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Simultaneous Determination of Sudan Dyes along with Dimethyl Yellow in Indian Curry Samples by Reverse Phase Liquid Chromatography

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Abstract

The synthetic organic azo dyes have been studied for their toxicity risk due to formation of suspected carcinogenic aromatic amines on reduction. Based on toxicity data, various unauthorized azo dyes are sometimes illegally used in food preparations either to enhance or to maintain the appearance of food products. Therefore suitable analytical screening and confirmatory methods are required for compliance verification of foodstuffs. Although several methods are available, no method for the simultaneous determination of all the four Sudan dyes along with dimethyl yellow in oil rich Indian curry samples has been reported so far. The present method utilizes a simple extraction step and simultaneous HPLC resolution of Sudan I, II, III, IV and dimethyl yellow in oil rich Indian curry samples. Analysis was performed on a reversed-phase LiChroCART(R) RP-18 column using the gradient mixture of acidified water, acetonitrile and methanol. The flow rate was 1.0 ml min^{-1} with λ max of 420 up to 4 min and then 500 nm, respectively to monitor dimethyl yellow and Sudan dyes. All the five colors showed good linearity at the concentrations of 0.25-25.0 mgL^{-1} with the regression coefficient from 0.997 to 0.999. The LOD ranged from 0.23-0.33 mgL^{-1} while LOQ varied between 1.19-1.70 mgL^{-1}. The intraday and interday precision gave good RSDs between 0.78 to 4.32%, and percentage recoveries ranged from 62.3 to 77.3%. The applicability of the method has been verified by analyzing fifteen curry samples procured from local markets.

Keywords: Dimethyl yellow; Curry samples; Sudan I, II, III, IV; Reversed phase HPLC

Introduction

The most widely used synthetic organic azo dyes have been studied for their toxicity risk [1]. The chromophoric azo group on reduction forms suspected carcinogenic aromatic amines under certain conditions [2]. Some of these dyes are used in food industry with the regulation of maximum permissible levels in a particular foodstuff [3]. Based on toxicity data, various azo dyes are unauthorized and are sometimes illegally used in food preparations either to enhance or to maintain the appearance of food products [4-6]. The adulteration of hot chili products with Sudan I, II, III and IV led the EU to adopt emergency measure [7]. The UK Food Standard Agency issued alert for various meat preparations in UK market for contamination with Sudan I [8]. The Agence Federale pour la Securite de la Chaine Alimentaire recalled curry samples contaminated with dimethyl yellow [9]. The Rapid Alert System for Food and Feed (RASFF) determined more than 0.50 mg dimethyl yellow per kg in curry powder in India [10].

Sudan I, considered to be a genotoxic carcinogen, is not permitted in foodstuffs [11,12]. Sudan II the dimethyl derivative of Sudan I have been shown to cause bladder carcinomas [13]. All the four Sudan dyes have been classified by IARC in the Group 3, i.e. not classifiable as to their carcinogenicity to humans. Dimethyl yellow, a Group-2B carcinogen [14-17] with hazardous potential, poses a risk to humans and animals [18-23]. The teratogenicity and mutagenicity study of this dye suggested a positive dose–effect relationship [24,25].

Undoubtedly, these synthetic dyes have major economic consequences for food industries and they constitute a potential risk to public health if they enter the food chain. Therefore suitable analytical screening and confirmatory methods are required for compliance verification of foodstuffs. Although several methods are available but they are limited either to the detection of Sudan I or dimethyl yellow alone; or the four Sudan dyes; or dimethyl yellow, Sudan I & II [4-6,26-30]. No method for the simultaneous determination of all the four Sudan dyes along with dimethyl yellow has been reported so far.

In the present paper we have developed and validated a simple reversed phase HPLC method for the simultaneous identification and determination of Sudan I, Sudan II, Sudan III, Sudan IV and dimethyl yellow in curry samples. The validation protocol included evaluation on detection limits, quantitation limits, linearity, accuracy (precision and trueness), recovery and selectivity. The proposed method requires minimal sample preparation, and provides a well-resolved analyte peak without much interference.

Materials and Methods

Reagents

AR grade glacial acetic acid was procured from Qualigens, Mumbai, India. Methanol and acetonitrile (HPLC grade) respectively, were purchased from Merck Limited, Mumbai, India and Fisher Scientific, Fair Lawn, New Jersey, USA. The standards of fat soluble dyes dimethyl yellow, Sudan I, Sudan II, Sudan III and Sudan IV were obtained from Fluka Sigma Aldrich St. Louis, MO, USA. All the other chemicals used were of highest purity available commercially.

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Apparatus

Chromatographic analysis was carried out with a Waters LC module (Waters Associates, Vienna, Austria) equipped with a dual pump (model # 510), Rheodyne injector with 20-µl loop and tunable absorbance detector model # 486. The chromatograms were recorded and processed by Waters Millennium® software.

Double beam spectrophotometer (Perkin Elmer Lambda Bio 20, Perkin Elmer Instruments, Switzerland) was used for spectrophotometric measurements using quartz cell of 10 mm path length.

Preparation of colour standards

The common name of the colours used, their Colour Index (CI) names, numbers and formulae are given in Table 1. Standard stock of each colour was prepared by dissolving 10 mg colour in 10 ml acetonitrile except Sudan III which was dissolved in chloroform. Subsequent working standards (0.25 to 25 ppm) were obtained by appropriate dilution with acetonitrile. A mixed standard was prepared by mixing appropriate aliquots from standard stock of each of the five colours. The standards were stored at 4°C in the dark and were stable at least for 2 months. The visible spectrum of each standard colour was obtained in order to know their respective λ_{max} (Table 1).

Preparation of samples

All the tested samples were obtained from the local market. The solid substances if any were removed from the curry samples and were stored under refrigeration till further processing. The samples were taken out from the refrigerator and mixed properly so that the oil present in them get properly mixed. 1 gm of sample in duplicate was taken and shaken with 1 ml of n-hexane so that the fat soluble dye comes into the hexane phase. The process was repeated two to three times so that all the colours get extracted into the n-hexane phase. The hexane phase was concentrated to 1 ml and shaken with 2 ml of acetonitrile. The tubes were centrifuged and acetonitrile phase was collected. The process was repeated each time taking fresh acetonitrile till the hexane layer became colourless. All the acetonitrile layers were pooled together.

The pooled acetonitrile layer was taken up and concentrated to dryness in a rotary evaporator. The residue was dissolved in 1.0 ml of acetonitrile and filtered prior to HPLC injection through a Millipore filter of 0.45 µm polyvinylidene fluoride (PVDF) membrane.

Chromatographic conditions

Analysis was performed on LiChroCART(R) 250-4 LiChrospher(R) WP 300 RP-18 column having particle size 5 µm with a LiChroCART(R) 4-4 guard column of LiChrospher(R) 100 RP-18 endcapped 5 µm size. The components of the mobile phase were filtered under vacuum through a membrane filter with a pore diameter 0.45 µm. The injection volume was set at 20 µl. The optimal mobile phase conditions consisted of 2% acetic acid: acetonitrile: methanol (150:200:650, eluent A) and methanol (eluent B) programmed on a linear gradient of 20 minutes with a flow rate of 1ml min^{-1}. From initial zero to 10 min, a gradient of 95% of eluent A: 5% eluent B was carried out. Between 12 to 17 min, 0% A: 100% B was followed and from 17 to 20 min the initial conditions of 95% A: 5% B was achieved. Finally, a 10 min equilibrium phase of the column was run to recover initial conditions of 95% A: 5% B. The UV/VIS detector was specifically programmed to monitor dimethyl yellow at 420 nm and four Sudan dyes at 500 nm wavelengths.

Common name	CI name	CI number	λ_{max}(nm)	Formulae
Dimethyl yellow	Solvent Yellow 2	11020	411	$C_{14}H_{15}N_3$
Sudan I	Solvent Yellow 14	12055	476	$C_{16}H_{12}N_2O$
Sudan II	Solvent Orange 7	12140	490	$C_{18}H_{16}N_2O$
Sudan III	Solvent Red 23	26100	504	$C_{22}H_{16}N_4O$
Sudan IV	Solvent Red 24	26105	514	$C_{24}H_{20}N_4O$

Table 1: Common names, CI (Color Index) names, CI numbers, λ_{max} and formulae of the standard synthetic fat soluble dyes studied.

Linearity and calibration standard

The linearity of the assay was checked by running duplicate set of each standard color and the calibration graph was obtained by plotting the peak area versus their concentration.

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and the LOQ were determined by the US Environment Protection Agency method [31]. Seven replicates of each dye at a concentration of 2.5 mgL^{-1} in curry samples (devoid of any dyes) were spiked and analyzed.

Peak area and retention time stability

To test peak area and retention time repeatability, a standard mixture of colors of concentration 10 mgL^{-1} was analyzed. Average peak area and retention times were calculated from eight individual runs and values were expressed as % RSD.

Recovery, repeatability, and reproducibility

Different concentration ranging from 25 µl to 500 µl of a standard mixture of dye of concentration 100 mgL^{-1} were spiked in blank curry samples (devoid of any dye) to obtain concentrations of 2.5, 5.0, 10.0, 25.0 and 50.0 mgL^{-1}, respectively. The recovery and repeatability were determined by performing the experiment in duplicates and the mean values were expressed with ± SD.

Statistical analysis

Results were expressed as mean ± SD (n=2). The SD, %RSD and coefficient of determinations (r^2) were determined using Microsoft Excel statistical software (Microsoft Corporation, Microsoft Office Excel 2007).

Results and Discussion

Optimization of the Separation

A lot of maneuvering with combination of organic solvent and acidified aqueous phase was required to achieve optimal resolution and peak symmetry. The peaks became sharp in the presence of acid but increase in concentration of methanol tend to merge the peak of dimethyl yellow with curcumin, one of the basic constituent of Indian curry sample. The mobile phase used by other investigators [4-6,26,27,29,30] could not clearly separate all the five dyes. The combination of water, methanol and acetonitrile also did not result in the clear separation. Hence different combinations of mobile phase were tried out and finally the combination of mobile phase presented in this investigation was found to be optimal that led to a satisfactory resolution of all the five fat soluble dyes with a distinct RT (Figure 1).

The resolution coefficient of all the dyes were greater than 1.5 except Sudan I (1.13) but its resolution remained un-affected. The efficiency factor of all the five dyes were less than 5 (Table 2).

Validation of the method

To support regulatory action, a method has to be accurate, sensitive and should be able to identify analytes with high selectivity. For this purpose, the analytical method evaluation including linearity, sensitivity/the limit of detection (LOD), the limit of quantification (LOQ), method precision and recovery was carried out. Dimethyl yellow, Sudan I, Sudan II, Sudan III and Sudan IV showed good linearity at the concentrations in the range of 0.25-25.0 mgL^{-1} with the regression coefficient from 0.997 to 0.999 (Figure 2). The values are typical for HPLC based determinations and acceptable for routine detection purposes. The HPLC eluted peak of standard dimethyl yellow, Sudan I and samples containing the respective dyes were matched (Figure 3).

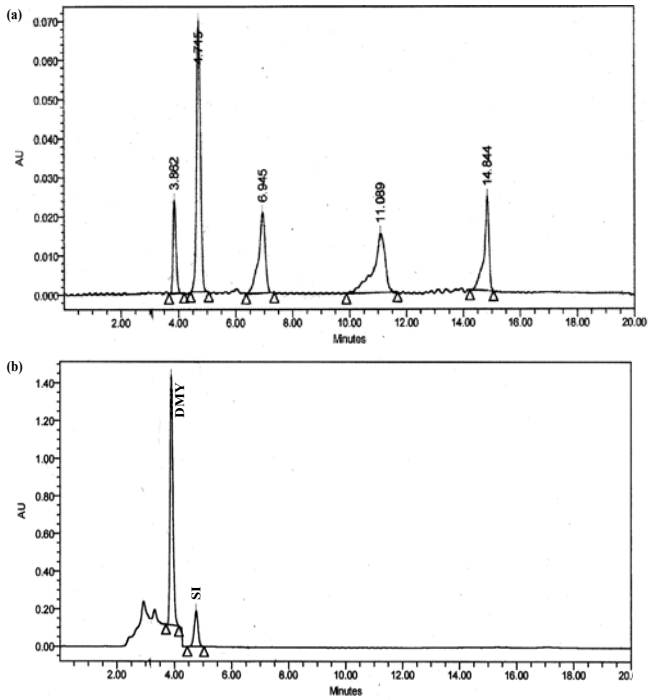

Figure 1: HPLC resolution of five fat soluble non-permitted colors encountered in standard (a) and dimethyl yellow (DMY) and Sudan I (SI) in curry sample (b) .

Dyes	RT(Min)	k'A[a]	R_s[b]
Dimethyl yellow	3.86	0.08	0.0
Sudan I	4.71	0.32	1.13
Sudan II	6.94	0.95	2.57
Sudan III	11.08	2.11	4.24
Sudan IV	14.84	3.16	4.45

The efficiency factor (k'A) and resolution coefficient were calculated as per the following formulae:

[a]$k'A = t_R - t_M / t_M$ (where k'A is the capacity factor, t_R is the retention time of the analyte and t_M is the time taken for the mobile phase to pass through the column).

[b]$R_s = 2[(t_R)_B - (t_R)_A]$ (R_s is the resolution factor, $(t_R)_B$ is the RT of peak B, $(t_R)_A$ is the RT W_A+W_B of peak A and W_A & W_B is the peak width of peak A and peak B, respectively).

Table 2: Retention time, resolution coefficient and efficiency factor of the five dyes using present mobile phase.

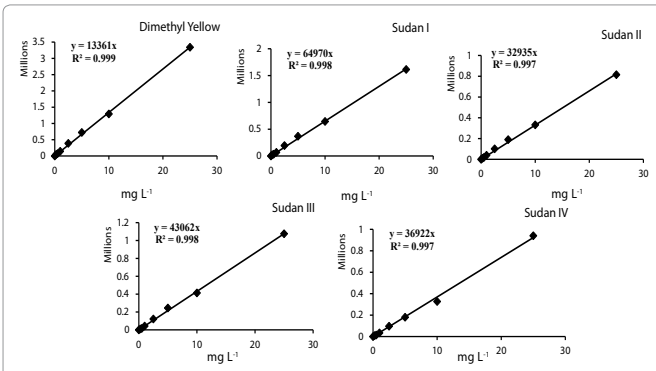

Figure 2: Calibration curves showing linearity of five fat soluble non-permitted dyes. Equation: *Y=b+mx*. Linearity range: 0.25–25.0 mg/L.

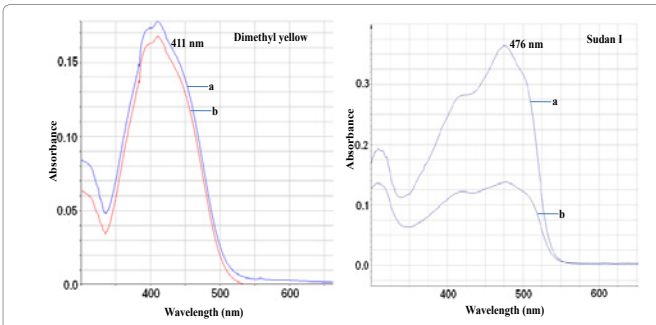

Figure 3: Spectral characteristics of individual Dimethyl yellow and Sudan I (a) and extracted dyes from curry samples (b). The horizontal and vertical axes represent wavelength (nm) and absorbance (A) respectively.

The LOD of the five studied dyes ranged from 0.23 (Sudan III) to 0.34 (Sudan IV) mgL^{-1}, whereas LOQ was found to be in the range of 1.19 (Sudan III) to 1.70 (dimethyl yellow) mgL^{-1}, respectively (Table 3). The studies carried out by Calbiani et al. [4] and Tateo et al. [28] showed the LOD and LOQ for Sudan I, Sudan II, Sudan III and Sudan IV in sauces samples and dimethyl yellow in curry samples to be less than 25 and 50 μgL^{-1} which were relatively more sensitive than the present study (0.23-1.70 mgL^{-1}) and may be attributed to use of LC-ESI MS/MS method which is far more sensitive than the present HPLC method. However, LOD and LOQ obtained by Cornet et al. [5] using DAD was of the magnitude of 0.2 to 0.5 and 0.4 to 1.00 mgL^{-1} for Sudan I, Sudan II, Sudan III and Sudan IV, which is similar to our present method using LC-UV/VIS detector (0.23-1.70 mgL^{-1}) for all the five dyes.

To test peak area and retention time repeatability, a standard mixture of colors was analysed. The relative standard deviations (RSD) of retention time were from 1.64% (Dimethyl yellow) to 3.19% (Sudan III), while the peak areas RSD was between 1.33% (Dimethyl Yellow) and 4.97% (Sudan III) (Table 4). The peak area and retention time repeatability of the developed methodology has a good level of reproducibility, with % RSD values of retention time and peak areas being within 5%.

Method accuracy was tested in terms of precision which gave good RSDs for both intra-day and inter-day precision. The intra-day precision (as RSDr) varied from 0.78% for Dimethyl yellow at 5.00 mgL^{-1} to 4.32% for Sudan III at a level of 5.00 mgL^{-1}. The inter-day

Dyes	Equation Y=b+ mx*	Linearity range (mg L^{-1})	Regression Coefficient	LOD (mg L^{-1})	LOQ (mg Kg^{-1} or L^{-1})
Dimethyl yellow	$1.73\times10^{4}+1.33\times10^{5}$	0.25-25	0.999	0.33	1.70
Sudan I	$1.32\times10^{4}+6.42\times10^{4}$	0.25-25	0.998	0.25	1.28
Sudan II	$0.90\times10^{4}+3.24\times10^{4}$	0.25-25	0.997	0.27	1.42
Sudan III	$0.38\times10^{4}+4.28\times10^{4}$	0.25-25	0.998	0.23	1.19
Sudan IV	$-0.57\times10^{4}+3.72\times10^{4}$	0.25-25	0.997	0.34	1.54

* Y=b+mx (where Y=linearity, b=intercept and mx=slope of the curve)

Table 3: Linear regression equation, linearity range, regression coefficient, limit of detection (LOD) and limit of quantification (LOQ) of the dyes.

Colours	Average retention time (min) *	*s*** (min)	RSD (%)	Average peak area* ($x10^{3}$)	*s* ** (peak area)$\times10^{3}$	RSD (%)
Dimethyl yellow	3.946	0.065	1.64	1307	17.4	1.33
Sudan I	4.770	0.092	1.92	632	2.73	3.44
Sudan II	7.041	0.189	2.69	333	8.54	2.56
Sudan III	11.277	0.360	3.19	421	20.9	4.97
Sudan IV	14.621	0.341	2.34	311	14.2	4.55

*Retention time and peak area are the average values derived from 8 individual runs of standard mixture of concentration - 10 mgL^{-1}.
***s* is the standard deviation

Table 4: Repeatability of peak areas and retention times of a standard mixture of the dyes.

Colours	Amount (mg L^{-1})	Intra-day precision*			Inter-day precision*		
		Average peak area	% RSDr	SE	Average peak area	% RDS_R	SE
Dimethyl yellow	5	723453	0.78	3267	715855	1.54	6374
	10	1296319	1.21	9079	1310675	1.55	11708
Sudan I	5	395516	4.19	9128	380424	2.46	5342
	10	644395	1.27	4724	618884	3.78	13510
Sudan II	5	189997	1.27	1396	190848	0.98	1083
	10	331961	2.72	5217	337181	1.41	2751
Sudan III	5	256179	4.32	6390	254170	4.19	6150
	10	413646	2.96	7072	420837	2.64	6430
Sudan IV	5	179689	0.62	644	179494	0.70	724
	10	317106	3.44	6307	325494	1.75	3299

* Data is derived from triplicate values

Table 5: Intra- and inter-day precision of dyes.

precision (as RDS_R) ranged from 0.70% for Sudan IV at 5.00 mgL^{-1} to 4.19% for Sudan III at a concentration of 5.00 mgL^{-1} (Table 5). Ertas et al. [6] reported 0.82-4.09% intra-day precision and 1.33-4.65% inter-day precision in red chilli pepper samples.

In order to evaluate the trueness of the method, recovery experiments were performed. Standard dyes spiked in curry samples (devoid of any dye) at five concentrations of 2.5, 5.0, 10.0, 25.0 and 50.0 mgL^{-1} showed a percentage recovery of 62.3% (Sudan IV) to 77.6% (Sudan II) (Table 6). The values indicate an adequate recovery rate and the recovery percentages found in the present study are close to those of Calibiani et al. [4] and Cornet et al. [5] where percentage recovery ranged from 51-86% and 62-97% in sauce & chilli tomato and cheese sauce samples, respectively.

In many studies, the extraction of dyes from food sample was carried out by acetone [4,30] or by acetonitrile [5,29]. Ertas et al. [6] used the solvent mixtures of acetonitrile, dichloromethane and methanol in his studies for extraction of dyes from chilli pepper. This type of extraction could not be followed with oil rich Indian curry samples as extraction with hexane was necessary prior to dye extraction to avoid interference in analysis. Thus, one treatment step has been used in our study, which ensured selectivity of dye extraction and avoided interference of food matrices.

Thus our method has advantage over other methods [4-6,29,30] where single step extraction with solvent or its mixtures have been carried out.

Application to real samples

The results of real market sample analysis revealed that most curry samples had blends of Dimethyl yellow and Sudan I. Dimethyl yellow in the curry samples was found in the range of a minimum of 2.16 mgL^{-1} to a maximum of 393 mgL^{-1}, whereas Sudan I varied from 4.63 mgL^{-1} to 264 mgL^{-1}. Sudan II, Sudan III and Sudan IV were not present in any of the curry samples (Table 7).

Conclusion

A simple and sensitive analytical method for the determination of five fat soluble dyes in a single run has been developed. HPLC resolution of the commonly encountered non-permitted dyes namely, Dimethyl yellow along with Sudan I, Sudan II, Sudan III and Sudan IV in oil rich Indian curry samples has been reported for the first time. The method

Dye	Amount added	Amount recovered	% Recovery	%RSD
Dimethyl yellow	2.5	1.89	75.5	1.12
	5.0	3.74	74.8	2.08
	10.0	7.56	75.6	2.53
	25.0	18.90	75.6	2.81
	50.0	38.20	76.4	1.48
Sudan I	2.5	1.91	76.3	3.62
	5.0	3.81	76.2	1.39
	10.0	7.31	73.1	2.23
	25.0	18.33	73.3	3.09
	50.0	36.80	73.6	2.31
Sudan II	2.5	1.94	77.6	3.56
	5.0	3.65	73.0	1.16
	10.0	7.01	70.1	2.42
	25.0	18.25	73.0	1.74
	50.0	36.48	73.0	2.42
Sudan III	2.5	1.74	69.4	3.87
	5.0	3.46	69.3	2.35
	10.0	6.83	68.3	2.28
	25.0	17.65	70.6	3.00
	50.0	35.85	71.7	1.78
Sudan IV	2.5	1.56	62.3	2.72
	5.0	3.31	66.2	3.74
	10.0	6.49	64.9	2.18
	25.0	15.74	63.0	1.68
	50.0	31.65	63.3	2.68

The samples were spiked with dyes at the concentrations of 2.5, 5.0, 10.0, 25.0 and 50 mgL^{-1}. Recovery of dyes was performed in duplicate and the mean data is presented

Table 6: **Recovery of individual dyes spiked in curry samples.**

Sample no.	Dimethyl yellow		Sudan I	
	Conc (mg L^{-1})	%RSD	Conc (mg L^{-1})	%RSD
1.	17.58	1.69	153.65	2.09
2.	131.39	4.95	147.06	3.05
3.	17.11	4.00	254.34	2.80
4.	2.16	3.87	6.36	3.55
5.	100.94	2.46	147.33	1.66
6.	4.79	3.61	4.63	4.59
7.	10.67	3.29	33.33	4.60
8.	12.15	2.19	65.01	4.06
9.	3.31	4.31	10.63	3.21
10.	392.85	2.19	165.64	4.41
11.	20.71	4.64	264.10	2.87
12.	302.36	1.75	167.14	1.94
13.	Nil	-	Nil	-
14.	Nil	-	Nil	-
15.	Nil	-	Nil	-

Data represent mean of duplicate values for each analyzed sample

Table 7: Determination of dimethyl yellow and Sudan I in curry samples collected from the local market.

utilizes an extraction step prior to dye extraction to avoid interference of complex food matrices and offers a combination of selectivity and convenience. The applicability of the method has been verified by analyzing fifteen curry samples procured from local markets.

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