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Survey and monitoring of the level of artificial dye Brown HT in food products and review of regulations and legal requirements for its use

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Abstract

Despite the many advantages that synthetic colors have over natural colors, their use is generally subject to restrictions from regulatory agencies. This research presents a simple method for identifying and quantifying the artificial color Brown HT in brown-colored food products using high performance liquid chromatography (HPLC). Furthermore, the technical regulations for the use of this color in the world are reviewed and discussed. Initially, separation and detection parameters were investigated and optimized. The method was validated under these optimized conditions. The linear range was determined to be 1.0 to 50 mg/L. The repeatability of the method within a single day and over several days was found to be 3.6% and 5.3%, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were determined to be 0.3 and 1 mg/L, respectively. After validating the method, 40 brown-colored food samples, including chocolate milk, cake, ice cream, chocolate desserts, etc., were prepared, and their color content was analyzed. Approximately 80% of the samples did not contain the artificial color. In some ice cream and cake samples, the color was observed in the range of not detected (ND) to 34.6 mg/kg. The percentage recovery in the analysis of real samples was within the satisfactory range of 95% - 99%. Regarding the current regulatory status of Brown HT, it is noted that, based on the 2010 European Food Safety Authority (EFSA) re-evaluation, the acceptable daily intake (ADI) was established at 1.5 mg/kg body weight/day, derived from long-term carcinogenicity and toxicity studies in mice

Keywords Brown HT, Synthetic dyes, Food samples, Regulations, High performance liquid chromatography.

1. Introduction

Brown HT (Fig. 1) is the disodium salt of 4,4'-[(2,4-dihydroxy-5-hydroxymethyl)-m-phenylene] bis (azo)] di-1-naphthalene-sulphonic acid. Brown HT, also called Chocolate Brown HT, Food Brown 3, and C.I. 20285, is a brown synthetic coal tar diazo dye. There are at least 10

synonyms for this compound. The most commonly used synonyms in published literature are Brown HT, Chocolate Brown HT and CI Food Brown 3 [1]. It presents very good stability in light and heat [2]. It is permitted for use in the UK under the Coloring Matter in Food Regulations and its review by the EEC authorities is pending. The



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principal uses of Chocolate Brown HT are in flour and sugar confectionery, canned meat, ice cream, soft drinks, puddles and sauces [3] (Table 1).

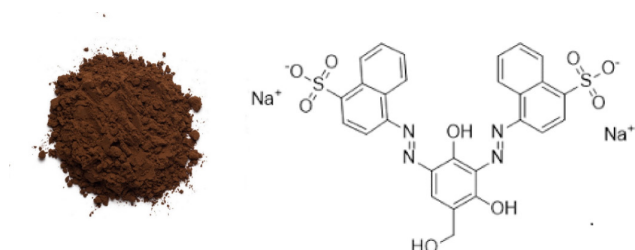


Fig 1. Appearance and chemical structure of Brown HT

Its E number is E155. It is used to substitute cocoa or caramel as a colorant. It is used mainly in chocolate cakes, but can also be found in desserts, cookies, candy, cheeses, teas, yogurts, jams, chocolate drinks, ice creams, fruit products, fish, wafers, breakfast cereals, and other products [4,5]. It is approved for use by the European Union [6]. It is banned in Australia, Austria, Belgium, Denmark, France, Germany, Norway, Sweden, Switzerland, Russia and the United States [7]. Similarly, according to INSO 740, this color is not on the list of permitted colors used in the food industry and therefore its use is prohibited in Iran [8].

In Codex Alimentarius Standard No. 192, the use of this substance is permitted in some food products (Table 1). Table 1 lists the food categories in which the use of the colorant is allowed, along with the maximum permitted use levels.

Also, several studies have reported that brown HT is associated with many health problems, including hepatorenal abnormalities, reductions in organ and body weight (BW), and detrimental effects on the gastrointestinal (GI) tract [12-14]. For example, Tawabul Islam et al (2024) demonstrated that consumption of chocolate brown HT, leads to the development and expansion of mammary tumors, even when consumed at low doses for an extended period of time [14].

In this regard, Brown HT has been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and 1984 and the EU Scientific Committee for Food (SCF) in 1975 and 1984. JECFA established an Acceptable Daily Intake (ADI) of 0-1.5 mg/kg body weight (bw)/day, while the SCF established an ADI of 0-3 mg/kg bw/day [15].

In the Iranian market, artificial Brown HT food-grade color is commercially available and used for coloring food products, although its use is not permitted according to the national standard INSO 740. However, no study has so far been reported in Iran aimed at developing a

liquid chromatography-based method for the monitoring and quantification of this colorant. Therefore, the present study was designed to establish an LC-based analytical method for the detection and quantification of Brown HT in selected food products from the market, where the use of this color is expected to be more likely. In addition, the potential adverse effects of Brown HT and the relevant international legal and regulatory status of this colorant are also reviewed.

2. Material and methods

2.1. Instruments

Chromatographic analyses were performed using a KNAUER HPLC system (model Euro Chrom, Germany) equipped with a K-1001 pump, a UV-Vis detector, and a K-2600 degasser. The separations were done in a C-18 chromatography column (250 mm x 4.6 mm, 5 μ m). The injection volume was 20 μ L and the column temperature was ambient temperature. Also, the flow rate of the mobile phase was 1 mL/min. The detection wavelength was 490 nm. The pH of the solutions was adjusted with a pH meter, model WTW Inolab (made in Germany). An ultrasonic water bath of 40 kHz with a power of 0.138 kW (Techno-Gas, Italy) was used to extract brown HT from food samples and also to degas the mobile phase. Deionized water was prepared by a water purification device manufactured by Aqua Max-Ultra Youngling Company (Seoul, South Korea). A centrifuge manufactured by Poya Electric Company of Iran was used to centrifuge the samples.

2.2 Chemicals and standards

Brown HT dye powder with a purity of 93% was purchased from Abyaz Chemical Company (Alborz, Karaj). Acetonitrile, sodium hydroxide, lead acetate, potassium ferrocyanide three hydrate and ammonium acetate were purchased from Merck, Germany. The base standard solution (1000 mg/l) of Brown HT was prepared by weighing 54 mg of the dye and making it up to volume with 50 ml of deionized water. Working standard solutions were prepared by suitable dilution of the base solution with distilled water. The mobile phase for liquid chromatography was prepared by mixing 30 percent of acetonitrile and 70 percent of ammonium acetate buffer. For the clarification of food samples, Carrez reagents were utilized to precipitate proteins and clarify the solution. Carrez solution I was prepared by dissolving 15.0 g of potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6] \cdot 3H_2O$) in 100 mL of distilled water.

Carrez solution II was prepared by dissolving 30.0 g of zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$) in 100 mL of distilled water.

Table 1. Permitted food category and maximum level of Brown HT based on Codex standard 192 [9].

BROWN HT				
INS 155	Brown HT	Functional Class: Colour		
FoodCatNo	FoodCategory	MaxLevel	Notes	Year Adopted
01.1.4	Flavoured fluid milk drinks	150 mg/kg	52	2017
01.6.1	Unripened cheese	150 mg/kg	3, 201, XS221, XS262, XS273, XS275	2023
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	150 mg/kg		2023
02.4	Fat-based desserts excluding dairy-based dessert products of food category 01.7	150 mg/kg		2023
05.2	Confectionery including hard and soft candy, nougats, etc. other than food categories 05.1, 05.3 and 05.4	50 mg/kg		2019
05.3	Chewing gum	300 mg/kg		2019
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	50 mg/kg		2019
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	50 mg/kg		2024
12.4	Mustards	300 mg/kg		2024
12.5	Soups and broths	50 mg/kg		2024
12.6	Sauces and like products	500 mg/kg	577 & XS302	2024
12.6.2	Non-emulsified sauces (e.g. ketchup, cheese sauce, cream sauce, brown gravy)	50 mg/kg	576	2023
13.6	Food supplements	300 mg/kg	539	2021
14.2.2	Cider and perry	200 mg/kg		2021
14.2.4	Wines (other than grape)	200 mg/kg		2021
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low alcoholic refreshers)	200 mg/kg		2021
15.1	Snacks - potato, cereal, flour or starch based (from roots and tubers, pulses and legumes)	200 mg/kg		2024
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	100 mg/kg		2024

2.3. Sample preparation

40 food samples including chocolate cake (8 samples), cocoa milk (5 samples), chocolate dessert (5 samples), chocolate ice cream (8 samples), Nescafé ice cream (8 samples), and cocoa powder (6 samples) were prepared from industrial and commercial samples.

The samples were first homogenized to obtain a uniform mixture. In the case of fatty samples, defatting was performed using hexane. Subsequently, 10 g of the defatted sample was mixed with 90 mL of distilled water, followed by the addition of 2.5 mL of Carrez solution I and 2.5 mL of Carrez solution II. The volume was then adjusted to

100 mL with distilled water. The mixture was transferred into two 50 mL Falcon tubes and centrifuged. Finally, the clear supernatant was filtered through a syringe filter, and 20 µL of the filtrate was injected into the HPLC system.

3. Results and discussion

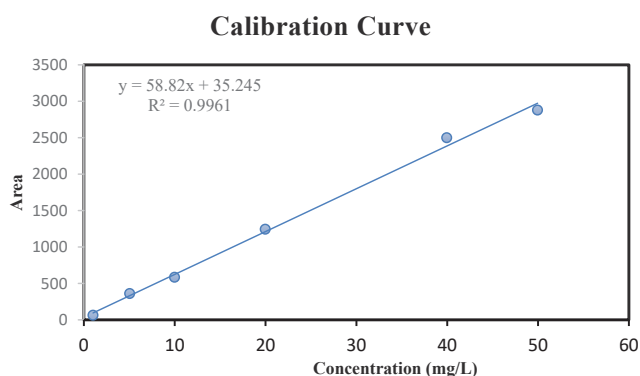
3.1. Validation of the test method

In Table 2, the validation parameters of the method under optimal conditions, including the limit of detection (LOD), limit of quantification (LOQ), percentage of relative standard deviation (RSD%), linear range and correlation coefficient of the calibration curve, are pre-

Table 2. Figures of merit of the proposed method

Repeatability percentage at 2.5 mg/L		LOQ (mg/L)	LOD (mg/L)	correlation coefficient	Linear range (mg/L)
Inter-day (n=6)	Intra-day (n=6)				
5.8	3.5	1.0	0.3	0.9961	1.0-50

sented. The proposed method was linear in the range of 1.0-50 mg/L and the correlation coefficient of the linearity method was $R^2 \geq 0.99$ (Figure 2). The relative standard deviation percentage of the method was obtained by performing six replicate analyses in one day and in several consecutive days using the standard solution of Brown HT with a concentration of 2.5 mg/L, 3.5 and 5.8, respectively. In addition, LOD and LOQ of the method were 0.3 and 1.0 mg/L, respectively.

**Fig 2.** Calibration curve of Brown HT

3.2 Analysis of real samples

In order to evaluate the efficiency of the proposed method, the collected samples were extracted and analyzed in triplicate. The obtained concentrations of Brown HT in different samples are given in Table 3. The concentrations of Brown HT obtained in different samples are presented in Table 3. Brown HT was not detected in some samples, while in the positive samples the concentrations ranged from 0.3 to 33.5 mg/kg. The accuracy of the method was further assessed by determining the recovery percentage in samples spiked at a concentration of 10 mg/L (Table 4). Figure 3 illustrates the chromatograms of the chocolate cake sample before and after spiking.

3.3 The last regulations about Brown HT dye in the world

The artificial color Brown HT (International Code E155) is an azo dye whose legal status varies worldwide due to differences in risk assessments. In the European Union, the European Food Safety Authority (EFSA) has authorized the use of this color in specific food categories such as bakery products, confectionery, chocolates, and dairy desserts. Similarly, Food Standards Australia New Zealand (FSANZ) has approved its use. Conversely, due to public health concerns, the United States Food and Drug Administration (FDA), as well as regulatory bodies in countries such as Canada, Japan, and some non-EU European nations, have strictly prohibited the use of this color in food formulations [16].

Based on EFSA's 2010 re-evaluation, an Acceptable Daily Intake (ADI) of 1.5 mg/kg body weight per day was established for Brown HT, derived from a long-term carcinogenicity and toxicity study in rats [15]. Exposure assessments indicated that average intake estimates at both the maximum permitted use levels (Category 2) and the maximum reported levels of use (Category 3) are generally below the ADI. However, for adults and children aged 1–10 years, upper percentile exposure estimates may exceed the ADI at the high end of the range. Consequently, countries where Brown HT is authorized are required to establish Maximum Permitted Levels (MPLs) in specific food categories to ensure that consumer exposure—particularly among children, who are more vulnerable due to lower body weight and higher snack consumption—remains within the established safety threshold.

Numerous toxicological studies have been conducted to evaluate the safety of this color, serving as the basis for regulatory decisions. In vivo and in vitro studies evaluated by EFSA have shown that Brown HT, at typical consumption levels, lacks genotoxicity and carcinogenic potential. However, long-term dietary studies on laboratory animals revealed that the administration of very high doses could lead to reduced growth rates, changes in body weight, and adverse effects on internal organs such as the kidneys and liver. These toxicological data assisted

Table 3. Results of Brown HT determination in the different samples

Sample type	Sample number (positive)	Detected range of brown HT (mg/Kg)
Chocolate cake	8 (4)	ND-33.5
Chocolate ice cream	8 (3)	ND-34.6
Nescafe ice cream	8 (2)	ND-32.5
Cocoa powder	6 (0)	ND
Cocoa milk	5 (0)	ND
Chocolate dessert	5 (0)	ND

ND: not detected

Table 4. Recovery results of Brown HT determination in the chocolate cake samples

Sample code	Brown HT added (mg/kg)	Brown HT found (mg/kg)	Relative recovery
1	-	10.0	-
	10	19.2	96.0
2	-	ND	-
	10	9.5	95.0
3	-	ND	-
	10	9.8	98.0
4	-	5.0	-
	10	14.5	96.7
5	-	ND	-
	10	9.9	99.0
6	-	21.0	-
	10	29.5	95.1
7	-	25.0	-
	10	33.5	95.7
8	-	ND	-
	10	9.6	96.0

ND: not detected

researchers in determining the No Observed Adverse Effect Level (NOAEL) and, by applying a standard safety factor, establishing the current ADI limits [10-14].

In addition to systemic toxicity assessments, a critical topic in clinical studies is the hypersensitivity reactions associated with azo dyes, including E155. Scientific evidence indicates that the consumption of this color can trigger allergic reactions, food intolerance, urticaria, and exacerbation of asthma symptoms in sensitive individ-

uals. This sensitivity is particularly prevalent in people who are intolerant to salicylates (such as aspirin). The existence of these scientific reports regarding sensitizing and allergenic effects is the primary reason for the precautionary approach and ban of this substance in countries like the US and Japan, as well as the strong emphasis on explicit labeling of this color's name and E-number on product packaging in countries where its consumption is permitted [10-14].

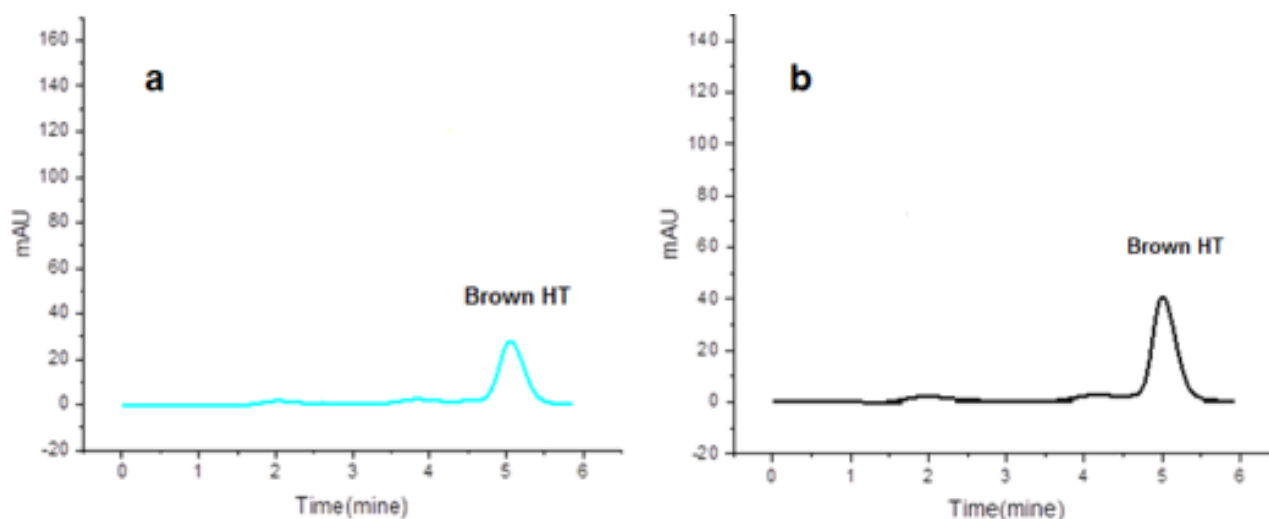


Fig 3. Chromatograms of chocolate cake sample (code 1) before (a) and after spiking with the control amount (10 mg/kg) of Brown HT (b).

4. Conclusion

In this study, the monitoring and determination of Brown HT in brown colored foods was studied using liquid chromatography. The developed method demonstrated high accuracy and precision under the optimized conditions, confirming its suitability for routine sample analysis. The concentration range obtained were in the range of 0.3-33.5 mg/kg in the samples. The color was observed in some samples of chocolate cake, chocolate ice cream and Nescafe ice cream. The results of this investigation and monitoring showed that Brown HT color was not used in any of the industrial samples with the standard mark, but it was observed in some samples prepared in the industry. This indicates the need for continuous monitoring and supervision of the industry because, according to National Standard No. 740 "Permitted Food Colors - Characteristics", its use in food materials and products is currently not permitted.

Given the widespread availability of Brown HT dye through online stores, as well as the fact that some samples tested positive for the dye, it is essential that wider sampling be conducted to accurately and comprehensively monitor this in candidate food products.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

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