NH_2$. The (**c**) derivatives were used as model ones in order to compare the properties of the new dyes. The synthesis of the derivatives with formula 1 was achieved experimenting with two different synthetic routes.

According to the first scheme (1) firstly the corresponding dichlorotriazine reactive dye was obtained, which at the second step reacted with allylamine (1^a derivatives), with allylic alcohol (1^b) and with ammonia (1^c derivatives).



Scheme 1.

According to the second route (Scheme 2) firstly the corresponding 2-monoallyl-4, 6-dichlorotriazine compound (a) was obtained, which at the next step reacted with the corresponding chromophore.

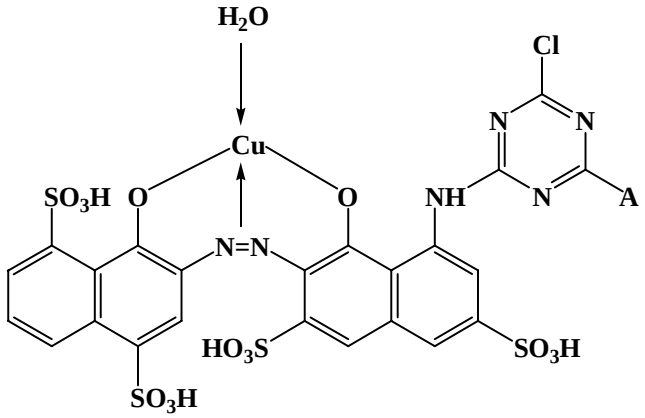


Scheme 2.

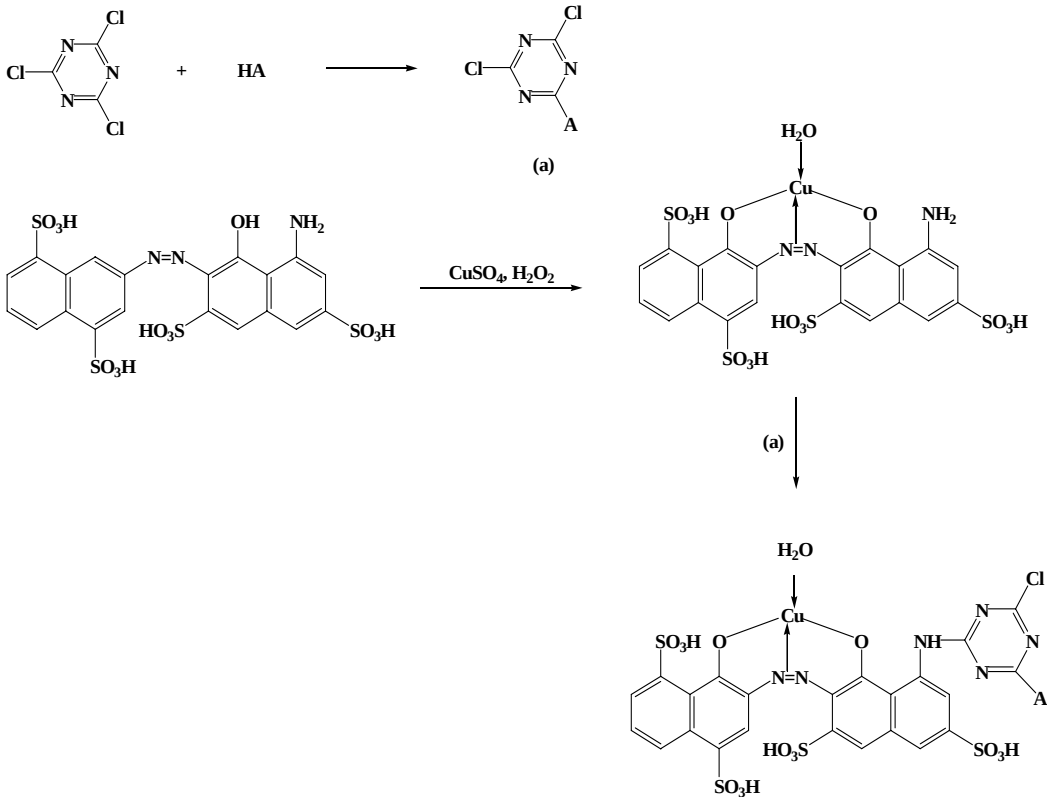
Table 1. Characteristic data for dyes with formula 1

Dye	λ_{max}^{abs} (nm)*	R_f	Dye	λ_{max}^{abs} (nm)	R_f
1.1^a	386	0.80^1	1.3^c	525	0.58^2
1.1^b	392	0.83^1	1.4^a	498	0.50^2
1.1^c	390	0.74^1	1.4^b	492	0.50^2
1.2^a	382	0.64^1	1.4^c	496	0.52^2
1.2^b	378	0.65^1	1.5^a	600	0.47^3
1.2^c	374	0.63^1	1.5^b	596	0.47^3
1.3^a	528	0.61^2	1.5^c	598	0.44^3
1.3^b	529	0.64^2			

* Solution in water; [1]- Silica gel plates $60F_{254}$ eluent system n-propanol-ammonia (1:1, v/v); [2]- Silica gel $60F_{254}$ plates and eluent system n-propanol-ammonia (2:1, v/v) and [3] - Silica gel $60F_{254}$ plates and eluent system n-butanol-acetic acid-H_2O (4: 1: 5,v/v).



Formula 2.



Scheme 3.

The course of the synthesis was followed by a quantitative thin-layer chromatography (TLC) using a "Camag" system comprising of a TLC Linomat IV device for sample application, a Scanner II and a SP 4290 Integrator. TLC analysis was performed on 20x20 aluminium–backed ready-made plates precoated with 0.2mm layers (Silica gel $60F_{254}$ products of Merck) following a procedure described before [5].

Scheme 1 was found to be more suitable for the synthesis of compounds 1.5, when for the rest of the dyes it was Scheme 2.

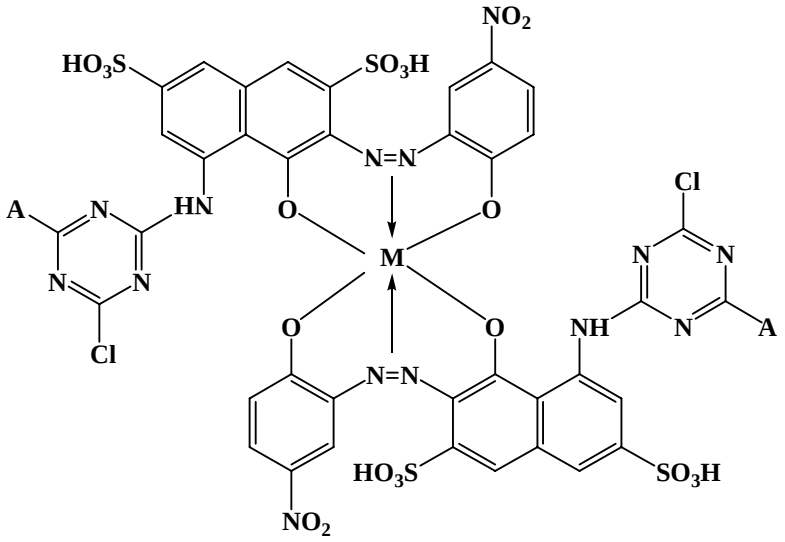
All the dyes were isolated in good yields, characterized and identified by TLC, UV/Vis, IR and ^{1}H-NMR spectra. The analytical data confirmed the structures. Some of these data are presented in Table 1.

The metallized dyes had their advantages and are of importance for the practice. The synthesis of more tolerant structures is of main importance as well. It was of interest to study the synthesis of structures similar to those shown above, where the basic chromophore is a metallized dye. Having in mind that among them the most applying are 1:1 copper (II) - azodye complexes, chromium (III) and cobalt (III) 2:1 –azo dye complexes, the dyes with such a structure were chosen. The first 3 derivatives (copper 1:1 complexes) are presented with formula 2, where A is - $NHCH_2CH=CH_2$ (dye *2.1*),-$OCH_2CH=CH_2$ (dye *2.2*) and $–NH_2$ (*2.3* a model dye).

The synthesis of the dyes is presented by Scheme 3, where HA are allylamine, allylic alcohol or ammonia.

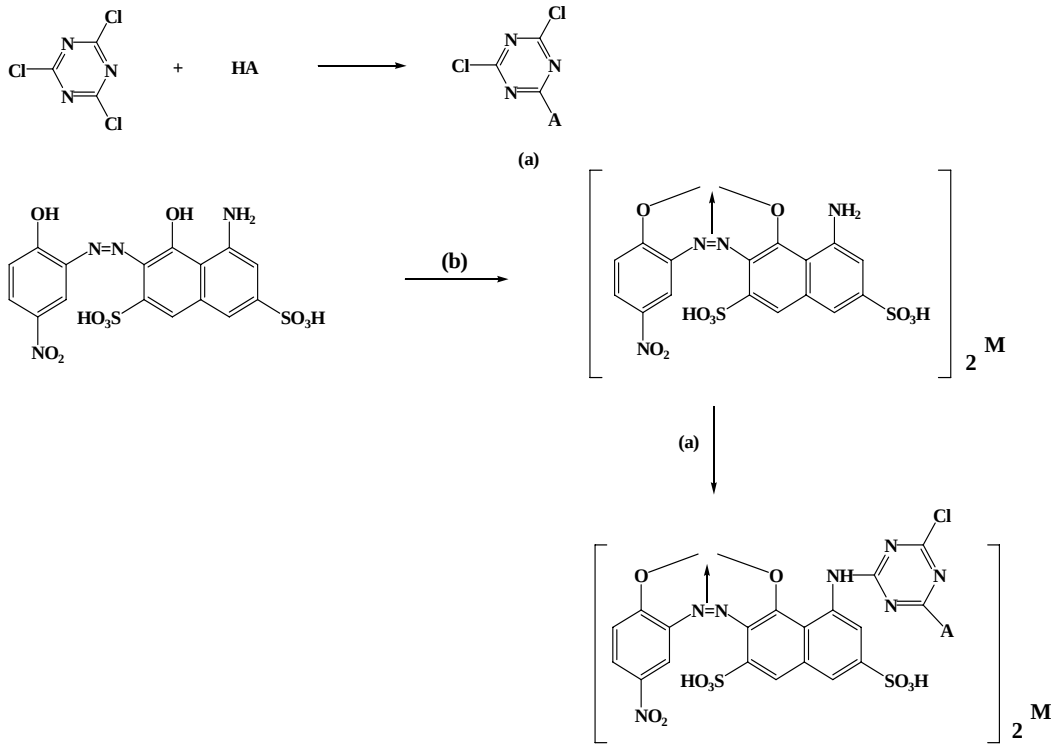
The synthesis was performed following the traditional procedure [4] and was monitored by a quantitative TLC [5]. The target compounds were obtained with good yields, they were characterized and identified by TLC, UV/Vis, IR and ^{1}H-NMR spectra. Some of the data are presented in Table 2.

The next 7 dyes have the general formula 3, where the meanings of the M (metal) and A are as follows:



Formula 3.

3.1 - M is Cr, A - $NHCH_2CH=CH_2$; *3.2* – M is Cr, A -$OCH_2CH=CH_2$; *3.3*- M is Cr, A- NH_2; *3. 4* - M is Co, A - $NHCH_2CH=CH_2$; *3.5* - M is Co, A -$OCH_2CH=CH_2$ and *3.6*- M is Co, A- NH_2. Their synthesis is presented by Scheme 4.



Scheme 4.

According to the scheme at the first stage by a reaction of cyanuric chloride with AH (allylamine, allylic alcohol or ammonia) the corresponding semi-products (*a*) were obtained. At the next stage, the products (a) thus obtained reacted with (*b*) - the corresponding complexes of Cr or Co with salicylic acid [3]. Four of the dyes (3.1, 3.2, 3.4 and 3.5) are new compounds, while 3.3 и 3.6 are model ones. The synthesis was monitored by a quantitative TLC [5]. The compounds were obtained with good yields, characterized and identified by the above-mentioned analytical methods. Some of the data are presented in Table 2.

Table 2. Characteristic data for dyes with formula 2 and 3

Dye	λ_{max}^{abs} (nm)	R_f
2.1	568	0.53[1]
2.2	568	0.50[1]
2.3	568	0.50[1]
3.1	588	0.69[1]
3.2	592	0.69[1]
3.3	590	0.70[1]
3.4	584	0.68[2]
3.5	584	0.70[2]
3.6	584	0.68[2]

[1]Silica gel $60F_{254}$ plates and eluent system n-propanol-ammonia (1:1, v/v); [2] Silica gel $60F_{254}$ plates, system n-butanol-acetic acid-H_2O (4: 1: 5,v/v).

Furthermore, by mixing dyes 3.1 and 3.4 in ratio 1:1 (dye *4.1),* 3.2 and 3.5 (dye *4.2*) and 3.3 and 3.6 (dye *4.3),* 3 black dyes were obtained.

Color Assessment of the Synthesized Dyes

Cotton fabrics were dyed with dyes 1.1÷1.5 at 3 % depth o.w.f., according to the standard procedure [3, 6]. Materials with an intense color (yellow for dyes 1.1÷1.2, orange with 1.3, red with 1.4 or blue with 1.5) were obtained. Using the "Data color" technique and the associated software, the color characteristics of the dyes were recorded. From these data we saw some movement to the blue-reddish color for dyes 1.1^{a}, 1.2^{a}, 1.3^{a}, 1.4^{a} and 1.5^{a} in comparison to the corresponding model dyes 1.1^{c} ÷1.5^{c}. The similar movement but to the blue-greenish color was registered for the dyes 1.1^{b}÷1.5^{b}. The percentage of exhaustion and fixation of the dyes were determined applying the standard procedure. Data obtained are presented in Table 3.

One can see from these data that the dyes with two active groups (chloro- and allylic) in the triazine ring had approximately a 10- 15 % higher percent of exhaustion and fixation on the fabrics, which make them ecologically more tolerant and more suitable for application.

With dyes 2, 3 and 4, wool fabrics were dyed and intensively colored in blue, violet and black materials were obtained. Similar investigations to determine the color characteristics of the dyed materials (using Data Color technique) were performed and the percentage of exhaustion and fixation of the dyes was determined. It was found between 85 and 90 % of exhaustion and 99 - 100 % of fixation for all the dyes containing allylic active group(s). These data are higher compared to those for the model dyes (2.3, 3.3, 3.6 and 4.3). Wash fastness for all the dyed materials was determined to be 5 / 5 /5.

The electrochemical behavior of dyes with formula 1.1- 1.2 into aqueous solution was studied. It was found that the bifunctional dyes were reduced more easily in water than the model ones, which again is an indication for their ecologically more tolerant application.

Table 3. Exhaustion (%) and Fixation (%) of the dyes with formula 1 on cotton

Dye	1.1^{a}	1.1^{b}	1.1^{c}	1.2^{a}	1.2^{b}	1.2^{c}	1.3^{a}	1.3^{b}	1.3^{c}	1.4^{a}	1.4^{b}	1.4^{c}	1.5^{a}	1.5^{b}	1.5^{c}
Ex.*(%)	82	80	74	86	88	70	85	85	70	82	80	75	90	88	74
F** (%)	96	95	85	97	98	92	99	99	90	99	98	90	99	99	90

*Exhaustion; **Fixation.

2. DYES AND PIGMENTS FOR POLYMERS

2.1. Ecological Problems

Production of synthetic polymers and their application is extremely intensive. This sets the question for their qualitative and lasting coloration. There are several methods of polymer coloration [9].

- Method of textile coloration
- Method of surface coloration
- Method of coloration “in mass”

According to the first method, the polymers are dyed in the same way as the textile materials. The coloration is achieved by fixing of the respective dye by hydrogen, ionic or/and van der Waals bonds to the material. The disadvantages of this method and the ecological problems are the same as the ones for the dyeing of the textile materials. Furthermore, the polymer must have appropriate functional groups in its molecule with which the dye can form bonds. Considerable part of the polymers is not up to this requirement. Such widespread applied polymers like polyethylene (PE), polypropylene (PP) and polyvinylchloride (PVC) do not have such groups and cannot be dyed in this way. Other polymers such as polyesters and polyamides, even though they contain functional groups in the polymer chain, due to the higher density of the polymeric structure, these functional groups are inaccessible for the dyes.

The disadvantages of the second method are that only the surface layer of the polymer is colored, some of its properties can be broken during the coloration, and it is vulnerable on mechanical impact.

According to the third method, the dyeing can be achieved during or after the processing of the products, when the molecules of the dye or the pigment diffuse or dissolve into the mass of the polymer. To carry out a qualitative and uniform coloration in this way definite requirements are put on the dyes and the polymers. The dye must be in fine dispersing form, mixed with different additives. The main disadvantage of this method is that the colored polymer is a mixture of a higher molecular and a lower molecular substance. With time, the lower molecular substance (the dye) migrated to the surface of the polymer, damaging the quality of the color. This can change some of the properties of the polymer, too. Besides, if the polymers do not have amorphous areas in which the dye’s molecule can diffuse, the coloration will not be possible.

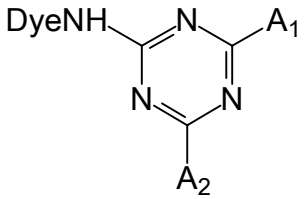
2.2. Approaches for Solving the Problems-chemical Method

The first information about chemical coloration of polymers has appeared in 1960’s. This method has economical and ecological advantages and can be realized in two ways: modification of polymers already synthesized or obtaining of self-colored polymers by copolymerization or polycondensation of a monomer with a color compound.

- *Modification of already synthesized polymer*

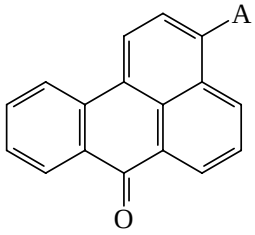
  An example of coloration by this method is the obtaining of self-colored polystyrene described in [9]. Another example is when the colorless *Vinylon* fiber was passing through a solution of a dye, which contained aldehyde groups, which reacted with the hydroxyl groups of the polymer thus the self-colored polymer was obtained.
- *Synthesis of self- colored polymers by copolymerization*

To apply this method, the corresponding dye or pigment must have functional groups able to participate in copolymerization, to be stable during the process, to not prevent the polymerization. The first polymerizing dyes of this type were patented by BASF in 1962 and were obtained by acylation or vinylation of some anthraquinone dyes [10]. The corresponding self-colored copolymers were obtained by the copolymerization with styrene, methylmetacrylate and acrylonitryl. During this period the information for the synthesis of self-colored polymers was summarized and published by Asquit [11], Mareshal [12], Guthrie [13] and others [14], including our earlier investigations on the synthesis and application of self-colored polymers [15-19]. For the first time, we demonstrated a principally new approach for introducing a polymerizing group in a dye molecule. As a base for the synthesis of such dyes, a proposal was made to use triazine reactive dyes with allylic- or acrylic group(s) as the substituents in the triazine ring. The dyes obtained had formula 4, where A_1 and A_2 are residues of allyl- or diallylamine, allylic alcohol, acryl- or methacrylamide.



Formula 4.

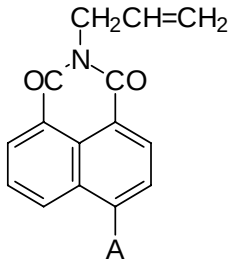
Firstly we studied two azo- and an anthraquinone chromophores only, and following the above mentioned idea, more then 30 polymerizable dyes and pigments were obtained. Later, the range of the chromophores was widened with benzanthrone (formula 5), whose derivatives were interesting with their bright colors, intensive fluorescence and good thermo- and light stability. Seven new polymerizing dyes with formula 5 were synthesized [20-25].



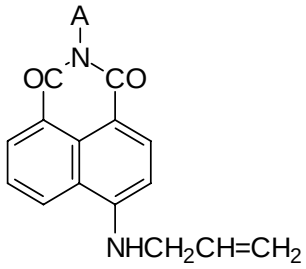
Formula 5.

The excellent color qualities and intensive fluorescence of the benzanthrone derivatives provoked our interest on the synthesis of some other polymerizing luminophores. Naphthalimide and 9-phenylxanthene dyes, which are among the most widely applied in different areas, were chosen.

Following this idea a group of polymerizing derivatives of naphthalimide with general formula 6 and 7, where A are residues of different aliphatic amines, were synthesized [26-31].



Formula 6.



Formula 7.

Their spectral properties were studied [27]. With all synthesized dyes self-colored copolymers of styrene, MMA and AN, with a stable to solvents color and an intense fluorescence were obtained.

Having in mind how important for the application the photo stability of the dyes is, it was of interest to study the stability of the obtained functional naphthalimide dyes.

In this present paper, we reported our recent investigations on the photo degradation of 10 dyes with formula 6, where meanings of A are as follows: *6.1* – Br, *6.2*- morpholino, *6.3*- piperidino-, *6.4*- piperazine, *6.5*-dimethylamino-, *6.6*- methyl amino-, *6.7*- diethyl amino-, *6.8*- ethyl amino-, *6.9*- allylamino- and *6.10*- NH_2.

To determine what the influence of the substituents on the photo stability of the dyes is, the solutions of the dyes in DMSO (2.10^{-4} g.mL^{-1}) were subjected to irradiation with UV light in a Suntest equipment (using a Xenon lamp 1.1 kW and λ = 290 nm). During the irradiation no change in the λ_{max} of absorption of the dyes was observed, which enable us to follow the kinetic of photo degradation spectrophotometrically by the method of the standard calibration curve. The data obtained at the end of the 2nd h are between 50 and 75 %, where the quantity of the dye in the solution before the irradiation was accepted 100 %. According to them we found that the photo stability of the compounds decreased in the order:

6.1⇒ 6.2 ⇒ 6.13 ⇒ 6.4 ⇒ 6.5 ⇒ 6.7 ⇒ 6.10 ⇒ 6.9 ⇒ 6.6 ⇒ 6.8

One can see from these data that the presence of an HN- fragment in the 4th position of the naphthalimide molecule (6.8, 6.6, 6.9 and 6.10) had a negative influence on the photo stability of the chromophore.

Table 4. Concentration of the dyes in copolymer (%)* during irradiation

Dye	6.1	6.2	6.3	6.4	6.5	6.6	6.7	6.8	6.9	6.10
Time 2h	97	93	95	95	92	90	91	87	90	90
Time 10 h	86	76	76	75	74	54	75	58	68	70

* The concentration of the dye in copolymer before irradiation was accepted 100 %.

Furthermore it was of interest to see the photo stability of the same dyes when they are incorporated into the polymer molecule. The copolymers of the above dyes with methyl methacrylate (MMA) were studied under the same conditions as the dyes- in solution of DMSO (2.10^{-3} g.mL^{-1}) and in films (plates with d = 90 mm and 2 mm thickness). The kinetics of photo degradation for 10 h was followed spectrophotometrically. The data obtained are presented in Table 4.

One can see from these data that:

- The photo stability of the dyes in copolymer is higher than that in solution and
- The structure- photo stability relationship followed the same order as in solution.

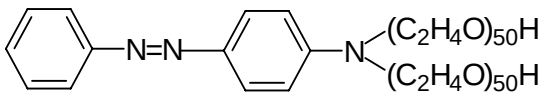
3. DYES FOR FOOD AND COSMETICS

3.1. Ecological Problems

It is well-known that for coloration of foods and cosmetics only a limited number of dyes and pigments is permitted. The ecological danger in this case is the most because the dyes and pigments get into the human organism directly, can undergo change and some toxic products can be obtained. In this connection looking for more tolerant dyes and pigments is of special interest.

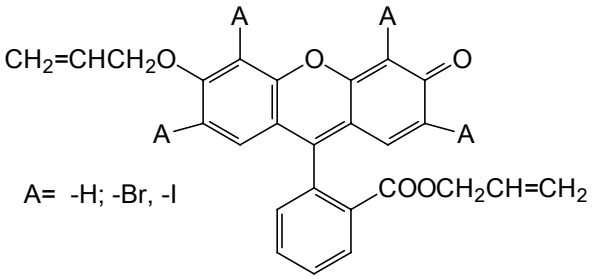
3.2. Approaches for Solving the Problems

It was established that if the molecular mass of an organic compound is over 1000, its penetration through the stomach walls and cells is very difficult or not possible [2, 32]. This fact has given a direction for synthesis of dyes with enlarged molecular mass. For example the Milliken dyes with a formula 8 were produced [2, 32].

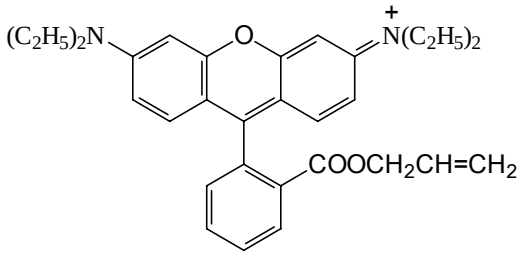


Milliken Yellow

Formula 8.



Formula 9.



Formula 10.

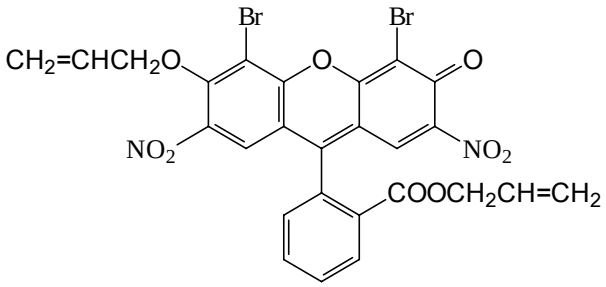
Derivatives of 9-phenylxanthene, due to their nice color and intense fluorescence have taken an important place among the dyes and pigments permitted and applied for coloration of food and cosmetics. In our earlier papers we demonstrated the synthesis of functional derivatives of 9-phenylxanthene [33-37]. They can be presented by formula 9 and 10.

The copolymers of these derivatives with MMA were obtained. Due to the chemical bonding of the dyes to the polymer chain, the copolymers thus obtained had an intensive color and fluorescence stable to solvents, which made them suitable for ecologically more tolerant application in some products like children's toys. Their spectral properties and photo stability were studied and it was found that the incorporation of the dyes into the polymer chain led to a considerable increasing of their photo stability [36, 37].

Polyacrylamide (PolyACA) is among the polymers widely applied in medicine, cosmetics, food processing industry and agriculture [38, 39]. It is well soluble in water which is important for the application. In a previous work of ours, the possibility of obtaining copolymers of acrylamide with some triazine herbicides and bactericides with more tolerant application, was established [40- 42].

Based on our experience, in the present paper we reported our study on the synthesis of copolymers of ACA with 5 dyes, derivatives of 9-phenylxanthene, with the view of their more tolerant application in the polymer form as food additives.

The dyes subject of the present study have the general formula 9, 10 and 11, where the meanings of A in formula 9 are: -H (dye 9.1); -Br (dye 9.2) and -I (dye 9.3).



Formula 11.

PolyACA is obtained easily by polymerization of acrylamide (ACA) in an aqueous medium, the polymer being water-soluble. That suggests a very good opportunity for application of the dyes in a water soluble polymer form. We performed the copolymerization at 50°C for 7 h in aqueous medium at two different concentrations of the corresponding dyes (0.1 wt. % and 0.2 wt %) in relation to ACA. The polymers thus obtained, brightly coloured and with an intense fluorescence are purified from unreacted monomers by three-fold reprecipitation from water solution with methanol. The latter is very good solvent for the dyes, but not for the polymer. After precipitation until a colourless filtrate was obtained, the polymers retained their color. This was an indication that the dyes were chemically bound to the polymer. The colored polymers thus purified were analysed.

The absorption UV/Vis spectra of all colored copolymers (solutions in formamide with concentration 2.10^{-2} g.mL^{-1}) were recorded and compared to those of the pure dyes in the same solvent (concentration 2.10^{-4} g.mL^{-4}). Neither hypso-, nor batochromic shift in their absorption maxima was established, thus showing that the basic chromophore did not change, neither during the polymerization nor as a result of its bonding to the polymer molecule. These results enable us to determine spectrophotometrically the quantity of the chemically bound dye using the method of the standard calibration curve. The data obtained are presented in Table 5.

Considering these results one can see that the percentage of the dye chemically bound in the copolymer, except for the dye 11, is satisfactory. Taking into account the fact that during the reprecipitaion of the polymers, lower-molecular fractions were removed as well, the real quantity of the dye reacted is higher and exceeds 50%. Thanks to the high color strength of the chromophores, the polymers reprecipitated were very intensely colored with bright fluorescence, and this intensity of theirs would be sufficient for the practice. The relatively lower activity (30 to 45% bound dye) of dye 11, could be due to the steric hinderence. More accurate conclusions however, could be possible after future investigations. The intrinsic viscosity [η] of all precipitated colored copolymers was determined. The same measurements were performed for PACA, obtained under the same conditions, but without a dye. These data showed that the participation of the dyes in the copolymerization process did not affect the molecular mass of the copolymer. These results enable us to consider that the dyes under study will be suitable for application in polymer form as food or cosmetic additives.

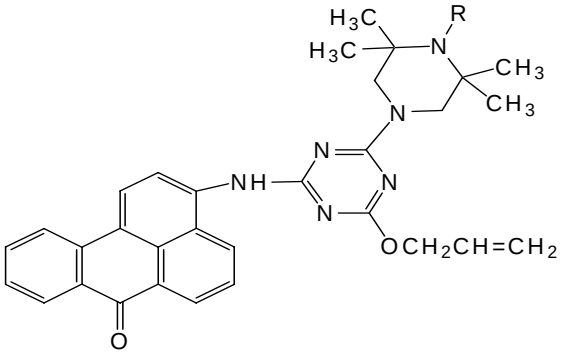
Table 5. Data for the chemically bonded in the copolymer dyes with formula 9, 10 and 11 (%)*

Copolymer with dye №	λ_{max}^{abs} (nm)	Chemically bounded dye at 0.1wt % initial concentration	Chemically bounded dye at 0.2 wt % initial concentration
9.1	460	56	74
9.2	534	57	80
9.3	542	52	65
10	570	72	72
11	538	30	45

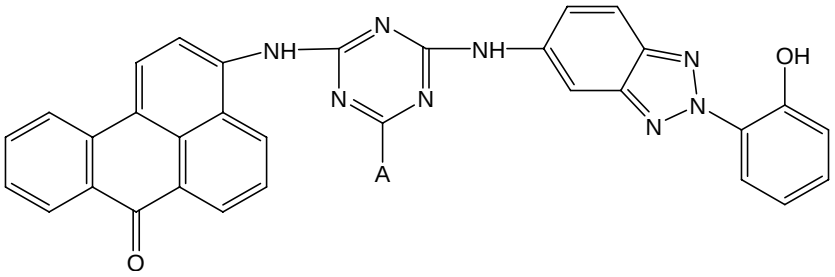
* initial concentration of the dye was accepted 100 %.

4. DYES AND PIGMENTS FOR "ONE-STEP" COLORATION AND STABILIZATION

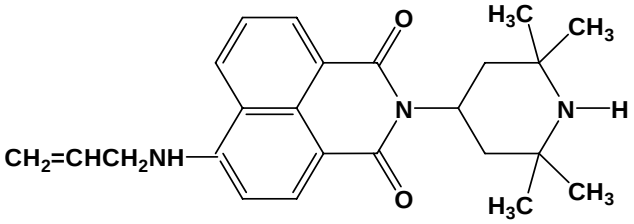
Along with the coloration, the photo stability of the materials is of great importance. Among different stabilizers used 2-hydroxyphenylbenzotriazole (HBT) and 2, 2, 6, 6-tetramethylpiperidine (TMP) derivatives are of interest [43]. Recently, the polymerizable stabilizers of different types were synthesized [44 - 48]. Their covalent bonding to the polymer chain provided stability towards solvents and a migration, both improving their environmental behavior, which is of importance for application in food and cosmetics. We reported before the possibility for "one-step" coloration and stabilization of polymethylmethacrylate (PMMA) and polystyrene (PSt), using polymerizable benzanthrone (formula 12 and 13) [24, 49] and naphthalimide (formula 14) [50] dyes, containing a polymerizable group and a stabilizer's fragment in the same molecule.



Formula 12.



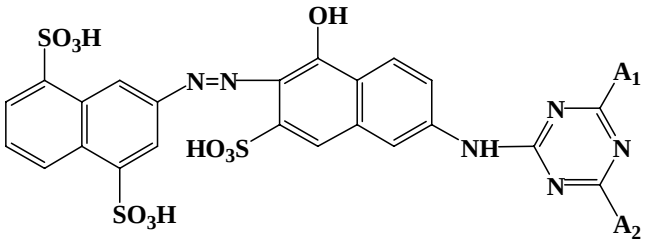
Formula 13. A is $–NHCH_2CH=CH_2$ or $–OCH_2CH=CH_2$ fragment.



Formula 14.

Bearing in mind these investigations and the fact that the azodyes are among the compounds with the lower photo stability, it was of interest to synthesize some azodyes, containing a TMP fragment in their molecule. To study the possibility for their application both for coloration and stabilization of polymers will be of interest as well.

The dyes reported in this paper have a general formula 15, where meanings of A are: A_1-tetrametylpiperidinilamino, A_2 -$NHCH_2CH=CH_2$ (15.1); A_1 -tetrametylpiperidinilamino, A_2 -$OCH_2CH=CH_2$ (15.2); A_1 –Cl, A_2 -$NHCH_2CH=CH_2$ (15.3) and A_1 –Cl, A_2 -$OCH_2CH=CH_2$ (15.4).



Formula 15.

The synthesis of the dyes was achieved according to the procedure described before [4, 24]. Our idea was to see what the influence of a stabilizer's fragment on the photo stability of the dye is. In this connection the solutions of the dyes were subjected to irradiation with UV light (in a Suntest equipment) and the kinetic of their photo degradation spectrophotometrically was followed. The data obtained are presented in Figure 1, where the numbers of the curves are 1- dye 15.1; 2- dye 15.2; 3- dye 15.3 and 4- dye 15.4.

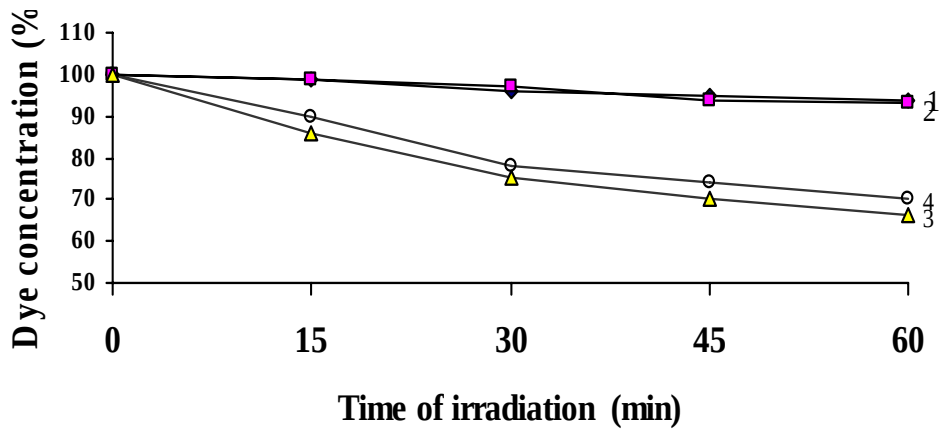


Figure 1. Dependence of dye's concentration (%) on the time of irradiation (min) 1-dye 15.1; 2- dye 15.2; 3- dye 15.3 and 4- dye 15.4.

One can see from these data that the presence of the TMP fragment in the dye's molecule led to the increasing of its photo stability by 25-30%.

Having in mind that the dyes had an unsaturated group in their molecule their ability to copolymerize with some monomers was studied. Polyacrylonitrile (PAN) is widely applied and its coloration and stabilization is of interest. Following the standard procedure the copolymerization of AN with the dyes with formula 15 (at 1% against AN) was performed in DMF solution at 70 °C. After 8h the orange-reddish colored polymers were obtained. After precipitation with hot water they retained their color, which was an indication for the covalent bonding of the dyes in the polymer. The influence of the dyes on the photo stability of the copolymers was studied, measuring the intrinsic viscosity [η] of the polymers before and after the irradiation. The data for the molecular mass calculated presented in Table 6 showed that the dyes 15.1 and 15.2 with a TMP fragment in the molecule had a very good stabilizing effect on the photo degradation of the polymer.

Table 6. Data for molecular mass of the copolymers of AN with the dyes with formula 15 before and after irradiation of UV light

Copolymer with dye	$Mn_o \times 10^3$ before irradiation	$Mn \times 10^3$ after 8h irradiation	S*
15.1	310	284	0.09
15.2	247	231	0.07
15.3	157	122	0.28
15.4	192	153	0.25
PAN without dye	338	295	0.15

$S^* = Mn_o / Mn - 1$ [48].

Based on the results obtained we can conclude that the dyes 15.1 and 15.2 having good photo stability and a stabilizing effect on the copolymers of AN can be applied successfully for one-step coloration and stabilization of these polymers with more tolerant behavior.

CONCLUSION

Based on this review and the results of our investigations, we can summarize that the polymer modifications of the dyes and pigments give definitely promising possibilities for their more tolerant and safe application, and the work in this direction is of great interest.

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Chapter 16

ENHANCED ANTHRAQUINONE DYE PRODUCTION IN PLANT CELL CULTURES OF *RUBIACEAE* SPECIES: EMERGING ROLE OF SIGNALING PATHWAYS

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ABSTRACT

Synthetic and natural analogues of 9,10-anthracenedione are well-known and widely used substances in the food and dye industries. Beyond their dyeing ability some anthraquinones are used as medicines as they exhibit beneficial effects in mammals and humans; moreover, anthracyline antibiotics have been applied therapeutically in the case of several malignant diseases. Total synthesis of anthraquinone derivatives using organic chemical methods is common; however, sophisticated biotechnological techniques provide alternatives for synthesis and overcome some environmental and economical concerns. The most frequently studied plant cell culture systems originate from members of the *Rubiaceae* family because these cell cultures are capable of producing high amounts of anthraquinone derivatives. Several methods to enhance the dye yield in order to obtain the best results have been and are still being used on a small-scale prior to applying them in large-scale industrial production. Numerous factors regulate the biosynthesis of anthraquinones in cell cultures like compartmentation, environmental stimuli (e.g. light, precursors) and endogenous (metabolic and developmental) factors. Since major points of the anthraquinone biosynthesis regulation are known in the *Rubiaceae* family, various possibilities have been raised to exploit these findings. Formation of hairy root and other transgenic cell cultures have proved to be useful tools to increase the anthraquinone production capacity; moreover, newer approaches (DNA and protein microarrays, proteomics) are promising techniques to define biosynthetic pathways to elucidate the unknown and rate limiting steps. Another effective approach to dye production enhancement in plant cell cultures is elicitation: exogenous stimuli induced gene activation. Various elicitors have been introduced during the past two

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decades affecting anthraquinone yields of measured cell cultures; however, the background of the influence on physiological events caused by elicitors is not fully understood. Elicitors are recognized by plant receptor(s) localized in the plasma membrane or cytoplasm. After elicitor signal perception, plant receptors activate effectors and signal transduction leads to the modulation of genes via second messengers. Altered gene expression might manifest itself in heightened production of antraquinone derivatives. An increasing knowledge of plant signal transduction enables us to choose a suitable elicitor, which activates/modulates the desired pathways for anthraquinone production leading to a more selective production of the needed compounds. This commentary summarizes results of the latest studies on elicitor induced signal transduction leading to anthraquinone production in plant cell cultures and discusses other relevant techniques on a comparative basis. Furthermore, major problems and a future outlook are also debated.

INTRODUCTION

Plants are unique sources of a wide range of metabolites including important natural dyes such as indigo, betalains, anthocyanins, flavonoids and anthraquinones. These specific molecules are produced by secondary metabolism of plant cells and, among other roles, they are important in survival of the plants amidst unfavorable environmental factors. Due to the genetic diversity of the plant kingdom, these specific compounds are produced by well-defined taxonomic groups, although molecules with similar chemical structure may be present in several non-related groups, also. Unfortunately, the majority of the secondary metabolites are not produced in all parts of the plants and are not produced in all developmental stages. Synthesis of the desired compounds often does not start until the plants are several years old. If the metabolites are accumulated in underground organs (e.g. rhizomes, roots), harvesting results in destruction of the whole plant; therefore, isolation of the valuable components is not always economic or acceptable from the ecological point of view.

To overcome the problems above, several efforts have been made in the past decades to establish plant cell cultures in order to produce secondary metabolites [1-2].

In spite of intensive research and its results, only a few secondary metabolites are produced industrially in large scale by plant cell cultures: the naphtoquinone pigment shikonin (by *Lithospermum erythrorthizon* cells, Mitsui Petrochemical Industry, Co. Ltd., Japan), the antitumor diterpene derivative taxol (by *Taxus cuspidata* cells, Bristol-Myers-Squibb Co., USA) and the 9,10-anthracenedione derivative pigment purpurin (by *Rubia akane* cells, Nitto Denko Co., Japan) [3]. Production of quinoid pigments seems to be the most successful application of plant cell cultures.

Anthraquinones (derivatives of 9,10-anthracenedione) are widely distributed molecules in living organisms, they are present in bacteria, fungi, lichens and several families of higher plants, such as *Rubiaceae*, *Rhamnaceae*, *Polygonaceae*, and *Leguminosae* [4] (Figure 1.)

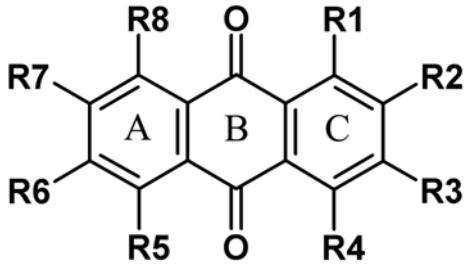

Figure 1. Chemical structure of anthraquinones.

Natural derivatives of 9,10-anthracenedione are well known and widely used chemicals to dye textiles in many regions of the world since ancient times (the oldest evidence is from 1350 B.C.) [5-6]. Extracts of *Rubiaceae* plants have been used for dyeing silk, cotton, jute, and other textiles, and to tint cosmetics and foods [5]. For better dyeing results and color modification of anthraquinones, numerous mordants have been applied as pre- or post-mordating agents, such as alum, copper sulfate, stannous chloride, ferrous sulphate, and potassium dichromate [5-6]. Recently, a wide range of synthetic anthraquinone derivatives has become available for dyeing purposes [7-8].

Beyond their dyeing ability, anthraquinones exhibit some valuable biological activities: their antimicrobial [9-10], antifungal [11], antimalarial [9], antithrombotic [12], antitumor [13], renal calculus eliminative [5], mutagenic and DNA binding [14], and antioxidant [15] features have been described. Moreover, some anthraquinone derivatives, called antracyline antibiotics, are applied in the therapy of different malignant diseases (doxorubicin and its derivatives, and mitoxanthrone and its derivatives). Unfortunately, they have some dangerous side effects, like congestive heart failure [16]. In the case of human malignant disease chemotherapy, one of the critical points is the selection of multidrug resistant tumor (MDR) cell lines in the organism, which are not responding to the applied drugs. To overcome the problems associated with anthracyline MDR cell lines, some new anthraquinone derivatives have been synthetized in the past decade [16-17].

Due to the diversified application of anthraquinones, their synthesis by organic chemical methods is common, although emission of toxic byproducts is unavoidable. Anthraquinone production by plant cell cultures is a more environmentally friendly method. Albeit this technology is suitable to generate only a few derivatives, its products are useful as basic molecules for further semi-synthetic processes of anticancer compounds [18]. One of the crucial factors of fermentation methods is the relatively high energy demand. As a consequence, several sub-methods have been developed in the past decades to increase the anthraquinone yield of plant cell cultures.

A number of plant cell cultures have been established in the past two decades from plant species of the *Rubiaceae* family with high capability of anthraquinone production: *Cinchona robusta* [19]; *Galium verum* [20], *M. citrifolia* [21], *M. elliptica* [22], *Ophiorrhiza pumila* [23], *R. akane* [24], *Rubia cordifolia* [25], *Rubia tinctorum* [2,26]. Moreover, some cell cultures produce anthraquinones also in bioreactors [27-30] offering the possibility of efficient production of these dyes in higher volumes (Table 1.).

Table 1. Examples of isolated anthraquinone derivatives from the *Rubiaceae* family

Compound	*Source plant cell culture*	R1	R2	R3	R4	R5	R6	R7	R8
Robustaquinone A	*Cinchona robusta*	OH	CH_3	H	OH	H	OCH_3	OH	OCH_3
Robustaquinone B	*Cinchona robusta*	OH	CH_3	H	H	H	OCH_3	OCH_3	H
1,3-dimethoxy-2-hydroxy-AQ	*Galium verum*	OCH_3	OH	OCH_3	H	H	H	H	H
Rubiadin	*Galium verum* *Morinda elliptica* *Ophiorrhiza pumila* *Rubia tinctorum*	OH	CH_3	OH	H	H	H	H	H
1,6-dihidroxy-2-methyl-AQ	*Galium verum*	OH	CH_3	H	H	H	OH	H	H
Alizarin	*Rubia akane* *Rubia cordifolia* *Rubia peregrina* *Rubia tinctorum*	OH	OH	H	H	H	H	H	H
Lucidin	*Ophiorrhiza pumila* *Rubia cordifolia* *Rubia tinctorum*	OH	CH_2OH	OH	H	H	H	H	H
Purpurin	*Rubia akane* *Rubia cordifolia* *Rubia tinctorum*	OH	OH	H	OH	H	H	H	H
Lucidin-ω-methyl ether	*Morinda citrifolia* *Morinda elliptica* *Rubia tinctorum*	OH	CH_2OCH_3	OH	H	H	H	H	H
Nordamnacanthal	*Morinda citrifolia* *Morinda elliptica* *Rubia tinctorum*	OH	CHO	OH	H	H	H	H	H
Lucidin-primveroside	*Morinda citrifolia* *Rubia tinctorum*	OH	CH_2OH	O-Pr*	H	H	H	H	H
Alizarin primveroside	*Morinda citrifolia* *Rubia tinctorum*	OH	O-Pr*	H	H	H	H	H	H
Munjistin	*Rubia cordifolia*	OH	COOH	OH	H	H	H	H	H
2-methyl-1,3,6-trihydroxy-AQ	*Rubia akane*	OH	CH_3	OH	H	H	OH	H	H
Pseudopurpurin	*Rubia tinctorum*	OH	COOH	OH	OH	H	H	H	H
1,6-dihydroxy-2-methyl-AQ	*Galium verum*	OH	CH_3	H	H	H	OH	H	H
1,3,8-trihydroxy-2-methoxy-AQ	*Cinchona robusta*	OH	OCH_3	OH	H	H	H	H	OH

*=primverose (6-*O*-β-D-xylopyranosyl-β-D-glucose); AQ=anthraquinone.
See also Figure 1.

SOME FEATURES OF BIOSYNTHESIS OF ANTHRAQUINONES IN THE *RUBIACEAE* FAMILY

To understand the secondary product formation of plant cell cultures in the *Rubiaceae* plants, a short overview is given here on their anthraquinone biosynthesis (for more details see [4]).

Essentially two basic biosynthetic pathways exist leading to anthraquinones in plants: the polyketide pathway and the chorismate/o-succinylbenzoic acid pathway [4,31]. The polyketide pathway is not involved in the anthraquinone formation in the *Rubiaceae* family.

The speciality of the anthraquinone biosynthesis in *Rubiaceae* plants is that A and B rings are formed via *o*-succinil benzoic acid (OSB) whereas ring C is formed from isopenteyl diphosphate (IPP) via the terpenoid pathway [31].

Initial molecules of A and B ring synthesis are phosphoenol-pyruvate and erythrose 4-phosphate. From these substances, the chorismic acid (its intermediary molecule is the sikimic acid) is formed via several steps. Isochorismate synthase (ICS) produces isochorismate from chorismic acid [32] and isochorismate in its turn is converted into OSB by OSB synthase in the presence of α-ketoglutarate and thiamine diphosphate. The OSB-CoA ligase activates the OSB by forming OSB-CoA ester and subsequently this ester goes through ring closure resulting in the formation of 1,4-dihydroxy-2-naphtoic acid, which gives the A and B rings of anthraquinones [33].

Formation of the C ring in the anthraquinones results from the cyclization via C-C bond formation between the aromatic ring of the naphtoquinone and isoprene unit IPP or 3,3-dimethylallyl diphosphate (DMAPP) [4]. IPP can be formed via two independent pathways: the mevalonic acid (MVA) or the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathways [34].

In the MVA pathway's first reaction, acetyl-CoA is converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) via several steps. HMG-CoA is converted into MVA, and next in a few steps the isopentenyl diphosphate is synthetized from the MVA [4,35].

In the MEP pathway, the initial step is the formation of 1-deoxy-D-xylulose-5-phosphate (DOXP) by the condensation of glyceraldehyde 3-phosphate and pyruvate [36]. The reaction is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) [37]. In the next reaction, DOXP is converted into MEP by the enzyme DOXP reductoisomerase (DXR). Then MEP is transformed into 4-cytidil diphospho-2*C*-methyl-D-erythritol (CDP-ME), and the CDP-ME is further changed into 2-C-methyl-D-erithritol 2,4-cyclophosphate (MEC). This molecule forms the IPP.

After its formation, IPP is converted by the enzyme IPP isomerase in both pathways into DMAPP, the activated monomer unit of isoprenoids [38 and references therein].

Most of the anthraquinones of the *Rubiaceae* family have substitutions in ring C and/or in ring A (Table 1). These substitutions might be introduced in late stages of anthraquinone biosynthesis and some antharquinones are stored in their glycoside forms, due to the glycosylation processes [4,39].

FACTORS AND METHODS LEADING TO ENHANCED ANTHRAQUINONE BIOSYNTHESIS AND YIELDS

Screening, Medium Optimization

The most common approach to enhance secondary metabolite production of plant cell cultures is selection of appropriate plants/cell strains [40]. This screening process may involve the selection of different organs/plants/biochemical variants/cultivars/species or *in vitro* cultured cell lines [40]. The latter approach has recently been applied to select *R. tinctorum* populations for optimal (e. g. low-level of genotoxic lucidin) anthraquinone composition [41]. In the case of anthraquinone derivatives, selection of high-producing cell lines is simple because of their color attribute, and lines could be selected by visual examination for cell aggregate cloning [42].

Another general technique is optimization of the culture medium composition [43]. Several studies have shown the positive effects of plant growth regulators on the antharquinone production, e. g. an increase of 1-naphtaleneacetic acid (NAA) level promoted the anthraquinone production in cell cultures of *Morinda*, *Galium*, *Cinchona*, *Rubia* [44-47]. Naturally, the developmental stage of cell cultures must be taken into consideration. Hagendoorn et al. [48] showed that the growth phase and the production phase could be affected distinctly with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). Abdullah and co-workers [27-28] described remarkable differences in anthraquinone production of *M. elliptica* cell cultures grown in “maintenance”, “production” and “growth” media. The “production” medium strategy enabled 5-fold anthraquinone yield in comparison to the “growth” medium.

The type and quantity of carbon source is also an important regulating factor of anthraquinone production. Generally sucrose has priority in use but not in all cases. Actually, in *R. cordifolia* culture glucose has a better effect [49]. The effect of sucrose is influenced by the phytohormone composition of the medium [46].

There are no general formulas for media because of the various demands of the different cultures; therefore it is advisable that the medium should be optimized according to their needs [43,45,50].

Medium optimization is the most essential step during establishment of plant cell suspension cultures. Combining it with the approaches above productivity might reach a 20-30 times higher rate in the case of compounds which were present in the initial cultures [51].

Techniques to Enhance Yields of Secreted Compounds into the Medium

Due to the chemical characteristics of anthraquinone derivatives, several cells secrete them (mainly in glycosidic form) into the culture medium [21,52]. This phenomenon could be utilized either by application of permeabilizing agents [53-54] resulting in 14-170-fold higher release of anthraquinones or by using a two-phase medium system facilitating the collection of less water-soluble anthraquinone derivatives into a non-polar phase [21,55]. Besides the two-phase liquid method, some authors have also applied adsorbents (eg. XAD resins) to ameliorate the separation of the released anthraquinone derivatives [54,56]. Immobilization of

cells is also an adequate method to enhance the production of secondary metabolites. Immobilization of *Cruciata glabra* cells resulted in a 5.6-fold increase in anthraquinone production and 34-fold higher anthraquinone release to the medium [57-58].

Direct Influence on the Biosynthetic Capacity

Our knowledge on the biosynthetic pathways gives several opportunities to enhance the antharquinone production of plant cell cultures [4]. A simple method is the precusor feeding to the cultures as intermediary molecules of the metabolic pathways such as chorismate, OSB (added to *Morinda* cell cultures) or pyruvate (precursor of the MEP pathway). Supplying them resulted in up to 4-fold increased anthraquinone production [4,59-61]. A further technique is the application of stimulators of different pathways such as proline (stimulator of pentose phosphate pathway leading to erythrose phosphate, a basic molecule of the shikimate pathway) or blocking competitive pathway's activity with specific inhibitors (like aminoindan-2-phosphonic acid an inhibitor of phenyalanine ammonia liase, the key enzyme of cinnamate biosynthetic pathway). Applying these methods, anthraquinone production of *R. tinctorum* cells was increased by around 50 percent [62]. Gliphosate, a partial inhibitor of chorismate biosynthesis, behaved controversially: in *M. citrifolia* cell culture it decreased the anthraquinone biosynthesis, while in *R. tinctorum* culture it enhanced the anthraquinone production [63].

Environmental Factors and Compartmentation

Taking into consideration the environmental factors of cell cultures (light, temperature, shaking, aeration etc.) their effect is obvious on culture metabolite production. Light intensity may have a principal role in association with anthraquinone production. Generally, illumination of the cell cultures reduces anthraquinone yield [22,64]; however, high-producing cell cultures are maintained for several years at dimmed natural light [65].

For anthraquinone production the compartmentation of cells is an important reality, at least four intracellular compartments are essential: plastids, endoplasmic reticulum, cytosol, and vacuolar system (e. g. for storage). All influences on differentiation status of the above compartments alter the secondary metabolite production ability of the cells. This fact should be considered during the process of designing the culture medium and engineering the fermentation strategy [4,66].

Plant Genetic Engineering

Plant genetic engineering approaches and techniques have made great progress during the past two decades and their use has led to the commercial introduction of several transgenic plants (e.g. various crops) in agriculture [51,67]. In the past years, tremendous advances have also been attained in metabolic engineering of plant secondary metabolism [4,68]. The major approaches of plant genetic engineering are summarized in Table 2. (for more details see [68-70] and references therein).

Table 2. Approaches of plant genetic engineering

Approach	Applied techniques	Reference
Discovery of new enzymes and genes of plant natural product biosyntheses	Enzyme purification, Peptide microsequencing, Screening of cDNA libraries, Establishment of expressed sequence tag, Whole genome sequencing, PCR	[68,69]
Identification and application of transcription factors involved in plant secondary metabilosm	RT-PCR, cDNA isolation, Dnase footprint analysis, Recombinant protein isolation	[70-73]
Inhibition and overexpression of biosynthetic enzymes	RNA interference, Overexpression of different genes, Overexpression of transcription factors	[69,74-76]
Investigation of enzymes involved in plant secondary metabolism	Protein crystallization, X-ray diffraction, Mass spectrometry	[68,77-78]
Combinational biosynthesis of plant natural products	Ligation of cDNA into plasmids and transfer into foreign organisms (bacteria, fungi, plants)	[79-81]

To enhance production of anthraquinone derivatives ICS gene (*ics*) was introduced into *M. citrifolia* and the cell line containing the *ics* gene in sense orientation showed higher ICS activity [4]. A DXS gene encoding cDNA was cloned from *M. citrifolia*, and its transcript level correlated with the antraquinone accumulation of the culture suggesting DXS small gene family regulation at the transcriptional level [82]. Unfortunately, there are only some relevant molecular biological works published in this field from *Rubiaceae*. This fact may be a result of the relatively low price of anthraquinone derivatives in comparison with other plant-derived compounds, e.g. taxol.

Among the extremely organized and genetically modified cultures, hairy root cultures are the best-studied. The hairy root development is based on the transfer of *Agrobacterium rhizogenes* T-DNA into the genome of infected plants. This T-DNA carries a set of genes encoding enzymes to regulate auxin and cytokinin biosynthesis. The altered balance of the hormones induces extraordinary root proliferation, called hairy roots (for details see [83] and references therein).

Several authors have demonstrated that anthraquinone derivatives are produced by hairy root systems from plants of the *Rubiaceae* family and the main compounds are very similar to the native roots. However, the yield of these compounds is often lower in the artificial root system than the measured amount in the native roots [29,46,84-85]. Other authors have established antharquinone producing transformed *Rubia* calli. They are good subjects for the investigation of the transferred cells' behavior under different stresses [86-88].

Elicitor Induced Anthraquinone Accumulation and Signal Transduction

The connection between plant secondary metabolism and plant defense mechanism is well-known, plants in reaction to unfavorable biotic/abiotic signals developed a set of defense

responses involving the production of secondary metabolites (phytoalexins, e.g. anthraquinones) [3,89]. For the plant defense response induction, the whole biotic/abiotic environment is not necessary but the presence of a special part of it is enough. Keen was the first person to use the term "elicitor" for substances, which can generate responses manifesting in phytoalexin production [90]. Nowadays, elicitors can be defined as substances of which small amounts induce or enhance the production of specific metabolites in a living cell system [91]. During the last decades, several elicitors were found; accordingly the classification of elicitors became necessary and they were classified on the basis of different approaches, such as molecular structure, specificity, origin etc. We can also include among elicitors some abiotic stress factors, such as shearing stress or temperature stress, in spite of the fact that no substances are added to the cell system [30]. Table 3 shows a classification of elicitors based on the papers of [3,89,91].

The first step in triggering elicitor induced defense reactions is the recognition of the elicitor by the plant cell "receptors". This highly specific recognition process is essential in response generation [92]. The physical/chemical linkage between the elicitor and its receptor causes conformational changes in the receptor's structure [3]. This structural alteration enables it to activate the effector molecules and via signal transduction pathways the appearance of the elicitor is transformed into particular signals expounded by the cell's metabolic and genetic apparatus. Receptors can be located in the plasma membrane, apoplast and cytoplasm, according to the molecular structures, which they recognize. The elicitor-receptor recognition is specific and characterized by high affinity, showing reversible and saturable binding. The above-mentioned specificity may manifest itself in altered anthraquinone production (both qualitatively and quantitatively) giving a key approach to influence the anthraquinone composition of cultured cells. Elicitors, jasmonic acid (JA) and salicylic acid (SA) act at particular sites of the signaling network regulating the formation of final outcome. Apart from the specific elicitor-receptor recognition, interactions of second messengers diversify also the induced responses in the cells [3,89].

Table 3. Classification of elicitors

Elicitor				
Abiotic	Physical	Wounding, Shearing stress, Temperature stress, UV irradiation		
	Chemical	Metal ions (europium, calcium, vanadium)		
Biotic	Chemical	Complex composition	Fungal cell wall extracts, Fungal spores, Bacterial culture extracts, Bacterial/Fungal homogenates	
		Defined composition	Polysaccharide	Alginate, Chitosan, Pectin, Chitin
			Oligosaccharide	Galacturonides
			Peptides	Glutathion, Systemin
			Proteins	Cellulase, Elicitins
			Lipids	Lipopolisaccharides, Syringolide
			Glicoproteins	Cryptogeins
			Volatiles	Volicitin

Activation of particular sections of the signal transduction and types of the plant defense phenomena differ in accordance to the elicitor types and the nature of their perception (e.g. the type and localization of receptors). However, elicitor induced signal transduction is a multi-component network involving several junctions and cross-talking points; accordingly the parts of this signaling system are not separated from each other but appearing side by side. Generally, they amplify/attenuate/modulate the other processes and finally this determines the induced gene expression patterns and the redistribution of metabolic fluxes [89].

The major parts and known key molecules of the signal transduction leading to increased production of secondary metabolites are indicated in Table 4. [3,89].

Table 4. Most important signaling components and some examples of their roles

Signaling event	Known roles
(receptor-induced) GTP binding protein (G-protein) activation	NADPH oxidase activation, Ion channel activation, Phospholipase activation, Anthraquinone pigment production
Ion fluxes/Ca^{2+} signal	Gene expression alteration, NADPH oxidase activation, Anthraquinone pigment production, Activation of calcium/calmodulin dependent molecules (protein kinases/phosphatases), Transcription factor activation/synthesis, Regulation/alteration/integration of other signaling events and tranfers signals to downstream events
Cytoplasmic acidification	PAL and HMGCoA gene transcription enhancement, Formation of oxidative stress
Oxidative burst	Direct antimicrobial effect, Cell wall reinforcement, enhancement of the synthesis of phytoalexins, Second signal messenger role –modulates/amplify other signaling events, Expression enhancement of defense genes (PAL)
Inositol phosphates/cyclic nucleotides	Calcium ion mobilization from intracellular stores, Regulation of plasmamebrane positive ion channels
Salicylic acid/Nitric-oxide	Hypersensitive response formation, Systemic acquired resistance formation, Gene expression modification, Influence on the synthesis of several secondary metabolites (e.g. anthraquinones)
Jasmonic acid	Gene expression alteration, Elicitor signal transduction, Second messenger of the different signaling pathways (modulator molecule)
MAP and other kinases/phosphatases	Regulation of different molecules (e.g. ion channels, transcrition factors) via phosphorilation/dephosphorilation
Abscisic acid /ethylene	Alteration of gene expression
Lipid signaling (oxylipins, phospholipases, and other lipid messengers)	Wound healing, Protein kinase and kinase cascade activation, Oxidative burst generation

GTP-Guanosine 5'-triphosphate; NADPH-Nicotinamide adenine dinucleotide phosphate (reduced form); PAL-Penylalanine ammonia-lyase; HMGCoA-3-hydroxy-3-methylglutaryl Coenzyme A; MAP-Mitogen-activated protein kinase.

Elicitation of plant cell systems is a simple and relatively cheap method to enhance production of desired compounds. In the cell cultures of plants belonging to the *Rubiaceae* family, several authors have applied elicitors to enhance the anthraquinone biosynthesis, such as fungal elicitors [26,65,93], chitosan [94-95], and signaling molecules such as JA

[26,86,96-97] or SA [26,86]. Elicitation with fungal elicitors and JA seems to be the most effective on anthraquinone yields and elicitation methods generally enable high anthraquinone production of the cells (up to 30-fold increase in the case of particular compounds). Our recent results have shown that fungal elicitors mainly increased production of glycosides while signaling compounds, like JA and SA, caused higher synthesis rates of aglycons. These findings may facilitate further advantages of selective dye turnout [26].

Other recently published data have also proved that several signaling events take part in elicitor induced anthraquinone production of *R. tinctorum* cell cultures, such as G-protein activation, oxidative burst, lipid signaling, calcium mobilization [95,98-101]. Understanding the above in connection with the biosynthetic routes is crucial in the conscious engineering of anthraquinone dye production. Exploration of the key reactions and rate limiting steps of signaling and biosynthesis, as well as using the regulator/modifier molecules, would give us the chance to obtain more effective, selective and economic processes in production of 9,10-anthracenedione analogue dyes. A new breakthrough can be expected from proteomic and metabolomic investigations. Detailed observation of protein content changes and metabolite composition after stresses or elicitor exposition may help to understand the role of regulators and interactions among the different signal transduction pathways.

CONCLUSION

Taking into consideration the valuable results in the enhancement of anthraquinone production, we can pronounce that the synthesis of 9,10-anthracenedione derivatives by plant cell cultures is successful and in several cases has economical and environmental advantages. Our main problem recently has been that production is not specific enough and several undesired derivatives are also produced in line with the other valuable ones. Tools to control the biosynthetic routes are not effective enough yet to narrow the turnout of the cells to one, maximum two main compounds, which might reduce the costs of the expensive separation procedures. Fortunately, new analytical methods provide fast scan, high selectivity and good productivity possibilities, so identification and quantification of the proper derivatives is a routine process even at a higher number of samples [102-103]. In the future, dye production by application of plant cell cultures should be a more prudential process and engineering should be more efficient once you know the target product and the affected key steps. The most relevant and progressive methods are genetic engineering and exploitation of elicitor-induced gene activation. The first method has small advantages in the case of anthraquinone production, the latter one has the benefits of several new advances, but the relationship between the elicitor type and the produced molecule has not yet been fully clarified. Based on our current knowledge, beyond the fully optimized culturing circumstances and application of the key precursors, over-expression of the key enzymes of the proper biosynthetic pathway(s) on its own is not enough to obtain optimal yields of cultures. This approach should be supplemented with the extinction of the capacity of competitive pathways by genetic modification or by applying specific inhibitors and/or by further stimulation of the over-expressed pathways with the proper elicitor. If the previous optimization steps were realized depending on the target molecule, further yield enhancing techniques (two-phase medium, immobilization) would be applied. A new approach in the additional processing of the

synthetized glycosidic molecules is the enzymatic hydrolysis with the native enzymes of plant cultures allowing the step of the additional hydrolysis to be replaced by the plant cultures, resulting in further cost reduction (Boldizsár et al. *in manuscript*).

Our hope is that in the coming years the above mentioned techniques will give the full possibility of producing more selectively the desired specific anthraquinone derivatives economically by using plant cell cultures.

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