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RESEARCH ARTICLE

STUDY ABOUT THE STABILITY AND SOME TECHNOLOGICAL PROPERTIES OF THE PIGMENTS SYNTHESIZED BY *EPICOCCUM NIGRUM*

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Abstract

A yellowish colorant complex, rich in carotenoids and flavonoids, synthesized by selected strain *Epicoccum nigrum* MIUG 2.15, was studied for its stability and some technological properties. Stability of watery and ethanolic extracts was evaluated indifferent conditions. The study was concerned with the behavior of the pigments for different pH values. The extracts bearing significant pH differences were experimented for temperatures of 5ºC, 20ºC and 60ºC and also for different types of light: solar, UV and in darkness. The components of the colorant complex were separated on ion chromatography, and analyzed the absorption spectrum of the isolated fractions were analyzed. The different separated fractions were studied for their antioxidant capacity and antimicrobial activity. The pigments complex biosynthesized by *Epicoccum nigrum* MIUG 2.15 proved stability in refrigeration and darkness conditions, and the better stability was found at ethanolic extracts in an alkaline medium. The isolated fractions proved a good antioxidant activity.

Key words: *Epicoccum nigrum* MIUG 2.15, carotenoids, flavonoids, pigments stability, antioxidant capacity

Introduction

Color is the first sensation that is received from food and this determines the first opinion of its quality. Foods have their own color on account of the pigments they contain. Generally, it is the better, using different technological process to maintain it.

However this is not always possible. We have to acknowledge that many naturally colored substances of the food complex are sensitive to partial degradation treatment taking place during the technological process to a higher extend than others that bring in a new characteristics and peculiarities dependent on the circumstances of the experiment. It is also obvious that the natural variety of materials used brought about great changes to their color. All these changes lead to similar coloring of foods. Only

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one modification is possible during such processes; while partially recovering characteristics of color, they may also be intensified to different degrees so that they should be accepted by the viewpoint of the consumer, fact which is of great concern for this study.

As a consequence specialists in the field of food science and technology are trying to improve loss of color accounted for by such changes during the technical preservation process by adding new colorants that could better match the appropriate color of the compound.

The tendency of using natural colorants comes, among other things, from the fact that, besides their technological actions, they are made of antioxidant compounds bearing in most cases, healthy effects as proved by recent studies and documented. At the same time, the range of food industry tending to make use of more healthy and safer foods is also trying to obviously improve them on the side of their benefits for human health.

The increasing development on the market of the use of pigment in foods, drinks, detergents and cosmetics, makes it necessary for the development of new production strategies for searching some new pigment sources.

Despite the fact that for several years, micro organisms are producing a wide varieties of pigments, the strategy of using them in food coloring is still relatively recent (Downham *at al*., 2000). Among microorganisms the fungi emphasis, displaying higher rates of growth and adaptation, allow getting secondary metabolites by means of biotechnological processes, thus a higher expectation of its use (Henry, 1996).

The strain *Epicoccum nigrum* MIUG 2.15 is one of these fungi, bearing a color compounds capacity of synthesis offered as a better source of food coloring (Bahrim *et al.,* 2005). The younger colonies are yellowish-oranges or oranges-reddish. Their colonies are quickly growing and are forming a sort mycelium (Figure 1).

It is easily cultivated, which makes it more attractive on the industrial level. They grow under submerged conditions or in solid state fermentation system or semisolids ones and have different water activity values (Pascualli *et al*., 1999, Bahrim *et al.,* 2005).

The first bibliographic data which give references about pigments of this fungal strain, date back to the beginning of the century. In 1911, Naumann described the red pigment production when this strain grew on top of roots. Previously, about 50 years later, some of the pigments present in the mycelium were identified in the cultural liquid, as β-carotene, γ- carotene, rodoxhantin, licopen (Gribanovski and Floppen, 1967) and antifungic compounds like flavipin (Bamford *et al*., 1961)

Many new items of research concerning these fungi were published during the late sixty years. The most recent studies have concentrated on the application of coloring of *Epicoccum nigrum*, in the biological control of fruit (Larena *et al.*, 2004) as also its synergy action using *Xanthophyllomyce dendrorhus* which favors the synthesis of astaxanthin (Echavarri *et al.,* 2004), a potent antioxidant.

Between other things, for the complex coloring to be accepted as food coloring it needs to be healthy. As to of what toxicities refers to up until today, there have been no documented cases of infections or allergies brought about in human beings or in animals, even if there is still a lot to be done and studied (Fassatiova, 1986).

On the other side of the technological interest of the complex it depends on its stability during the process conditions and type of food storage, the same as it does on its solubility and capacity to diffuse and mix with food.

The aim of this study was to verify the stability of watery and ethanolic extracts of yellowish-oranges colorants, synthesized by a *Epicoccum nigrum* selected strain, and to evaluate some technological properties of antioxidant and antimicrobial capacity.

Materials and Methods

Materials:

- Fungal bio-product. *Epicoccum nigrum* MIUG 2.15, selected strain, is member of Industrial Microbiology Collection (coded MIUG) of the Applied Microbiology Department of the Bioaliment Platform of

the Faculty of Food Science and Engineering of "Dunarea de Jos" University of Galati Romania (Figure 1).

Figure 1. *Epicoccum nigrum MIUG 2.15 morfocultural characteristics*

This strain produces a complex of carotenoid:flavonoid coloring agents in a 20:1 ratio (orange colorant) (Bahrim and Socaciu C., 2005). Cultivation of moulds for pigment biosynthesis was performed in the Bioaliment´s Laboratories in the solid state fermentation system for 10 days, at 25°C and in darkness (Soptica and Bahrim, 2005). The fermentative medium thus obtained was dried at 40°C and milled and the resulting powder like koji was transferred for this study.

- Reagents: Milli-q water, ethanol, chlorhydric acid, sodium hydroxide, methanol, ethyl ether, dichloride methane, sodium thiosulphate, ethyl acetate, acetone, 2.2- azinobis-(-3 ethilbenzthyazol-6sulfonate, potasic thiosulphate, sodium acetate, 2,4,6- tris- 2 piridyl-s tryazin, ferric clorure, 2,2- diphenil- 1- picrylhidrazyl, AXD2 resin
- Appliances: Chromatographic column of glass (25x0.46 cm), Heidolph WB 2000 rotavapory, Beckam DU 650 spectrophotometer, Kontron-Centrikon T-124 centrifuge, Agimatic- E agitator, Philips L 565 lamp, UV lamp.

Methods:

- Pigments extractions and separations the extraction was made by following the method established by Bahrim and Soptica, 2004, using as extractants water and 96% ethanol. According to the data published it is believed that the pigments produced by *Epicoccum nigrum* MIUG 2.15 are a mixture of pigments of natural carotenes and phenolic compounds (Bahrim *et al*., 2005). Let us consider the polarized difference between both pigment groups, which specialists tried to separate relying upon most of the chromatographic techniques of columns commonly used for the separation of phenolic pigments (Di Stefano and Cravero, 1990). So, the ethanolic extracts, before eliminating ethanol and after having been redissolved in water, they were fractionated by chromatographic column on resin XAD2, using as a consecutive form 3 different effluents: ethyl ether, ethyl acetate and methanol. The separation proved evaluating the absorption spectrum UV- VIS of each fraction, previously evaporated and redissolved in water.
- Extracts stability evaluation: it was evaluated that the extract stability at different pHs (3.00, 6.00, 9.00). The effect of temperature was also studied, maintaining the extracts at 5ºC, 20ºC and 60ºc. A partial study was done on the stability of light practicing its stability upon exposure to solar light, UV light of 256 nm to darkness.

Due to logistic impediments it was impossible to study all the possible combinations of the factors studied below.

- Stability in environmental temperature: the three pHs were practiced and effect of darkness and solar and UV lights.
- Stability at refrigeration temperature (rooms at 5ºC): the pHs and the effect of darkness and solar light.
- Stability at 60ºC: the pHs in darkness (heater) and natural solar light (thermostatic and bath).

The tests were made three times. The graphic representations were considered as value means and standard deviations. The LSD (Least Significant Difference Test) test as to detect different

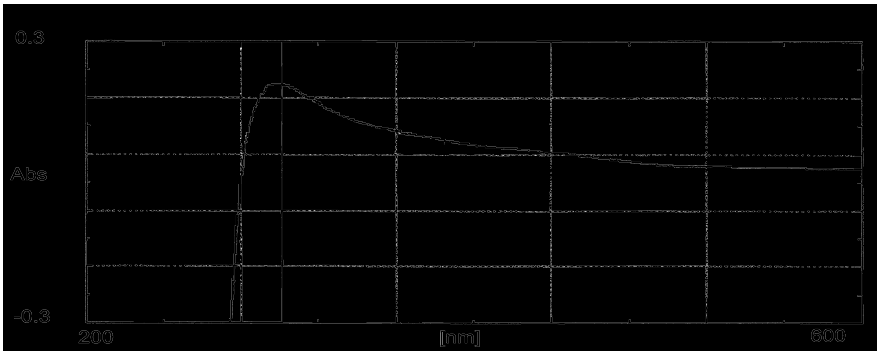
differences was used. The stability was monitored during 21 days, registering the spectrum absorption at UV-VIS (200-600 nm) of each extract and noting the wavelength of maximum absorption at every moment and the absorption at 430 nm, wavelength of maximum absorption of the extracts recently obtained.

- Antioxidant capacity of pigment fractions determination: it was interesting to evaluate the antioxidant activity of each fraction by different means and facing possible applications of the pigments object of study in the food industry. The antioxidant capacity of the fractions evaluated applying three chemical methods related to the power of pure reactor by FRAP (Benzie, 1997) method, and with the capacity to capture or block different radicals (scavenger), like ABTS and DPPH methods (Brand *et al.*, 1995).

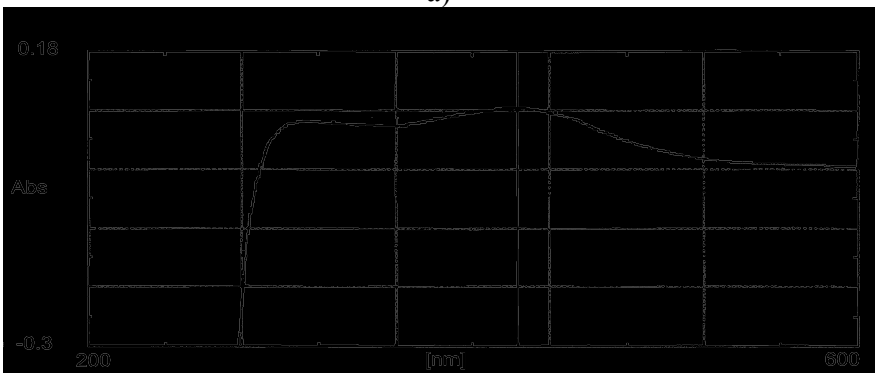
Results and discussions

The separation of the pigments synthesized by *picoccum nigrum* MIUG 2.15

The purpose of this separation is to place each group of pigments in its most adequate state depending on the properties of its own characteristics that can bear different technological concerns. In the same time some properties due to isolated fractions was also evaluated. The absorption spectrum of the corresponding extracts of each fraction indicate that it achieved in separating, at least at grain level, two different groups of pigments (Figure 2). The ones those are isolated in fractions by ethyl ether and ethyl acetate, and those that are carried away by methanol. Although the spectrum UV-VIS would not be enough to determine the types of compounds present in each case, it was perceived that the first fractions shelter the pigments of natural phenolic (maximum absorption around 280nm) and that of methanol the carotenoides (maximum absorption in visible).



a)



b)

Figure 2. *Absorption spectrum of isolated fractions in the chomatographic column: a) fraction of ethyl ether and ethyl acetate; b)methanol fraction*

Antioxidant capacity (CAO) of fractioned pigments

The CAO was evaluated by the technology approaches as combining in an additive the coloring capacity, the stability and the preservation, not only at a microorganism level but also at a chemical level blocking oxidative reactions deteriorating the food. The relative results of the CAO evaluation, clearly state the manifest that the most active fraction was that of methanol, which presented the highest values in the three methods used (Table 1). In general, the three fractions gave satisfactory values of CAO and the different amounts could be accounted by the presence of different compounds at each fraction, and/or the difference of pigment concentration at each fraction. These factors need to be studied in further studies.

Table 1. *Antioxidant activity of the Epicoccum nigrum MIUG 2.15 pigment separated by column chromatography*

Pigments fraction	Antioxidant activity		
	ABTS, µg trolox	DPPH, µg trolox	FRAP, mM Fe^{2+}
Ethyl ether	1.754 ± 0.115 [b]	1.188 ± 0.1744 [b]	0.883 ± 0.071 [b]
Ethyl acetate	1.344 ± 0.146 [a]	0.928 ± 0.1042 [a]	0.658 ± 0.0612 [a]
Methanol	2.575 ± 0.241 [c]	1.283 ± 0.1562 [c]	1.348 ± 0.012 [c]

[1]Values at the same column with a different letter are statistically different for a p value of 0.05 according to the LSD test.

Environmental conditions upon pigment comportments

Firstly, it is important to indicate that the absorption spectrum of watery and ethanolic extracts were very similar in displaying essential quantitative differences. The extract recently obtained showed maximum absorption around 430 nm, corresponding to a yellowish- slightly orange color present. A certain pH effect was found with the extracts color, above all with the intensity of the color, which decreased when its pH was tested. Initially, we thought that these variations could be only associated by using chlorhydric acid as an acidly agent. This first impression was eliminated for two reasons: 1) the effect was unequal watery and ethanolic extracts; 2) the use of other acids produced similar effects.

In general, the results proved that the stability of the extracts was dependent on the pH, extraction, temperature and the action of the light.

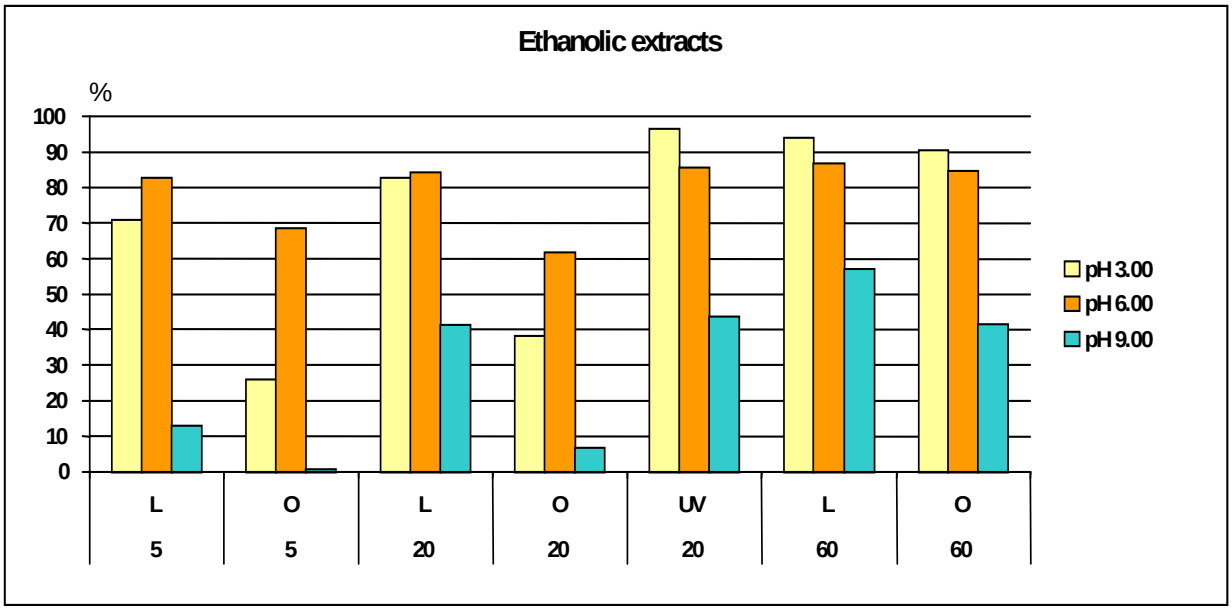
Generally, the ethanolic extracts were more stable than the watery ones, with the exception of alkaline extracts. These results were expected the same was as the extraction with alcohol to reduce the risks of contamination, which was of less favorable development for degrading reactions. Apart from the conferred stability by ethanol, in the most unfavorable conditions of temperature the losses are superior at 90%.

The pH effect on the stability of the extracts

In general, a better stability was achieved on the extracts when these were adjusted to alkaline pHs observing sudden decreases of the color intensity on a lot of extracts adjusted to acid pH. Jarred with the effect of ethanol as a debated protector, was observed that the acid ethanolic extracts were more stable that those watery homologous. Besides refrigeration, storage of extracts at ambience temperature, suffered a very small loss of color than those homologous watery ones (Figure 3).

The temperature effect on the stability of the extracts

The results proved reasonable, excepting of the watery extract stored by refrigeration found lower than expected as compared to pH acids and the presence of light (Figure 3). In general, the loss of color took place slower in the refrigerated extracts, as compared to its being quicker and more sudden when extracts were stored at 60ºC. The notes indicate that the isolated pigments are thermo-degraded which could mean a certain limitation in its technological applications. The stability of the extracts at lower temperatures is adequate for its use in manipulated products, conserves and consumed cold.



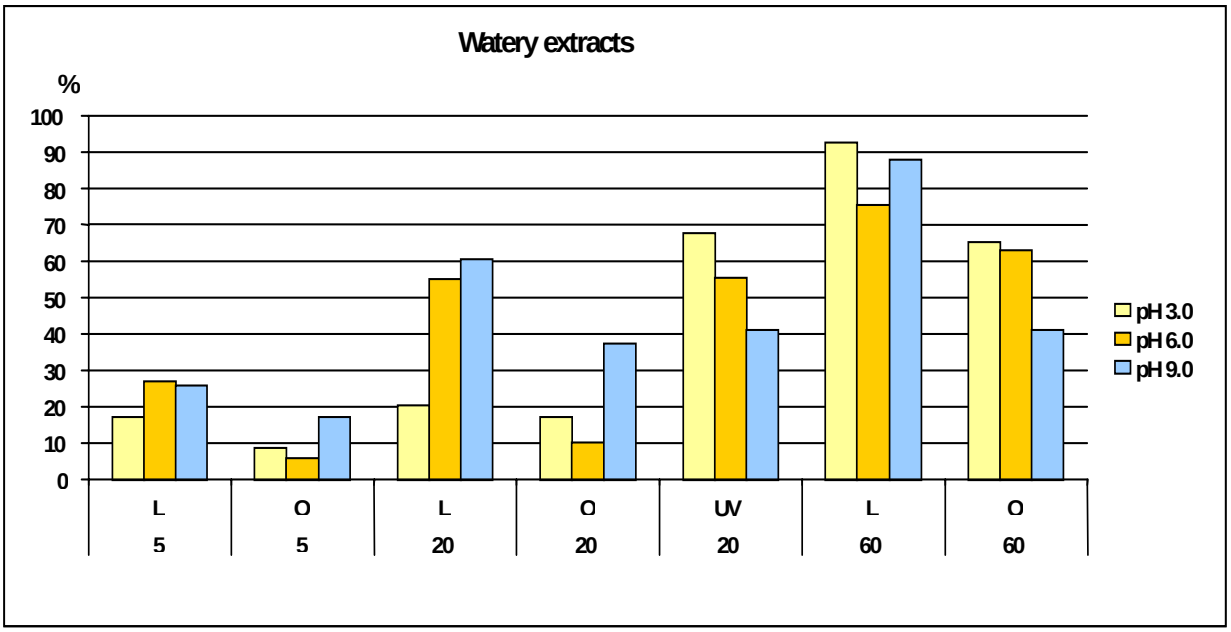
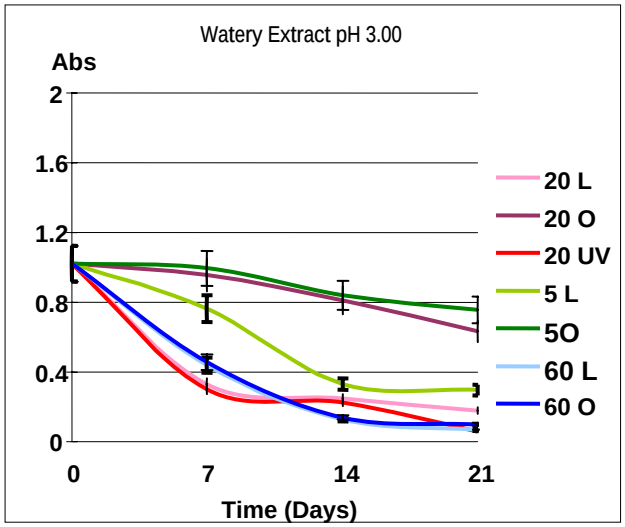


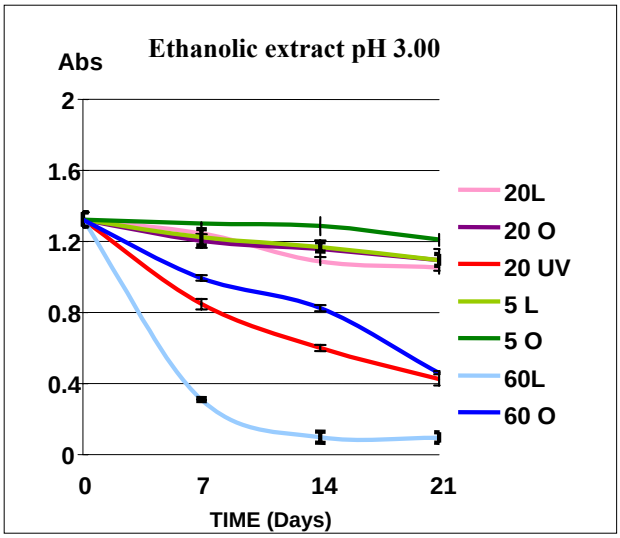
Figure 3. *Color losses of watery and ethanolic extracts after 21 days of storage, expressed like % absolute absorbance losses at 430 nm. L -solar light, O-darkness, UV- UV light, the practiced temperatures 5°C, 20°C and 60°C*

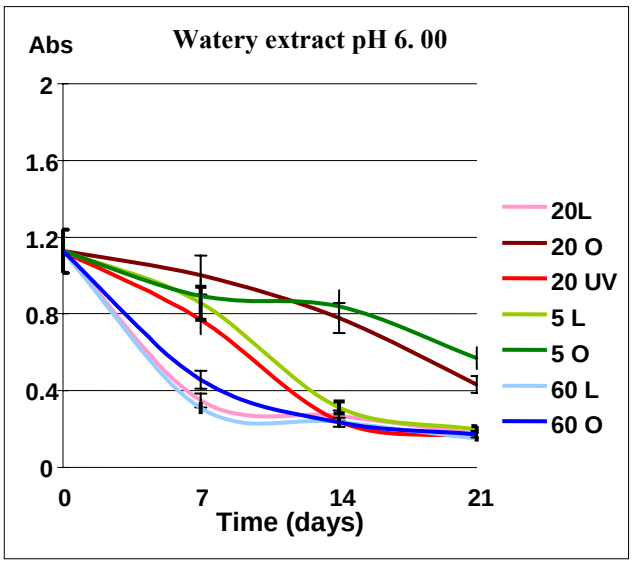
The light effect on the stability of the extracts

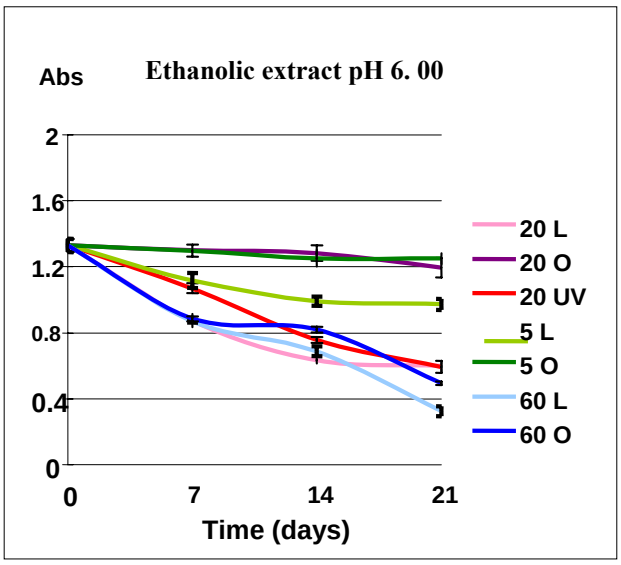
As it was expected, the extracts stored in darkness displayed higher stability than the ones stored at light, which phenomena is commonly associated with photosensitivity of most of the natural pigments. Once again, the watery extracts were found less stable or more sensitive to light than alcoholics ones. The extracts were not very stable to the continuous exposure to UV light, although in most cases the stability was similar to that shown of solar light (Figure 4).

To sum up, the highest percentage of color loss was registered at highest temperatures, in the presence of light and pHs acids. The rates of losses went up to 90%. Nevertheless, losses were minimum reaching values of 5 to 17 %, under most favorable conditions (5ºC and darkness) ant refrigeration temperatures, in the absence of light. It may be concluded that the strain *Epicoccum nigrum* MIUG 2.15 produces a mixture of pigments of average stability, although it depends on the conditions of extraction and of storage, something that must be clearly taken into account if one wants to apply the research to the food industry









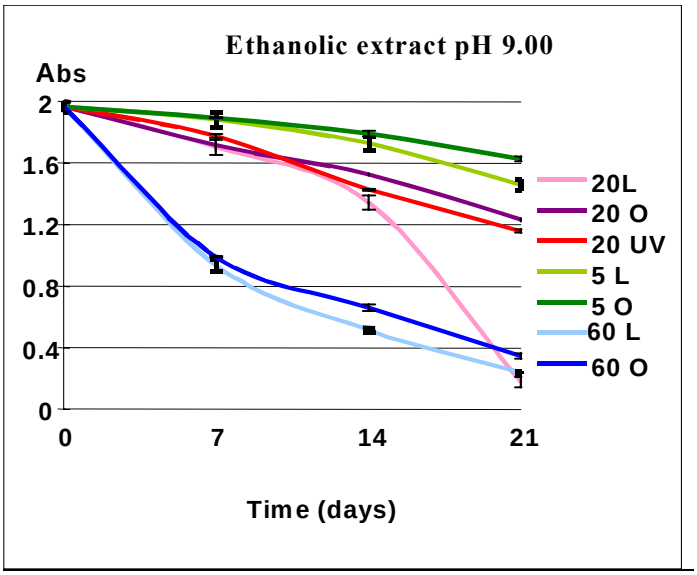


Figure 4. *Extract's absorbance evolution at 430 nm for the studied conditions*
L -solar light, O-darkness, UV- UV light, the practiced temperatures 5°C, 20°C and 60°C

Conclusions

The selected strain *Epicoccum nigrum* MIUG 2.15 represents a certain possibility to become a new fountain of natural food colorant, which besides its coloring ability displays technological properties of additional interest. However, for this to happen the following things must occur:

- A better characterization of synthesized pigments of the fungi;
- Check its harmlessness
- See how the food matrix behave

For a successful extraction it is recommended that it be possible to precede the same in an alcoholic means and avoid the too acidly pHs.

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