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Synthetic colorants: Analysis in pediatric over the counter drugs OTCs by HPLC and exposure risk to children

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ABSTRACT: Synthetic colorants (SCs) are a class of pharmaceutical excipients, which could have harmful side effects especially in a vulnerable population like children. This study aimed to develop and validate a simple reverse phase high performance liquid chromatography (RP-HPLC) to quantitate eight SCs e.g. Tartrazine, Indigo carmine, Sunset yellow, Allura red AC, Carmosine, Ponceu 4R, Fast green FCF, and Brilliant blue FCF in pediatric over the counter medications (OTCs) and to assess the daily colorants intake through OTCs administration. After a solid phase extraction (SPE) using ChromabondHR-XAW cartridges, a gradient chromatographic elution was carried out using C18 column, and diode-array detector. Mobile phase composed of 80 mM ammonium acetate (pH 7.0) and methanol:acetonitrile 70:30 (v/v) solutions. Validation was performed according to ICH guidelines. The developed method was applied to quantify SCs in pediatric OTCs (syrups, suspensions, gummies, and chewable tablets) marketed in the Gaza Strip. Separation was completed within 18 minutes. The recovery rates of colorants were in the range of 72.01 - 117.15%. The developed method was linear with a correlation coefficient (R2 > 0.9989). Limits of detection (LOD) and limits of quantitation (LOQ) ranged between 0.007 - 0.12, and 0.02 - 0.36 (µg/mL), respectively. The method showed unique selectivity, accuracy, and precision. Application of the developed HPLC method to assay synthetic colorants in different OTCs was successful. Assessment of daily colorant intake showed that the patients were exposed to 45% of the acceptable daily intake (ADI) of sunset yellow through an OTC. The ratio of SCs should be reduced to a minimum in pediatric formulations.

KEYWORDS: Synthetic colorants; HPLC-DAD; validation; OTCs; ADI.

1. INTRODUCTION

Colorants are excipients utilized fundamentally by pharmaceutical industry to make the products more appealing to consumers. Synthetic colorants (SCs) are widely applied in comparison to natural due to high chemical stability under different conditions, great dye ability, and inexpensive production [1,2]. Its' application is strictly regulated, due to potential side effects like allergic and asthmatic reactions, carcinogenic, and mutagenic properties, and worsening symptoms in ADHD children [3,4]. Accordingly, acceptable daily intake (ADI) values of colorants (Table 1) were established [5]. Properties of food, drug, and cosmetic (FD&C) colorants and lakes as excipients are mentioned in pharmacopeia but there is no official assay [6]. Unlike food products, the literature research demonstrates insufficient analysis of SCs in pharmaceuticals [7-9]. Prior to analysis, various extraction and clean up steps were performed e.g. solid phase extraction (SPE) [10], QuEChERS [11], cloud point extraction [12], and membrane filtration [13].

For quantitation many techniques were applied e.g. thin layer chromatography TLC [7], UV- visible spectrophotometry [13], derivative spectrometry [14,15], densitometry [16], capillary electrophoresis [17], reversed phase high performance liquid chromatography (RP-HPLC) [8,18], ion pair liquid chromatography (IP-LC) [19], voltammetry [20] and differential pulse polarography [21].

SPE technique of various sorbents were applied to extract colorants like Strata X-WA[22], aminopropyl-modified silica [10], Oasis WAX [23], Oasis HLB [24], polyamide resin [25], amberlite XAD [26,27]. Mixed mode ion exchange polymeric sorbents were utilized to purify organic ionic compounds selectively [28,29].

OTCs could be a source of SCs to which children are exposed. Two surveys were published about the exposure risk among children due to ingestion of colorants through medications[9,30]. The aim of study was to develop and validate a method based on SPE and RP-HPLC to analyze SCs simultaneously in pediatric

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OTCs. The validated method was then applied to determine SCs in real samples marketed in the Gaza Strip, and the daily intake of SCs by children through OTCs administration was assessed.

2. RESULTS AND DISCUSSION

2.1. Synthetic colorants in OTC samples

Synthetic colorants in studied samples were identified in TLC [7]. Identification was confirmed by comparing retention time of samples with standards. Description of samples and distribution of colorants are given in Table 2. Synthetic colorants were declared in 38% of marketed OTCs, which were imported brands. It is well established that, excipients may lead to severe side effect in some circumstances. Regulation of pharmaceuticals' labeling should be updated in Palestine according to international standards[31].

Colorants in pharmaceuticals are not a pre-requisite for syrups. They are not recommended in anti-histaminic formulations. Most pediatric allergy syrups are dye free. Carmosine and ponceu 4R are not classified as FD&C or even as drug and cosmetic (D&C) [31]. Formulations contained further colorants of D&C category -declared in some OTCs – were not assessed in this paper.

2.2 Sample preparation

Sample preparation by dilution and pH adjustment was satisfactory. Suspensions required further heating and shaking to liberate colorants. Purification of colorants from matrices is a crucial step for analysis. Mixed mode anion exchange cartridge are applied primarily to purify weak acids (pka< 5) and strong acids like sulfonic acid (pka < 1) from aqueous matrices. Synthetic colorants have at least two sulfonate groups and were purified by such sorbent from foodstuffs and beverages [22]. Syrup and oral suspension dosage forms contain active ingredient(s) along with a series of excipients like water, alcohol, co-solvent, sweetening agents, flavor, colorants, suspending agents, wetting agents, surfactants, buffering agents, and preservatives. In sample preparations, dilution process was required to reduce the viscosity of solutions. Gummies contain gelatin as main excipient, and other formulation binders like wax and agar. Destruction of this matrix through heating at 50 °C was satisfactory to liberate the water soluble colorants. In chewable tablets, colorant lakes of aluminum were present. Liberation of colorants required masking of aluminum by edetate.

2.3 SPE extraction

Pediatric samples in the study have components such as active ingredient (Ibuprofen), excipients (like benzoate, citrate, citric acid, edetate, synthetic colorants) and ions which can interact with mixed mode anion exchange by ionic bond at selected pH. Considering these interactions, manufacturer's recommended protocol was modified. Washing the cartridge with an acid in organic solvent (2% formic acid in methanol) was important in this case to elute weak acids from the sorbent and leaving strong acids bonded. Elution step depends on neutralizing the sorbent with ammonia (1-5% v/v) in organic solvent[22,23]. The optimum elution was with 5% ammonia in methanol. The efficacy of developed procedure was proved by comparing peaks of standards prepared in water, with the peaks of the standards prepared in blank samples (Figure).

2.4 Chromatographic system

HPLC-UV/VIS is mostly applied for analysis of colorants [32]. Separation of colorants performed at pH 7 and elution depends on hydrophobic interaction with stationary phase and presenceof ionizable groups. More polar colorants are eluted first followed by less polar. Azo dyes were eluted first followed by triarylmethane dyes. Fast green FCF has polar characteristic due to phenolic OH group and was eluted before brilliant blue FCF. Gradient elution and the composition of mobile phase was optimized to have good resolution and sharp peaks.Ammonium acetate as a chemical modifier has the advantage of improving the separation of ionizable compounds and pH 7 kept colorants neutral according to their pKa values [29]. The presence of acetonitrile in mobile enhances elution of colorants and the peaks become sharper.The resolution of peaks was good with no overlapping. Figure shows chromatograms of a standard mixture.

2.5 Method validation

Validity of the developed method was examined according to ICH guidelines [33].

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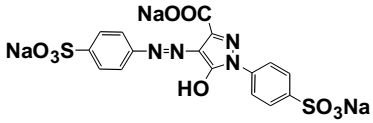
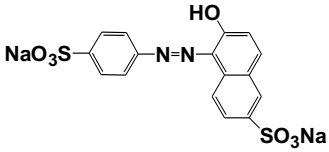
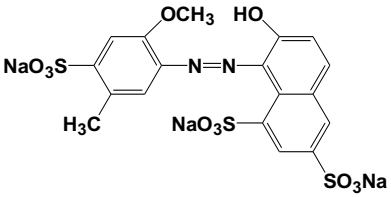
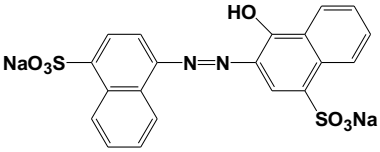
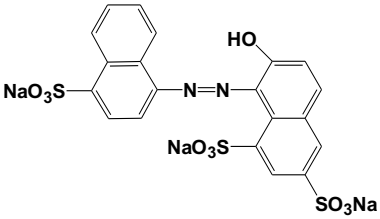
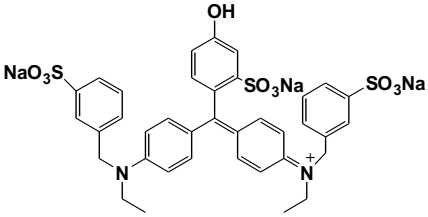
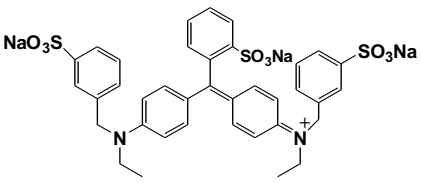
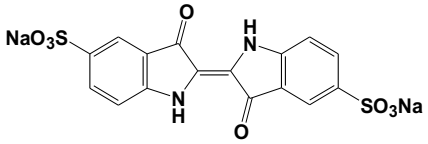
Table 1. Structures, physical properties, and ADI of studied synthetic colorants.

Colorant, E numbers[a]	Structure	Log P	pKa	ADI[b]
Tartrazine E 102		-10.17	9.4	0-7.5
Sunset yellow E 110		-1.18	10.36	0-4
Alura red AC E129		-0.35	12.3	0-7
Carmosine E 122		n.a[c]	n.a[c]	0-4
Ponceu 4R E124		n.a[c]	11.24	0-4
Fast green FCF E 143		-3.22	n.a[c]	0-25
Brilliant blue FCF E 133		-4.94	n.a[c]	0-6
Indigo carmine E132		n.a[c]	3.72	0-5

[a] European community number
[b] Acceptable Daily Intake ADI (mg/kg body weight/day) according to JECFA[5].
[c] Not available.

Table 2. Pediatric OTCs samples and its' colorants.

OTCs Type (Dosage forms)	No. samples Total (declared colorant)	Colorants
Cough/cold/ congestion (Syrup and oral suspension)	13 (2)	tartrazine, sunset yellow, carmosine , allura red AC, brilliant blue FCF, ponceu 4 R , fast green FCF
Allergy (Syrups)	2 (1)	allura red AC , carmosine
Fever reducer / pain reliever (Syrup and oral suspension)	5 (1)	tartrazine, carmosine, allura red AC, brilliant blue FCF
Fever reducer / pain reliever (Chewable tablet)	2 (2)	allura red AC aluminium lake, indigo carmine aluminum lake
Children's multivitamins (Chewable tablet)[a]	2 (2)	allura red AC aluminum lake, sunset yellow aluminum lake, indigo carmine aluminum lake
Children's multivitamins (Gummies)[b]	2 (2)	tartrazine, sunset yellow, brilliant blue FCF, allura red AC

[a] One formulation includes three different colored chewable tablets
[b] Two formulations include three different colored gummies.

2.5.1 Selectivity

Interference liabilities of pharmaceutical active ingredients and excipients were verified by analyzing comparable blanks of studied syrups and oral suspensions by the developed procedure. No interference peaks were recorded at the retention times of the colorants (Figure).

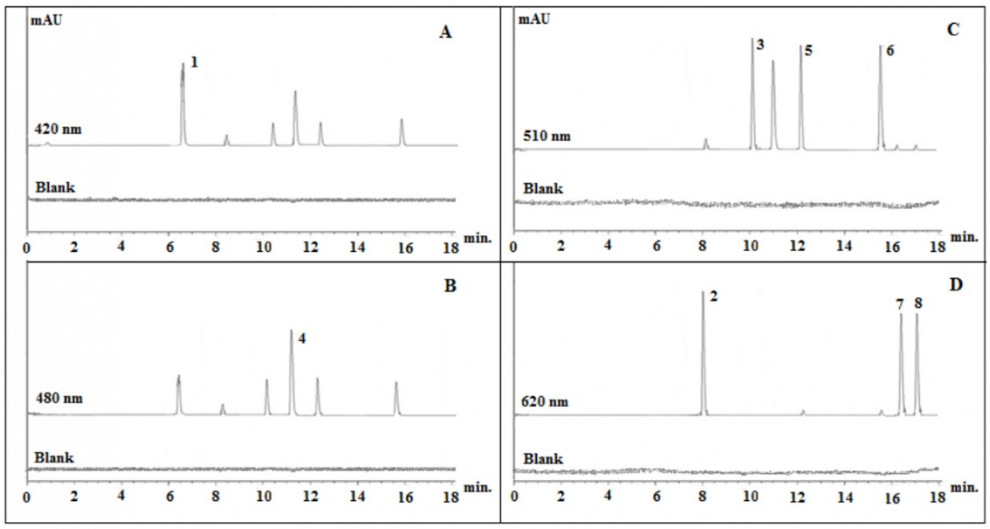


Figure. HPLC chromatograms A, B, C, and D of mixed synthetic colorant standard solution (10 µg/mL) against blanks measured at 420, 480, 510, and 620 nm, respectively. 1-Tartrazine, 2- Indigo carmine, 3-Ponceau 4R, 4-Sunset yellow, 5- Allura red AC, 6- Carmosine, 7-Fast green FCF, 8-Brilliant blue FCF.

2.5.2 Linearity and range

Linearity of colorants - except indigo carmine - was proved over the range 1-100 µg/mL with a correlation coefficient (R^2) 0.9989-0.9996 (Table 3). For Indigo carmine, the range of the calibration curve was 0.5-50 µg/mL. The amount of colorants recommended for oral pharmaceuticals should not exceed 0.01%[3].

Table 3. Retention time and regression data of SCs at selected wavelength.

Colorant	Linear range (µg/mL)	λ_{max} (nm)	t_R[a] (min)	Regression equation[b*] (Mean±SD), (n = 5)	LOD[c] (µg/mL)	LOQ[d] (µg/mL)
Tartrazine	1-100	420	6.7	Y = 68.19±1.09X + 9.87±0.65	0.03	0.09
Indigo carmine	0.5-50	620	8.1	Y = 148.87±1.36X + 11.15±0.32	0.007	0.02
Ponceu 4R	1-100	480	10.1	Y = 24.96±0.59X + 5.87±0.32	0.12	0.36
Sunset yellow	1-100	480	11.0	Y = 55.96±1.10X + 3.03±0.67	0.04	0.11
Allura red AC	1-100	510	12.2	Y = 71.73±0.91X - 6.13±0.82	0.04	0.11
Carmosine	1-100	510	15.6	Y = 65.83±0.91X - 10.03±0.78	0.05	0.14
Fast green FCF	1-100	620	16.4	Y = 89.83±1.07X - 7.63±1.21	0.04	0.13
Brilliant blue FCF	1-100	620	17.1	Y = 110.03±1.06X + 15.03±0.60	0.02	0.05

[a] Retention time
[b] Regression equation Y= aX ± b where Y is average peak area, X is colorant concentration (µg/mL), a is slope and b is intercept, SD: Standard deviation, *: Regression coefficient R^2 values were > 0.99.
[C] LOD Limit Of Detection
[d] LOQ Limit Of Quantitation

2.5.3 Limit of detection (LOD) and limit of quantation (LOQ)

LOD, and LOQ are listed in Table 3. LOQ achieved in developed method regarding dilution process ranged 0.02-0.36 (µg/mL), which is enough to evaluate colorants in pharmaceuticals.

2.5.4 Accuracy

The results of recovery are presented in Table 4. Percent recovery values of colorants ranged from 72–117%. Reported recovery rates of colorants in sport drinks, juices, beverages and drugs using SPE and d-SPE in the literature were 71-127% [22,24]. The modified SPE extraction procedure included a washing step with 2% formic acid in methanol, which was essential for purification.

Table 4. Recovery data of colorants using spiked syrup samples

Colorant	Recovery% (Mean±SD)		
	2 (µg/mL)	25 (µg/mL)	50 (µg/mL)
Tartrazine	95.38±2.96	101.03±2.19	117.15±6.41
Indigo carmine	72.01±2.84	75.36±1.62	76.83±2.74
Ponceu 4R	88.76±1.34	94.33±2.67	92.91±3.06
Sunset yellow	102.38±2.96	107.19±2.77	114.31±4.32
Allura red AC	97.57±2.97	101.42±4.36	106.53±3.16
Carmosine	91.65±3.49	90.15±3.12	92.11±1.47
Fast green FCF	87.48±2.76	89.86±3.11	90.32±3.54
Brilliant blue FCF	92.37±2.88	98.43±2.46	98.93±3.11

SD: Standard deviation of three determinations.

2.5.5 Precision

Precision is the measure of how close the data values to each other for a number of measurements under the same analytical conditions [33]. The developed method was precise (Intra- and inter-day studies), since %RSD was less than 2% (Table 5).

The developed analytical method demonstrates its versatility by successfully applying to various pharmaceutical dosage forms beyond multivitamin tablets and capsules [9]. The extraction procedure effectively purifies synthetic colorants (SCs) alongside excipients and active ingredients in OTCs. Using the developed method, analysis of eight SCs in pharmaceutical dosage forms was done with accessible RP-HPLC-DAD instrumentation.

Šuleková et al. introduced two isocratic HPLC methods for analyzing five SCs in multivitamin tablets and capsules [9]. Lehmkuhler et al. targeted five SCs using RP-HPLC coupled with photodiode detection and gradient elution. The analysis covered OTC forms for children and pregnant women, with percent recoveries from 71 to 127% [30]. Khanavi et al. presented an eco-friendly chromatographic approach with a C8 column and phosphate buffer for quantifying eight SCs in drinks, syrups, candies, and more. Polyamide sorbent aided purification, showing quantification limits from 0.52 µg/mL in allura red to 5.79 µg/mL in quinoline yellow [25]. Our modified method provided a simultaneous one-run solution for the colorants in different matrices, better LOQ values and high analytical recoveries.

Table 5. Intra- and inter-day precision assays of colorants.

Colorant	RSD%					
	Intra-day (n=5)			Inter–day (n =3)		
	10 (µg/mL)	30 (µg/mL)	50 (µg/mL)	10 (µg/mL)	30 (µg/mL)	50 (µg/mL)
Tartrazine	1.32	0.98	0.87	1.06	1.13	1.21
Indigo carmine	1.28	1.01	0.97	1.21	0.87	0.91
Ponceu 4R	1.03	0.95	0.92	1.48	1.35	1.51
Sunset yellow	0.79	0.89	0.67	1.08	0.84	0.86
Allura red AC	1.81	1.11	1.00	1.31	0.79	0.83
Carmosine	1.29	0.92	0.94	1.93	1.31	1.01
Fast green FCF	1.06	0.86	0.88	0.87	0.92	0.79
Brilliant blue FCF	1.10	1.02	0.99	0.89	0.71	0.90

RSD: Relative Standard Deviation

2.6 Analysis of OTC samples

The developed method was applied to pediatric OTCs. The results are shown in Table 6 and Table 7. Allura red AC is the most abundant colorant in samples. In cold/cough/congestion syrups and pain reliever/fever reducer syrups and oral suspension allura red AC ranged from 44.34±2.55 to 174.02±3.91 (mg/kg). Brilliant blue FCF was at concentration below 32.14±1.60 (mg/kg). Formulation for treatment of allergy should not contain colorants. A plenty of antihistaminic marketed syrups were dye free. Only two allergy syrups were colored. Carmosine and ponceu 4 R were detected in 3 samples. Both colorants are banned in USA. Incorporation of Ponceu 4R in syrups was stopped locally according to updated regulations [34].

2.7 Daily colorant intake

Many studies had concerend SCs daily intake [30,35]. In this study daily intake of SCs was calculated and the results are listed in Table 8. The results show that the problem of exposition to SCs for children is serious. For example; daily intake of carmosine by administering sample 4 saturated 26.35% of ADI. When a child aged 4-6 years administers pain-relieving syrups 17 and 18 at their maximum recommended daily dose of 40 mL, he is consuming approximately 1.59 mg/kg bw/day of carmosine and 1.80 mg/kg bw/day of sunset yellow, respectively. This intake corresponds to around 39.74% of the ADI for carmosine and 45.05% of the ADI for sunset yellow. Syrups and oral suspensions are prescribed to children generally due to difficulties in tablets or capsules intake. Children are a vulnerable group that is exposed to many sources of colorants by ingestion of different bright colored foodstuffs, and beverages [36-38]. These results indicated that, OTCs could contribute considerably to risk exposure of children to colorants.

In contrast to syrups and oral suspensions for pain relieving/fever reducing, exposure to colorants through chewables were negligible (%ADI saturation <3). The percent ADI of colorants by multivitamins gummies and chewables was < 2%. The results showed that OTCs (syrups and oral suspensions) could be an important source for the total exposure of children to colorants. Therefore, to protect children's health, the amount should be reduced to a minimum.

3. CONCLUSION

This study has developed an accurate method to analyze eight SCs simultaneously in a variety of pediatric OTCs available in the Gaza Strip. The procedure includes simple pretreatment of sample followed by SPE extraction. The proposed method proves its efficiency in purifying SCs from the pharmaceutical matrices, demonstrating high sensitivity and selectivity. The method was applied successfully in analysis of SCs in OTCs. It's worth noting that only a limited number of studies have concentrated on colorant levels in pharmaceuticals, and this particular aspect remains insufficiently investigated. The study underscores that certain OTCs like syrups and oral suspensions for pain relief, fever reduction, and cold/cough/congestion treatments could contribute up to 45% of ADI for some examined cases. In light of these findings, there's a notable need for updating registration regulations within the Palestinian pharmaceutical sector concerning excipient labeling and the use of colorants.

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Table 6. Data of colorant's content in OTCs (syrups and oral suspensions, n=2).

No.	Active ingredients	Color of OTC	Colorant	Colorant content (mg/kg) (Mean±SD)	Colorant concentration (µg/mL) (Mean±SD)
	Cough/cold/congestion syrup and oral suspension				
1	Guaifenesin, Dextromethorphan, Phenylephrine	Red	Allura red AC	174.02±3.91	210.57±4.73
2	Guaifenesin, Dextromethorphan	Red	Allura red AC	81.04±2.44	101.30±3.05
3	Chlorpheneramine, Dextromethorphan	Red	Allura red AC	68.65±0.59	89.93±0.77
4	Acetaminophen, Chlorpheniramine, Pseudoephedrine, Dextromethorphan	Red	Carmosine	771.39±2.90	944.51±3.57
5	Triprolidine, Pseudoephedrine	Orange	Tartrazine	81.80±8.51	95.71±9.96
6	Triprolidine, Pseudoephedrine, Gauifenesin	Yellow	Tartrazine	46.04±4.00	59.85±5.21
7	Ambroxol	Orange	Tartrazine	60.02±1.03	76.83±1.32
8	Dextromethorphan, Gauifenesin	Orange	Tartrazine Sunset yellow	55.45±2.67 136.02±7.09	69.37±3.34 170.02±8.86
9	Acetaminophen, Guaifenesin, Dextromethorphan, Phenylephrine	Purple	Allura red AC Brilliant blue FCF	92.87±8.15 15.36±0.87	116.09±10.19 19.20±1.09
10	Acetaminophen, Diphenhydramine, Phenylephrine	Purple	Allura red AC Brilliant blue FCF	44.95±2.91 7.78±0.91	57.54±3.73 9.96±1.17
11	Dextromethorphan, Gauifenesin	Purple	Allura red AC Brilliant blue FCF	71.24±1.77 1.62±0.34	88.34±2.19 2.01±0.42
12	Triprolidine, Dextromethorphan	Red	Ponceu 4R	64.17±2.17	78.93±2.68
13	Chlorpheneramine, Phenylephrine	Green	Fast green FCF	173.64±6.92	210.11±8.38
	Allergy syrups				
14	Chlorpheniramine	Red	Carmosine	75.20±4.38	87.23±5.08
15	Diphenhydramine	Red	Allura red AC	83.60±1.13	100.32±1.36
	Fever reducer/pain reliever syrup and oral suspension				
16	Acetaminophen	Pink	Allura red AC	73.47±0.82	92.57±1.03
17	Acetaminophen	Red	Carmosine	567.84±20.55	715.28±25.48
18	Ibuprofen	Orange	Sunset yellow	628.61±8.44	810.91± 10.97
19	Ibuprofen	Red	Allura red AC	44.34±2.55	55.42±3.19
20	Ibuprofen, Acetaminophen	Purple	Allura red AC Brilliant blue FCF	161.56±7.01 32.14±1.60	190.65±8.27 37.93±1.84

SD: Standard Deviation

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Table 7. Data of colorant's content in OTCs (Chewable tablets and gummies, n=2).

No.	Active ingredients	Color of OTC	Colorant	Colorant content (mg/kg) (Mean±SD)	Colorant concentration (µg/tablet) (Mean±SD)
			Fever reducer/ pain reliever chewable tablets		
21	Acetaminophen	Purple	Brilliant blue FCF lake	396.85±4.96	515.90±6.45
22	Ibuprofen	Purple	Indigo carmine lake	316.45±17.11	395.56±21.39
			Multivitamin chewable tablets		
23	Vitamin A, C, E, D3, B1, B2, B6, B12, Folic acid, Niacin, Sodium.	Purple	Allura red AC lake	850.11±90.37	1028.63±109.35
			Indigo carmine lake	315.52±21.55	381.78±26.08
24	Vitamin A, C, E, D3, B6, B12, Folic acid, Niacin, Flouride	Red	Allura red AC lake	708.32±13.89	878.32±17.22
		Purple	Allura red AC lake	978.91±91.25	1213.85±113.15
			Indigo carmine lake	397.85±15.68	493.33±19.44
		Orange	Sunset yellow lake	1610.54±31.85	1996.4±39.49
			Multivitamin gummies		
25	Vitamin A, C, E, D3, B2, B6, Folic acid, Niacin, Thiamin, Biotin, Pantothenic acid, Iodine, Zinc, Sodium	Red	Allura red AC	67.96±3.05	169.9±7.62
		Purple	Allura red AC	78.54±13.12	196.35±32.8
			Brilliant blue FCF	5.11±0.44	12.78±1.10
		Orange	Sunset yellow	95.21±2.52	238.03±6.30
26	Vitamin A, C, E, D3, B12, Folic acid, Biotin, Pantothenic acid, Iodine, Zinc.	Red	Allura red AC	67.58±1.47	168.95±3.68
		Yellow	Tartrazine	73.51±5.14	183.78±12.85
		Green	Brilliant blue FCF	12.85±0.89	32.13±2.23

SD: Standard Deviation.

4.MATERIALS AND METHODS

4.1 Chemicals, reagents and apparatus

Standards of synthetic colorants (tartrazine, sunset yellow, ponceu 4R, carmosine, allura red AC, fast green FCF, brilliant blue FCF, and indigo carmine), ammonium acetate (≥98%), aqueous ammonia solution (28-30%), formic acid (98-100%) p.a grade were purchased fromMerck (Darmstadt, Germany).

HPLC grade methanol, acetonitrile, and water were acquired from Merck (Darmstadt, Germany). Chromabond®HR-XAWSPE cartridges (weak basic secondary and tertiary ammonium anion exchanger with base material polystyrene-divinylbenzene copolymer of pH stability 1–14, 3 mL, 60 mg) were purchased from Macherey-Nagel GmbH&Co (Dueren, Germany).

For sample preparation the following apparatus and materials were used: SPE vacuum manifold from Supelco (Sigma-Aldrich, Darmstadt, Germany), N-EVAP nitrogen evaporator from Organomation Associate, Inc. (Massachusetts, USA), analytical balance from Boeco (Hamburg, Germany), pH meter from Hanna Instrument Inc. (Woonsocket, USA), vortex mixer from Fischer Scientific (Waltham, Massachusetts, USA), centrifuge from Tehtnica (Na Plavžu, Slovenia), and cellulose disposable syringe filters (0.45 µm) from Agilent (California, USA).

4.2 Preparation of standard solution

Standard stock solution of each synthetic colorants at 100 µg/mL concentration were prepared by dissolving required amount of standards and dissolving in 100 mL volumetric flask with water (HPLC grade). Working standard solutions were prepared freshly by mixing appropriate volumes of stock solutions

and dilution with water to produce a concentration of the calibration range. Standard solutions were kept stable for two weeks in dark place at 4°C.

Table 8. %ADI saturation by OTCs administered at the recommended dose.

No.	Colorant	Maximum daily dose[a]	Daily intake mg	%ADI saturation[b]
1	Allura red AC	5 ml, 6 times	6.32	5.01
2	Allura red AC	5 ml, 6 times	3.04	2.41
3	Allura red AC	5 ml, 6 times	2.70	2.14
4	Carmosine	5 ml, 4 times	18.89	26.35
5	Tartrazine	2.5 ml, 3 times	0.72	0.53
6	Tartrazine	2.5 ml, 3 times	0.45	0.33
7	Tartrazine	2.5 ml, 3 times	0.58	0.43
8	Tartrazine	5 ml, 6 times	2.08	1.52
	Sunset yellow		5.10	7.08
9	Allura red AC	5 ml, 6 times	3.48	2.76
	Brilliant blue FCF		0.58	0.53
10	Allura red AC	5 ml, 6 times	1.73	1.37
	Brilliant blue FCF		0.30	0.28
11	Allura red AC	5 ml, 6 times	2.65	2.10
	Brilliant blue FCF		0.06	0.05
12	Ponceu 4R	2.5 ml, 4 times	0.79	1.09
13	Fast green FCF	2.5 ml, 4 times	2.10	0.47
14	Carmosine	2.5 ml, 6 times	1.31	1.82
15	Allura red AC	5 ml, 6 times	3.01	2.39
16	Allura red AC	5 ml, 4 times	1.85	1.47
17	Carmosine	10 ml, 4 times	28.61	39.47
18	Sunset yellow	10 ml, 4 times	32.45	45.05
19	Allura red AC	5 ml, 4 times	1.11	0.88
20	Allura red AC	5 ml, 4 times	3.81	3.03
	Brilliant blue FCF		0.76	0.70
21	Brilliant blue FCF lake	1.5 tablet, 4 times	3.10	2.87
22	Indigo carmine lake	1.5 tablet, 4 times	2.37	2.64
23	Allura red AC lake	One tablet daily	1.03	0.82
	Indigo carmine lake		0.38	0.42
24	Allura red AC lake	One tablet daily	0.88	0.69
	Allura red AC lake		1.21	0.96
	Indigo carmine lake		0.49	0.54
	Sunset yellow lake		1.20	1.67
25	Allura red AC	Three gummies	0.51	0.40
	Allura red AC	once daily	0.59	0.47
	Brilliant blue FCF		0.04	0.04
	Sunset yellow		0.71	0.99
26	Allura red AC	Two gummies	0.34	0.22
	Tartrazine	once daily	0.37	0.27
	Brilliant blue FCF		0.06	0.06

4.3 Chromatographic system

HPLC apparatus was composed of Prominence LC-20AB solvent delivery unitequipped with UV-Vis diode array detector (SPD-20A) and vacuum degasser (DUG-20A5R). LabSolution Software was used for data acquisition and processing from Shimadzu (Kyoto, Japan).

Chromatographic separation was carried out using Shim-pack® VP-ODS (250 × 4.6 mm, 5 µm particle size) from Shimadzu (Kyoto, Japan) as stationary phase. Mobile phase was composed of solution A which is 80mM ammonium acetate solution (pH: 7.0) and solution B which is 70:30 (v/v) methanol:acetonitrile solution. A gradient elution was performed at a flow rate of 1.0 mL/min and at room temperature. The

gradient program started with 10% solution B as the initial step and increased to reach 40% solution B in 10 min and then increased again to 70% B in 16 min. Finally, the composition was kept 2 min at this ratio. The column was conditioned for 5 minutes before each analysis. The samples were injected using Rheodyne manual injector and 20 µL loop. The column temperature was maintained at 25 °C. The detector was operated at 420 nm for tartrazine, 480 nm for ponceau 4R and sunset yellow, 510 nm for carmosine and allura red AC, and 620 nm for indigo carmine, brilliant blue FCF, and fast green FCF.

4.5 Samples

Several types of pediatric OTC medications were collected from local pharmacies in Gaza Strip. Synthetic colorants were declared in a few preparations on the label of dosage forms. Identifications were performed using thin layer chromatography [7], and through comparing with peaks of colorant by HPLC. Samples were cough/cold/congestion syrups, allergy syrups, pain/fever reliever oral suspensions, syrups, and chewables, children's multivitamins gummies, and children's multivitamins chewable tablets. Blanks used in the study were comparable dosage forms without colorants. Absence of synthetic colorants were confirmed by HPLC.

4.6 Sample preparation

4.6.1 Syrup and oral suspension

Three milliliters of syrup were transferred in 50 mL conical centrifuge tubes, diluted with water, pH adjusted to 7 by NH_3 solution (0.1 M), and diluted to the mark of 30 mLwith water. Samples were put in ultrasonic bath at 50±5 °C for 20 min and were frequently mixed by vortexing. Then centrifugation followed at 5000 rpm for 5 min. 3 mL of the clear solution were subjected to SPE extraction.

4.6.2 Children's multivitamin gummies

Samples contain gummies of three different colors per bottle. 2.0 g from each colored gummies were separately put in conical centrifuge tubes, and water was added to volume of 30 mL mark. The procedure continued as described in syrup and oral suspension.

4.6.3 Children's chewable tablets

Five tablets per colored chewable tablets were grinded by mortar and pestle. 1.0 g of powder were put in conical centrifuge tube followed by 0.1 g disodium edetate and diluted to 25 mL with NH_3 (0.25% w/v). Samples were vortexed and extraction was performed ultrasonically at 50±5 °C for 20 min. After centrifugation at 9000 rpm for 5 min. 2.5 mL of supernatant were transferred into 5 mL volumetric flask, pH was adjusted to 7 with formic acid (5% v/v) and the volume was completed to 5 mL with water. 3 mL were loaded on the cartridge for SPE extraction.

4.7 SPE extraction

Chromabond®HR-XAW SPE cartridges were placed in positions of vacuum manifold and the procedure was performed with a flow rate 20 drops per min. Cartridges were conditioned by 2mL methanol followed by 2 mL water. Three milliliters of diluted samples were loaded onto cartridge and slowly aspirated into the column. Washing after sample loading proceeded with 2 mL water, then 2 mL of 2% formic acid in methanol, and finally 2 mL methanol. The cartridges were dried using negative pressure (about 700 mbar for 1 min) and the elution was performed using 5 mL ammonia in methanol (5%, v/v). Eluates were evaporated under nitrogen at 40 °C. Residues were reconstituted in 2 mL, 80 mM ammonium acetate and vortex, filtered using syringe cellulose membrane filter and analyzed using HPLC.

4.8 Method validation

The method validation was performed according to the International Conference on Harmonization (ICH) guidelines [33].

4.8.1 Selectivity

Selectivity was carried out by comparing the chromatograms of blank and spiked samples to evaluate interference liabilities of components in pediatric OTCs (Active ingredients and excipients) with target colorants. No interferences were observed at corresponding retention times.

4.8.2 Linearity and range

Calibration curves of standards were constructed by plotting colorant concentration against average peak area. The standard solution mixture at concentrations of 0.5, 2.5, 10.0, 50.0, and 100 µg/mLwere injected in triplicate under chromatographic conditions mentioned above. The calibration curve and correlation coefficient were evaluated (n=5).

4.8.3 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated using the following formula
LOD = 3.3 σ / S and LOQ = 10 σ / S, σ: Residual standard deviation of regression line, S: Slope of the regression line.

4.8.4 Accuracy

The accuracy was examined by analyzing spiked blank samples with each colorant at 2, 25, 50 µg/mL levels. The concentrations were calculated using the calibration curve and the percent recovery was calculated using equation 1.

Eq. 1 Recovery% = Calculated concentration (µg/mL) × 100 /Theoretical concentration (µg/mL)

4.8.5 Precision

Intra- and inter-day precision were examined by preparing spiked samples at concentration 10, 30, 50 µg/mL per colorant, and analyzed. Intra – day precision was assessed by performing five repetitions of each level during a single day and the inter – day precision by three repetiotions of each level per day over three different days.

4.9 Analysis of OTC samples

Different OTC samples marketed in the Gaza Strip were analyzed on SCs content by the developed and validated method as mentioned in sections 4.3-4.7.

4.10 Daily intake of SCs

Equation 2 was used to calculate daily intake of SCs for syrup and oral suspension. For chewable tablets and gummies equation 3 was used. Daily intake calculation was performed regarding the recommended dose for 4-6 years children weighed 18 kg as given by the manufacturer [37]. The intake data of colorants were then compared with the respective acceptable daily intake and ADI% was calculated according to equation 4. Percent ADI showed the extent of saturation of ADI for 4-6 years old child by OTCs intake.

Eq. 2 Colorant daily intake (mg/day) = Conc (µg/mL)×Daily dose (mL)/1000
Eq. 3 Colorant daily intake (mg/day) = Conc (µg/mL)×Daily dose (Tablet)/1000
Eq. 4 ADI% = Colorant daily intake/ADI × 100

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