Evaluation of physical and chemical characteristics of yellow colorant produced by *Epicoccum nigrum* MIUG 2.15 in crude extracts and emulsions

ROMICĂ CREŢU **, GABRIELA BAHRIM*, DIMA STEFAN **, MIHAELA OLTEANU ***

** Dunarea de Jos University of Galaţi, Faculty of Food Science, 111 Domneasca Street, 800201Galati, Romania, e-mail:*

*** Dunarea de Jos University of Galati, Faculty of Science, 111 Domneasca Street, 800201Galati, Romania, e-mail:*

***University of Bucharest, Physical Chemistry Department, Bd. Regina Elisabeta, Romania

Abstract

The fungal colorants produced by an Epiccocum nigrum selected strain showed a great potential as an economical source of natural pigments reason of their edible characters and easiness for pigment biosynthesis, coupled with the attractive luminosity, chromo and hue of the pigments. The fungus Epiccocum nigrum was isolated from air and the cultivation of moulds for pigment biosynthesis was performed in solid state fermentation system, in darkness conditions.

The fungal colorants were characterized through UV-visible spectroscopy; HPLC and colour attribute analysis (Tristimulus Colorimetry). This complex of pigments is rich in carotenoids and flavonoids. The carotenoids content was of 948.48 mg/100g powder and the flavonoids content was of 52 mg/100g powder. As a result of the same process were also some compounds known as poliphenols (the anthocyanins type). The colour characteristics (CIE L^*, a^*, b^*, C^*_{ab}, h_{ab}) of the pigments in extract solution were $L^* = 93.745$, $a^* = -20.541$, $b^* = 91.991$, $C^*_{ab} = 94.256$ and $h_{ab} = 102.59$.

The oil-in-water emulsions with fungal colorant were instrumentally evaluated during storage at room temperature through UV-visible spectroscopy, microscopically observation, conductometric and turbidity measurements. The emulsions with these colorants are milkiness. The experimental results of colour differences and its components in the case of coloured emulsions (at pH = 7) were: $\Delta L^* = 26.67$, $\Delta a^* = 0.33$, $\Delta b^* = -8.04$, $\Delta C^*_{ab} = -7.99$, $\Delta h_{ab} = -4.9$, $\Delta H^*_{ab} = -0.87$ and $\Delta E^*_{ab} = 27.86$.

From the results presented in this paper, it should be noted that the colour shade of emulsions containing fungal colorants was stable during storage under normal light for 35 days, the colour of emulsions remaining almost unchanged.

Keywords: immobilization, food emulsions, tristimulus coordinates, *Epicoccum nigrum*, fungal colorants

Introduction

The fungus *Epicoccum nigrum* could be a good source of yellow colorants for the food products such as some emulsions. *Epicoccum nigrum is* commonly isolated from air, soil, a large variety of plants, human skin, insects, foodstuff, and textiles. The *Epicoccum* genus contains one species. It considered saprophytic and in some cases can appear as an opportunist, being a secondary invader of plants. *Epicoccum nigrum* grows rapidly and produces woolly to cottony colonies on potato dextrose agar at 25 °C. The colonies are sometimes yellow or yellow to orange. The fungus *Epicoccum nigrum* has been proved to produce polyphenolic pigments, along with carotenoids to a lesser extent. The fungus produces pigments which turn the colour of the medium to orange yellow. These pigments are

diffusible in the continuous or immiscible liquid phase of the some food products such as emulsions. Spore formation may not always occur; the stress may be induced it [1, 2]. Emulsions play a key role in the food industry. From dairy products, such as whipped cream and cheese, to baked goods and condiments, emulsions help to form the matrix on which these products are constructed. Some typical food emulsions are mild cream, ice cream, butter, margarine, salad dressing, and meat emulsions [3]. In practical emulsions the droplet size is about micro- or submicro-meter. As it is well known, several processes can realize the emulsions instability: Ostwald ripening (transfer of material from small droplets to larger ones), sedimentation, aggregation, coalescence and partial coalescence. The partial coalescence may be a first step of true coalescence.

The effects of the numerous factors and were studied process variables. Usually, an overall effect of various factors was followed and their influence on dispersion and characteristics of emulsions was concluded. In systematic investigations of a particular oil/emulsifier/colorant/water system as many various factors as possible should be taken into account [4, 5]

Colour is one of the main organoleptic characteristics used to establish the quality and acceptability of food products and food emulsions [6].

In the naturally dyes category for adjustment and/or correcting decolouration of food products based on emulsions the red and yellow colorants are admitted. The necessity of these operations derives from technological and sensory reasons.

Consequently, in this emulsions can be immobilized natural and/or synthetic colorants using the emulsification method. The consumers concern over the use of synthetic additives in food products had as a result a growing interest for new sources of natural pigments in the food industry, over the last years. However, some factors such as pH, temperature, light or oxygen can limit the usage food colorants. In the case of stockpiling and transport of emulsified food products the environment factors, such as pH, can alter the product without immediate noticeable change in the food emulsion aspect [7-9].

In our previous papers the usage of natural colorants as potential antioxidants in lipid peroxidation were studied also [10].

In this study the physical-chemical characteristics of some yellow colorant biosynthesised by *Epicoccum nigrum* in crude extracts and the model systems of oil-in-water and water-in-oil emulsions with this new fungal colorant were investigated. The influence of emulsifier type and concentration, the effect of oil viscosity and concentration, the agitation time and intensity also were investigated. According to these objectives the stability of emulsion types in terms of the emulsifier nature and the emulsifier and colorant concentration, the pH of the solution, the presence and contribution of some hydrocolloids (Arabic gum), as well as the colour evaluation were analysed.

Material and Methods

Reagents and apparatus

Fungal colorant (F.C.) synthesised by *Epicoccum nigrum* MIUG 2.15 selected strain of Industrial Microbiology Laboratory Collection (acronym MIUG) rich in carotenoid and flavonoid pigments was obtained after solid state fermentation and pigments extraction in 96% ethanol (0,05 g powder/10 mL ethanol) using a stable powder achieved after fermentative dried and milled fermentative media.

All reagents were analytical grade or pure. The oil used as lypophyl phase of the emulsions was sunflower oil (Prutul S.A., Galati, Romania). The emulsifiers Polyoxyethylene

(20) Sorbitan monostearate (Tween 60), Sorbitan monooleate (Span 80) and the hydrocolloid (arabic gum) were produced at PROLABO, France.

The water for the experiments was deionised. The citric acid, $C_6H_8O_7 \cdot H_2O$, 0.1 M and sodium acid phosphate, $Na_2HPO_4 \cdot 2\ H_2O$, 0.2 M (from Sigma–Aldrich Chemicals Co) used as tampon solution (pH = 2.2 – 8.0) were used for adjustment of pH of the emulsions.

An UV-Visible spectrophotometer (DOUBLE BEAM PC 8 SCANNING AUTO CELL UVD-3200 LABOMED, INC) was used to perform the absorbance and transmittance measurements. For that purpose, quartz cells with a path length of 10 mm were used. Digital pH Meter equipped with a combined glass-calomel electrode and 1 mm cells were also used for the experimentation (the pH values were measured at room temperature).

Emulsions were observed by using optic microscope and microscope images were captured with a video camera (VP-L907, Samsung) and transferred for analysis to the USB port of a Pentium IV, 1200 MHz computer. The type of the emulsion (oil-in-water or water-in-oil) was identified by means of electrical conductivity measurements (Digital Conductivity Meter) and the drop size by optical microscopy (Zeiss Microscope).

All vessels and glassware were cleaned and consecutive abundant rinsed with deionised water.

Pigment extracts characterisation through HPLC chromatography

The analyses were performed by an Agilent 1100 HPLC system consisting of a binary pump, an autosampler, and a temperature controlled column oven.

A separation system in gradient was applied, using two solvents: solvent A: acetonitrile: methanol, 9: 1 with TEA buffer and solvent B: ethyl acetate 100% with TEA buffer; following the eluent program: minimum 0-16 min, solvent B from 0 to 60 %; minimum 16-40 min, solvent B = 60%; minimum 40-42 min, solvent B from 60 to 0 %.

Emulsion preparation

The O/W and W/O emulsions were prepared at 20 ± 2 ^{0}C with Span 80 solution (for the W/O emulsions) or Tween 60 solution (for the O/W emulsions) using a high-speed blender (Ultraturrax homogenizer: T-309, France) at 2000 rpm. The soluble surfactant (Tween 60) was initially dissolved into the aqueous phase, while the oil soluble surfactant (Span 80) was initially dissolved in the sunflower oil. The F.C. was dissolved in ethanol (96%). The water and oil phase were put in contact for pre-equilibration before preparing the emulsion.

Emulsions containing surfactants, natural colorants and arabic gum solutions at varied volume ratios (Table 2) were prepared by mixing the pre-equilibrated phases [11]. The emulsification time was of 15 minutes. Some of the emulsions with SDS in water as aqueous phase were also prepared in the same way. In all emulsions, the volume fraction (φ) of oil was between 0.25 and 0.5.

Colour determination

The colour was studied by a "Tristimulus Colorimetry" method. To obtain the tristimulus values, recommendations made by the Commission Internationale de l'Eclairage: CIE 1964 (x, y) system (CIEXYZ), CIE 1976 ($L^*a^*b^*$) space (CIELAB), CIE 1986 and CIE 1995 were applied, using as references the CIE Standard Illuminant type D65/10^o visualizing field and bidistilled water as a reference solvent. From transmittances values is possible to calculate the CIE tristimulus values (X, Y, Z). The $L^*a^*b^*$ system, recommended by the CIE in 1976, expresses colour in a three - dimensional space. The CIE $L^*a^*b^*$ system for colour specification was applied following the most recent CIE recommendations [12 - 15].

Mathematical transformations the CIE tristimulus values allow the three coordinates to be transformed to describe the F.C. and emulsions colour. Such coordinates relate to lightness (L^*) and the two others relate to chromaticity (a^*, b^* in the CIE $L^*a^*b^*$ System).

Results and Discussions

***Epicoccum nigrum* MIUG 2.15 pigments in crude extracts characterization**

After pigments extraction, the extracts were clarified by centrifugation (15 min/6000 rpm), diluted 1/10 (w/v) with ethanol 96% and than spectrophotometric analyzed.

The spectrum of these F.C. was recorded between λ=280 nm and λ=780 nm, and at pH 7.00 a maximum absorbance was obtained around 429 nm. Figure 1 shows the visible spectrum of F.C. The F.C. chemical analysis shows a carotenoids content of 948,48 mg/100g powder (by using UV/Vis method) and the flavonoids content was of 52 mg/100g powder (by using Folin-Ciocâlteu method).

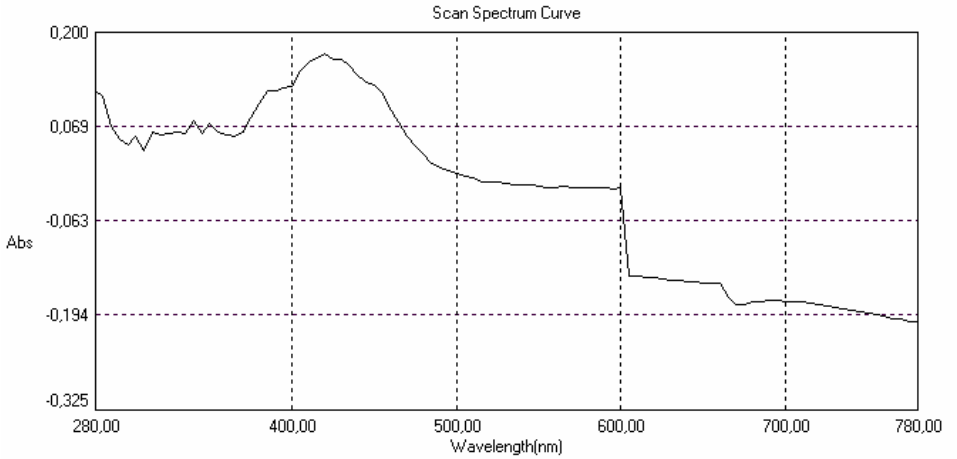

Figure 1. UV/Visible spectra of the F.C. extract samples from fungal source

The HPLC analysis of F.C. shows compositional details of the pigments (Table 1). Figure 2 shows signals on the 1 – 30 min range (at 190 - 498 nm).

Table 1. The HPLC parameters of F.C. produced by *Epicoccum nigrum* MIUG 2.15

tr	λ max	Semnal attributions
2.05	240, 430	Flavonoids and carotenoids
3.45	240-260, 430	Flavonoids and carotenoids
5.00	240,	Flavonoids and phenolic acids
12.8	240	Flavonoids and phenolic acids
14.4	280	Phenolic acids
29.5	500-550	Poliphenols (anthocyanins type)

(extract in 96% ethanol)

Through HPLC analysis are visible two signals groups: specific signals of the polar groups (flavonoids and rhodoxantine) and free forms specific signals of the phenolic acids (tr = 13-15 min.).

Influence of emulsifier nature and F.C. colorant concentration on emulsions formation and stability

The stability of the emulsions with F.C. prepared using the technique discussed above was investigated. The fungal colorant is a novel natural colorant applied for the first time to prepare food emulsions in the experiment described in this paper. The influence of some factors on the stability of F.C. and food coloured emulsions were studied during storage.

Using SDS as emulsifier reduces interfacial tension faster than Tween 60 at the same concentration, because SDS at the interface is much faster adsorbed [16].

Some type of emulsions thus prepared and the reagent used are given in Table 2. The type of these emulsions was confirmed by means of electrical conductivity measurements. As can be observed in Table 2, the conductance value for the emulsion III is higher than that for emulsions IV. Arabic gum (2%) was added in the aqueous phase to improve the viscosity and stability of emulsions. The somewhat creaming observed in this emulsion can be attributed to droplet aggregation.

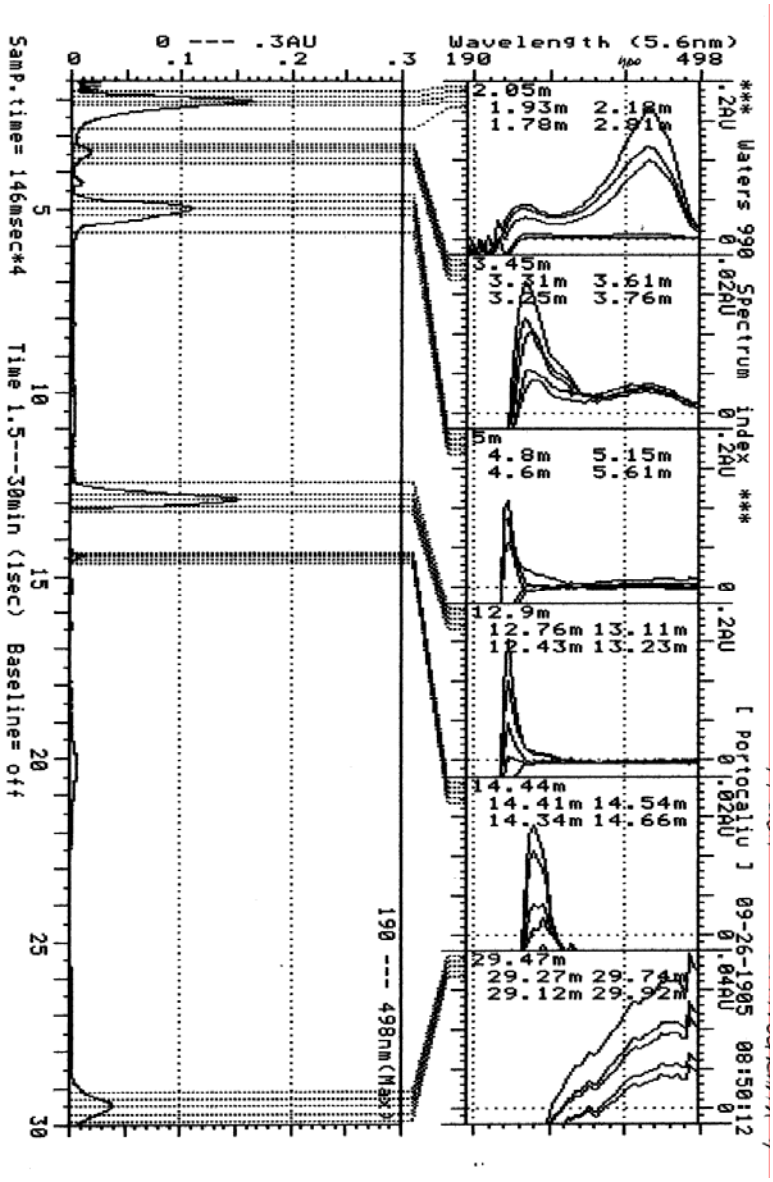

Figure 2. HPLC chromatogram of yellow colorant from *Epicoccum nigrum* MIUG 2.15

The emulsions indicated in table 2 are of a milky appearance because the average droplets size is large (results are shown). However, the turbidity and droplets size distribution in diluted emulsions are not changed after 1 hour.

Particle size of all emulsions was measured after samples were stored overnight and after that, 7 days at room temperature. The most frequent occurring droplet diameters were constant during emulsification. These are influenced by the emulsifier concentration. The results obtained are in general agreement with the data already known. As a representative example the percentage distribution vs. droplet size for the emulsion I is reported in Figure 3.

Table 2. The type of some emulsions, operating conditions and characteristics

The type of emulsions	Ingredients	Composition, %	Conductance, μS	Operating conditions	
				T*, ^{0}C	A**, rpm
Emulsion I W/O	Sunflower oil	2.5	35	20 ± 2	2000
	Span 80, 1% sol. (w/v)	5			
	Distilled water	85			
	Fungal colorant (v/v)	2.5			
	Arabic gum, 2% sol. (w/v)	5			
Emulsion II W/O	Sunflower oil	2.5	25		
	Span 80, 1% sol. (w/v)	5			
	Distilled water	85.5			
	Fungal colorant (v/v)	2			
	Arabic gum, 2% sol. (w/v)	5			
Emulsion III O/W	Sunflower oil	5	60		
	Fungal colorant (v/v)	1.5			
	Tween 60, 1% sol. (w/v)	10			
	Arabic gum, 2% sol. (w/v)	5			
	Distilled water	78.5			
Emulsion IV O/W	Sunflower oil	5	55		
	Fungal colorant (v/v)	1.5			
	Tween 60, 1% sol. (w/v)	10			
	Arabic gum, 2% sol. (w/v)	4			
	Distilled water	79.5			

*Temperature; **Agitation

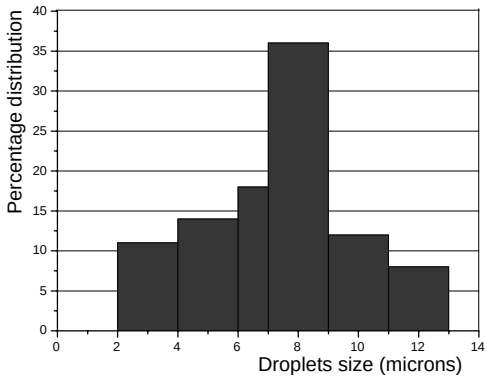

Figure 3. Droplets size distribution in the cases of the emulsion I

Figure 4 shows typical microscope images of emulsion with F.C. formed in the conditions above discussed. As may be observed in figure 4, the immobilization of colorants in emulsions was accomplished.

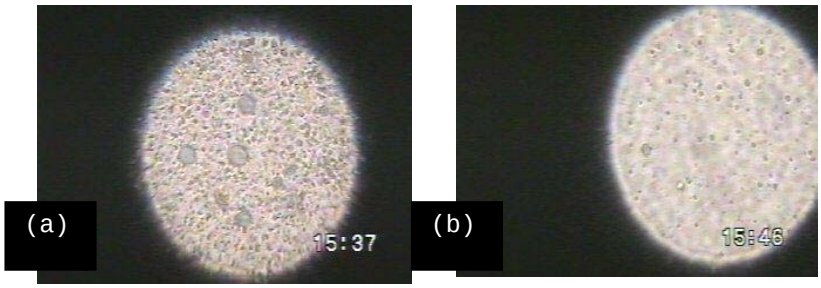

Figure 4. Images of W/O emulsions with food colorants:
a) emulsion I; b) emulsion I – diluted

In practice, the prepared emulsions were diluted 1:100. Then, the transmittance of the diluted emulsion was measured at room temperature with the UV - VIS spectrophotometer (Table 3).

Table 3. The characteristics of emulsion - food colorant systems

Sample	Characteristics	Storage time, days				
		1	3	7	14	35
I	λ_{max}, nm			630		
	Absorbance	0.477	0.450	0.416	0.419	0.309
	Transmittance %	33.3	35.3	38	38.2	49.1
	Turbidity index*, cm^{-1}	1.1	1.04	0.97	0.96	0.71
II	λ_{max}, nm			480		
	Absorbance	0.251	0.215	0.234	0.249	0.206
	Transmittance %	55.8	60.8	58.2	56.3	62.1
	Turbidity index*, cm^{-1}	0.58	0.50	0.54	0.57	0.48
III	λ_{max}, nm			648		
	Absorbance	1.339	0.481	0.371	0.384	0.946
	Transmittance %	4.5	33	42.2	41.2	11.2
	Turbidity index*, cm^{-1}	3.10	1.11	0.86	0.88	2.19
IV	λ_{max}, nm			639		
	Absorbance	0.273	0.204	0.183	0.207	0.291
	Transmittance %	53.2	62.5	65.5	61.9	51
	Turbidity index*, cm^{-1}	0.63	0.47	0.42	0.48	0.67

* $\tau = -\ln\left(\frac{\%T}{100}\right)/L,\ (cm^{-1})$, were τ and T are the turbidity index and the transmittance respectively, and L is the path length

The table 3 displays a good agreement between the results, concerning the studied systems stability and obtained by these methods. In the same time, nevertheless, a soft decrease of the emulsions stability takes place after 3 days (or 7 day for sample I) from preparation. The destabilization which followed the first day can be explained by what is called depletion flocculation.

Influence of pH on emulsion - colorant systems stability

The pH values variation in the time for the coloured emulsions stored in normal light at room temperature were measured and the data are presented in figure 5.

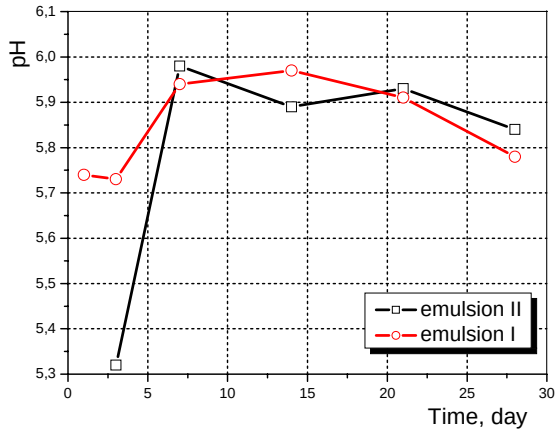

Figure 5. The variation of pH emulsions during storage at room temperature in the cases of the W/O emulsions with F.C.

From this figure should be noted that the pH value of emulsion II ranged between pH=5.32 and pH=5.98, while in the case of emulsion I was between pH=5.73 and pH=5.97. From FC, the pH values were somehow higher.

Colour evaluation

The table 4 and 5 show the results obtained for objective colour evaluation carried out in CIE systems (for F.C. and the most stable emulsion with these colorant).

The L^*, a^*, and b^* coordinate axis define the three dimensional CIE colour space. Thus, if the L^*, a^*, and b^* coordinates are known, then the colour is not only described, but also located in space. The colour CIELAB coordinates L^*, a^* and b^* for the most stable emulsion are given in Table 5. The graphic representation (results not shown) of this colour space with Illumining D, was made from the corresponding L^*, a^* and b^* values, which are given in the mentioned table.

Table 4. The experimental results of samples obtained according to CIE 1964 (x, y) coloured system (CIEXYZ)

Sample	Tristimulus values			Chromaticity coordinates			Dominant wavelength λ_d, nm	Excitation purity p_e, %
	X	Y	Z	x	y	z		
CF	72,639	84,681	13,556	0,425	0,496	0,079	571,5	0,792
Emulsion I	71,687	73,454	77,404	0,322	0,330	0,348	561	6,84

Table 5. CIELAB coordinates of C.F. and the coloured emulsion I

Sample	Chromatic coordinates			a^*/b^*	$(a^*/b^*)^2$	C^*_{ab}	h_{ab}
	L^*	a^*	b^*				
CF	93,745	-20,541	91,991	-0,223	0,050	94,256	102,59
Emulsion I	95,5501	0,8239	6,9161	0,11913	0,01419	6,96499	83,206

In the table 4 and table 5 colour coordinates L^* represents the lightness and a^* and b^* indicate the change in hue from red to green and from yellow to blue. Also, C^*_{ab} represents chroma, a correlate for saturation and h_{ab} is the hue angle, a useful quantity in specifying hue numerically.

Colour difference in the cases of the F.C. and/or emulsion I (ΔE^*_{ab}) is calculated using coordinate geometry as the length of the line joining the positions of the two colours (Table 6)

Table. 6. The results for the CIELAB colour difference (D65/10^0)

pH	ΔL^*	Δa^*	Δb^*	ΔC^*_{ab}	Δh_{ab}	ΔH^*_{ab}	ΔE^*_{ab}
5.32*	1,8051	21,3649	-85,0749	-87,291	-19,384	-8,630	87,735
4 - 7	26,67355	0,330873	-8,03713	-7,996	-4,906	-0,874	27,860

* ***the values were calculated for initial determinations of the chromatic coordinates between F.C. and emulsion***

Most of the greatest total differences (ΔE^*) were measured between fungal colorant solution and coloured emulsion I at pH 5.32. In the case of emulsion I, increasing the pH further to 7.00 had as a result the reduced yellowing effects ($\Delta b^* = -8{,}037$ and $\Delta E^* = 27{,}86$).

Conclusions

Acacia gums, natural products have emulsifying and stabilizing properties because the glycoproteins and the arabinogalactan-proteins fractions are fixed at the interface oil/water; glucuronic acids develop negative charges around each oil droplets and create electric repulsion forces, cancelling the Vand der Waals attraction forces. Added to electric repulsion there is also the steric repulsion due to the flexibility of gum molecule adsorbed to the surface of the globule.

From these results, during the storage at room temperature under normal electric light, for approximate 35 days, it can be concluded that the surfactant emulsion – food colorant systems were stable. This experiment has shown that some colorants from microbial origins can be used to prepare oil-in-water and water-in-oil emulsions using emulsification/external gelation method. The immobilization of such colorants in food emulsions by this technique is a good method. The food emulsions prepared with arabic gum were more stable than other emulsions. The results suggest that the colour evolution for fungal colorant followed approximately the same variation as in the cases of the emulsions. The use of fungal colorant as potential food colorants in slightly acid products should therefore be considered, at least in products with limited storage time which are kept at room temperature.

We therefore recommend that future studies should identify more efficient techniques of immobilization and stability of food colorants in emulsions. Colours can also be described and located in CIELAB colour space using an alternate method, that of specifying their L^*, C^*, and h^* coordinates. The coordinates L^*, a^* and b^* showed a good relationship between the colour of samples.

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