Detection and Determination of *Para*-red in Chillies and Spices by HPLC

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Summary: A rapid High Pressure Liquid Phase Chromatography (HPLC) method was developed for the detection and determination of *para*-red in red chilli powder and other related food materials. The detection was made at 488 nm using a UV-VIS detector. The chromatographic separation was achieved on a reverse phase C_{18} column with isocratic elution by using a mobile phase consisting of deionized water (200 ml containing 0.34 g KH_2PO_4) and HPLC grade methanol (800 ml containing 1.01 g tetraethylammonium bromide). The flow rate of mobile phase was maintained at 1 ml/min. The retention times were ranged between 15 to 20 min. Toluene was used for extraction to prepare sample. The standard was *para*-red. The method was in-house validated based on following criteria: Limit of detection (LOD), Limit of quantification (LOQ), recovery, repeatability and linearity. LOD and LOQ were upto 1 mg/kg and 3 mg/kg, respectively. The recovery values ranged betweem 97-99 %. Repeatability and linearity obtained for *para*-red standard solutions were 1.91 % (RSD, n = 7) and $r^2 > 0.98888$ (in the range of 1.0 to 100 mg/l), respectively.

Introduction

The industrial dye *para*-red [1-(4-nitrophenylazo)-2-naphthol] (Fig. 1) belongs to the group of azo dyes. According to the IARC (International Agency for Research on Cancer), azo dyes have been classified as potential carcinogenic substances. On oral up-take the azo dyes can be reduced to the partially carcinogenic amine-components same as that of metabolic components of Sudan dyes which belong to the group 2 [1]. Sudan dyes are banned as food additives in the Europe. *Para*-red is chemically similar to Sudan-I and is illegally used for imparting red color in hot chilli and its related food products. First time Sudan-I was found in red chilli imported in France from India. As a reaction the EU issued Decision 2003/460/EG [2] imposing condition on import of hot chilli and hot chilli products to be tested for Sudan-I. The decision was amended in January 2004 (2004/92/EG) to include Sudan-II, III and IV. Other dyes under scrutiny are *para*-red, Sudan orange G, Sudan Red 7B, and Butter Yellow [3]. IARC establish various measures concerning the analytical control of hot chilli and other related products at import or already in the market. More precisely the decision applies to fruits of the genus *Capsicum*, dried and crushed or ground [4]. Products, which tested positive, have to be destroyed. Due to the related expenses a reliable testing method is inevitable. The EU announced the limit of detection for Sudan-I and other similar dyes in the range of 0.5-1.0 ppm using the HPLC method [2]. For the time being any food containing dyes above these limits must be withdrawn from market.

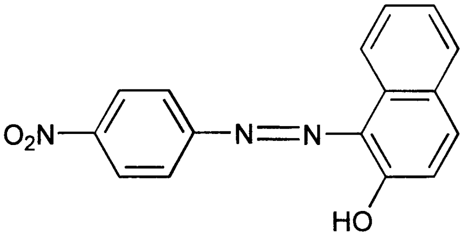

Fig. 1: Structure of *para*-red: [1-(4-nitrophenylazo-2-naphthol)].

Since 2003, a number of methods have been put forward by using HPLC. The mode of detection, UV, PDA or HPLC/MS directly impacts the limit of detection. A method was used in a regional agency suggesting toluene as extraction solvent for Sudan-I from chilli, and methanol for re-dilution of the dried extract. Methanol was used as HPLC mobile phase on C_{18} column [4]. In this case, GC/MS was suggested to confirm the identity of the peak. In another method, benzene as an extraction solvent and HPLC with C_{18} column, UV at 436 nm and 100 % methanol as mobile phase was suggested for *para*-red determination [5]. Other methods

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proposed for determination of synthetic food colorants included HPLC technique coupled with a Dionex AD 20 absorbance detector [6] and determination of colorant matters mixtures by solid-phase spectrophotometry [7]. A collaborative trial from Food Standards Agency [4] showed two methods, 145a and 145b, concerning the application of HPLC to evaluate Sudan I in chilli powder. Recently, two methods based on the use of gel permeation chromatography-liquid chromatography-tandem mass spectrometry interfaced with electron spray ionization (GPC-LC-ESI-MS/MS) were devised for the determination of *para*-red and nine banned azo dyes in hot chilli products [8, 9]. Other recent assays of *para*-red in food stuffs introduced the use of HPLC and liquid chromatography linked with tendem mass spectrometry and isotope dilution methodology [10, 11]. Efforts are still underway among scientists to develop an analytical method for these dyes to improve the consistency of the results. In this paper, we present a method which combines an easy extraction procedure with toluene and analysis on HPLC for determination of *para*-red upto 1 ppm in red chilli powder and other related food materials.

Results and Discussion

With the presented method, 25 samples of red chilli powder, pickle and spices, bought from the local market have been analyzed for *para*-red, out of which four tested positive. Toluene was used for extraction to prepare sample and *para*-red standards due to maximum solubility and oily nature of *para*-red dye. The method was in-house validated using red chilli powder based on following criteria: limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability and linearity in red chilli powder.

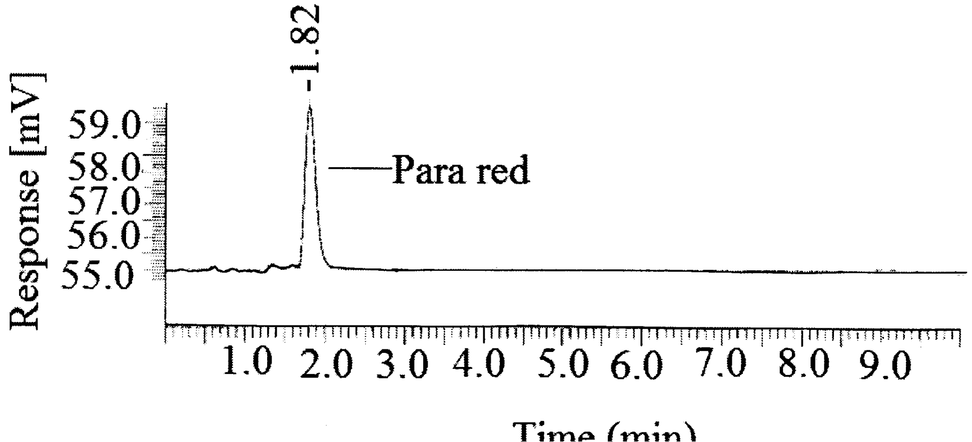
Extraction recovery was verified by spiking the sample with pure *para*-red (1 and 2 mg/kg). Recovery values were ranged from 97-99 %. The LOD and LOQ were 1 mg/kg and 3 mg/kg in sample, respectively. The repeatability and linearity were 1.91 % (RSD, n = 7) and $r^2 > 0.98888$ (in the range of 1.0 to 100 mg/l), respectively.

The UV-detector was set at 488 nm due to maximum absorption of *para*-red at this wavelength. The retention times were ranged from 15 to 20 min. *Para*-red was identified in the sample by virtue of its retention time and comparison of its wavelength scanned with reference to the working standard solutions. The areas of any identified peaks at 488 nm from the test portions extract and working standard solutions chromatogram were recorded and were used in calculation to find the concentration of *para*-red if present. Results of the analysis of 25 samples are presented in the Table-1.

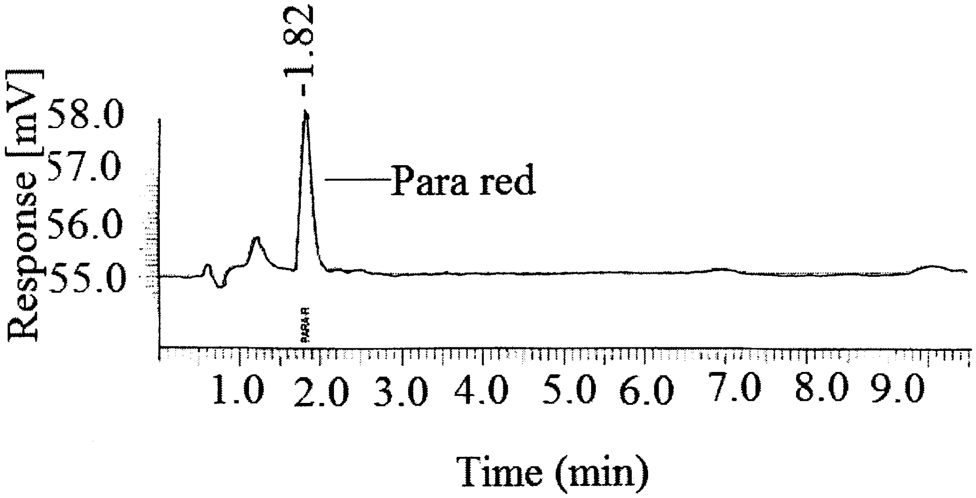
Table-1: Analysis of *Para*-red.

Sample No	Name of Sample	Results
1.	Pickle	- ve
2.	Pickle	+ ve (75 ppm)
3.	Red chilli powder	- ve
4.	Red chilli powder	- ve
5.	Red chilli powder	- ve
6.	Red chilli powder	- ve
7.	Red chilli powder	+ ve (100 ppm)
8.	Spices	- ve
9.	Mix Spices	- ve
10.	Red chilli powder	- ve
11.	Red chili powder	- ve
12.	Red chilli powder	- ve
13.	Mix Spices	- ve
14.	Red chilli powder	+ ve (200 ppm)
15.	Red chilli powder	- ve
16.	Red chilli powder	- ve
17.	Red chilli powder	- ve
18.	Red chilli powder	- ve
19.	Red chilli powder	- ve
20.	Red chilli powder	- ve
21.	Red chilli powder	- ve
22.	Red chilli powder	- ve
23.	Red chilli powder	+ ve (120 ppm)
24.	Chilli whole	- ve
25.	Tabasco Sauce	- ve

One major problem in the previously described methods was the sample or matrix interferences which in the present method have been solved by adding tetraethylammonium bromide (TEAB) and potassium dihydrogen phosphate (KH_2PO_4) in the mobile phase. A possible complex formation between *para*-red and ionic reagents like TEAB delayed elution time of *para*-red (R_t >10 min) Fig. 2. Other sample interferences were eluted before *para*-red thus reducing the risk of sample or matrix interferences. This can also eliminates the possible risk of interferences caused by carotenoids etc. present in *Capsicum* as they also absorb in the range of some of the dyes; making this method superior to the other methods described earlier. Same studies were carried out in these labs using non-ionic mobile phase ($MeOH:H_2O$; 80:20) where *para*-red was eluted with the sample interferences (R_t < 5 min) (Fig. 3) which not only gave false positive results but also causes inaccurate calculation of the area of peak, thus resulting in unreliable quantitative results.



a = Para red std



b = Para red std + sample

Fig. 2: HPLC elution profiles in non-ionic medium.

Experimental

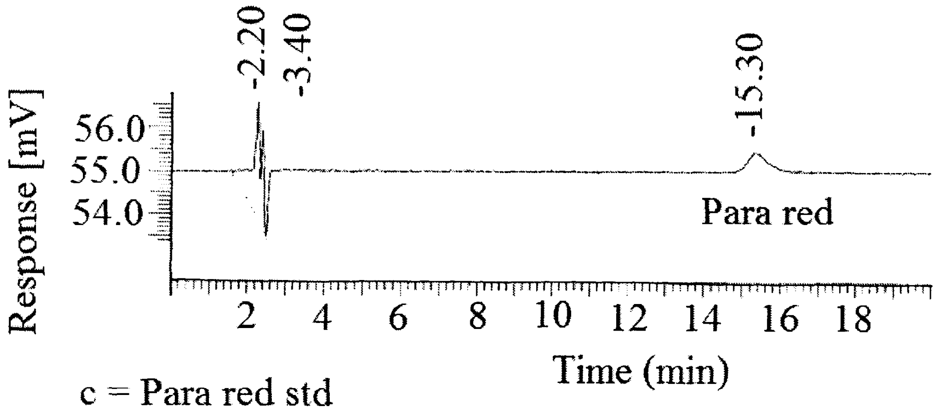
Materials and Methods

- The experiments were conducted on red whole chilli, chilli powder, mix spices, pickles and tobasco sauce bought from the local market.
- Methanol HPLC grade (Sigma-Aldrich)
- *Para*-red (95 %, Aldrich, code 10,099-4)
- Tetraethylammonium bromide (TEAB)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Water (de-ionized)
- 0.45 μ filter membrane

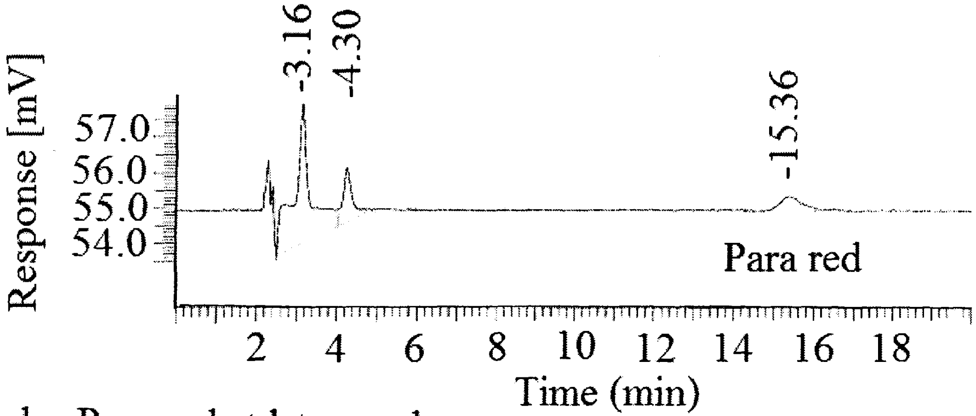
Mobile Phase
a = Non Ionic Mobile phase MeOH:H_2O; 80ml:20ml
b = Ionic Mobile Phase: MeOH:H_20; 80ml (containing 1.01 g tetraethylammonium bromide): 20ml (containing 0.034 g KH_2PO_4)

HPLC Conditions

HPLC Perkin Elmer (Perkin Elmer, Norwalk CT, USA Model- 200) was used that was equipped with a Rheodyne injector (20 µl loop), binary pump series 200 and tuneable UV absorbance detector performing detection at λ = 488 nm. 20 µl of each



c = Para red std



d = Para red std + sample

Fig.3: HPLC elution profiles in ionic medium.

standard solutions were injected sequentially. Same volume of test portion extracts were injected. Sample extracts were injected in duplicate and bracketed with standard solutions. Sample spiked with *para*-red were also injected. The column used for chromatography was Waters® ODS-1 (C_{18}) (4.6 mm × 250 mm, 5 μm), stainless steel and fitted horizontal. The mobile phase consisted of deionized water 200 ml containing 0.34 g KH_2PO_4 and HPLC grade methanol 800 ml containing 1.01 g tetraethylammonium bromide and filtered through 0.45 μ membrane filter. The flow rate of mobile phase was maintained at 1 ml/min. Acquisition software used was Total Chrom TM Workstation Perkin Elmer. The retention times were ranged from 15 to 20 min.

Para Red Stock Solution

0.1000 g of *para*-red was weighed and transferred to a 100 ml volumetric flask with 10 ml of toluene. The solution was sonicated and volume was made up to the mark with HPLC grade methanol with a concentration of 1000 mg/l. This solution has a concentration of 1000 mg/l.

Para Red Working Solutions

Standard solutions with concentrations of 1.0 mg/lit, 5.0 mg/l, 10.0 mg/l, 20.0 mg/l, 50.0 mg/l, 100 mg/l of *para*-red in methanol were prepared. The solutions were protected from light as far as practicable by wrapping in aluminum type foil.

Extraction Procedure for Sample

An amount of about 5 g, exactly weighed sample, was taken in a thimble and extracted with 250 ml toluene in Soxhlet assembly for two hours. The solid samples were finely ground by an electric blender while wet chilli-containing food products (*e.g.* relishes, chutneys and pickles) were homogenized as possible prior to extraction. Extraction was carried out on heating mental. The extraction was repeated thrice, every time recovering the toluene that was evaporated on rotary evaporator. The concentrated extract was dissolved in toluene, filtered and transferred into a 50 ml volumetric flask. The volume was made up with HPLC grade methanol.

Calculation

The amounts of *para*-red were determined by the following expression;

$$\text{Concentration (mg/kg)} = \frac{\mathbf{A}}{\mathbf{B}} \times \mathbf{Y} \times \frac{\mathbf{50}}{\mathbf{W}} \times \mathbf{D}$$

where

A = mean area of an identified peak in the test portion extract

B = mean area of peak of interest in the working standard solutions

Y = concentration (mg/l) of the suitable working standard solutions

W = weight of the sample

D = dilution factor (if any)

Conclusion

The presented HPLC method has been used for the routine analysis of *para*-red. The method is suitable for identification of *para*-red in chillies and other food products. Among 25 samples of red chilli powder, pickle and spices screened, four tested positive for *para*-red. Limit of detection (LOD) and Limit of quantification (LOQ) were upto 1 mg/kg and 3 mg/kg respectively. The recovery values were ranged between 97-99 %. Repeatability and linearity obtained for *para*-red standard solutions were 1.91 % (RSD, n = 7) and $r^2 > 0.98888$ (in the range of 1.0 to 100 mg/l), respectively. The use of toluene as an extraction solvent on other foods containing chilli, such as sauces, sausages, and other matrixes is also an advantage.

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