

Evaluation of Total Carotenoids in Chilly: A Guide to Forensic Examination of Chilly Powder by Spectrophotometry

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Abstract

The frequent use of dried chilli powder in dacoity and murder cases is routine and quite common practice in criminology. The main active ingredient is "Capsaicin" a flavonoid which is highly pungent and causes lachrymatory effects. The next most important ingredient is "Capsanthin" a carotenoid which imparts colour to chilly (Capsicum). Capsicum is normally classified by its extractable colour and this important factor determines the quality of the final product. The extractable colour was determined by American Spice Trade Association (ASTA) which refers to the international standard for measuring the extractable colour by Spectrophotometry.

Our study was carried out in three stages. Stage 1 involves the utility of various solvents namely methanol, acetone, hexane, chloroform and ethyl acetate and the contribution of these solvents for efficient pigment extraction was examined thoroughly and comparatively. Stage 2 involves the utility of ASTA method for the measurement of total extractable colour of Capsicum powder by UV-Vis Spectrophotometer. Stage 3 involves the identification of any added commercially available dyes by Thin Layer Chromatography and Spectrometry.

Technical Application: The uniqueness of total extractable carotenoids property is made use of in this study and helps in connecting the crime with the criminal in murder and dacoit cases and also in trade mark violation cases of comparing questioned and standard brand samples and to find out the presence of adulterants such as commercially available dyes.

Keywords: ASTA; Extractable colour; Carotenoids; Spectrometry.

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Introduction

Chillies are the fruits or berries of plants belonging to the genus Capsicum of the Nightshade family, Solanaceae.

The five available species are Capsicum annum L., Capsicum frutescens L., Capsicum chinense Jacq., Capsicum baccatum L., and Capsicum Pubescens R & P. Capsicum annum is the most wide spread in terms of household consumption and industrial processing. It is a source of biologically active compounds such as flavanoids capsaicinoids,

carotenoids capsanthin & capsorubin, vitamins, proteins, fibres and mineral elements. It is normally divided into two types sweet or mild pepper and hot or chilli pepper. Paprika extracts are extensively used in food industry as natural flavoring but also as a colouring agent for many foods such as spicy foods.

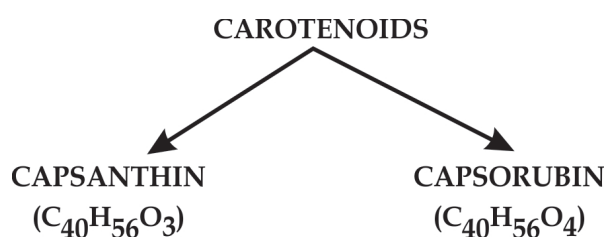
In this context, we conducted studies on three stages. First stage involves the utility of various solvents in the category namely alcohols, ketone, hydro carbon solvents, chlorinated hydro carbon solvents and esters and their contribution for

maximum and efficient pigment extraction was examined thoroughly. Systematic and routine chemical analysis was carried out to find out the natural carotenoids in chilly. Second stage involves the measurement of total extractable colour in terms of American Spice Trade Association (ASTA) unit (total carotenoids).

Third stage involves the interpretation of ASTA unit in different brands of chilly powder preparations and to find out the presence of any added commercially available dyes apart from natural carotenoids.

Carotenoids – Basic Concept

Paprika extract primarily consists of Carotenoids-natural pigments namely Capsanthin and Capsorubin, flavanoids namely capsaicin, ascorbic acid and fixed fruit oil & ascorbic acid.



Turmeric: The active constituent or turmeric is Curcumin, is the colouring pigment present, which imparts yellow colour. It is 1,7bis(4-hydroxy-methoxy phenyl)-1,6-heptodiene-3,5dione and also known as natural yellow 3 and it is a poly phenolic compound.

The majority of carotenoids exhibit absorption in the visible region of spectrum, between 400 and 500nm. Because they obey the Beer-Lambert law i.e., absorbance is linearly proportional to the concentration, absorbance measurements can be used to quantify the concentration of total carotenoids in chilly samples.

ASTA refers to the international standard for measuring the extractable colour of chilli pods, powder and other chilly powder preparation namely sambar powder and are determined by the Spectrophotometric method.

Paprika is normally classified by its extractable colour (total carotenoids) and this important factor determines the quality and price of the product. The factors that normally affect the colour of the dried chillies include cultivation, stage of maturity at harvest and subsequent curing, fruit drying conditions and the final moisture content.

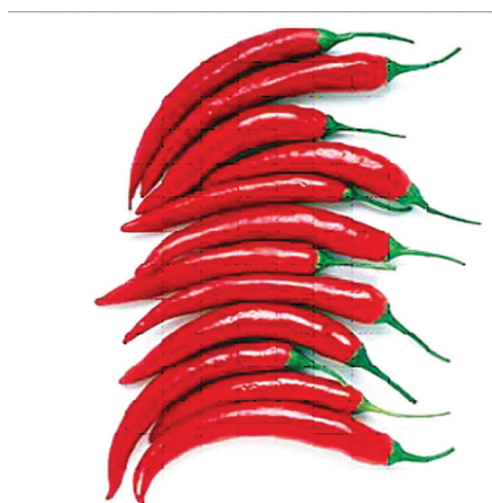


Fig. 1: Red Chilly.



Fig. 2: Chilly Powder



Fig. 3: Chilly Masala Powder (Sambar Powder)



Fig. 4: Turmeric Powder

Protocol

Forensic Science Laboratories are frequently called upon to examine the presence of chilli powder encountered in murder or theft cases and to compare the same with the suspected one. A

routine chemical examination followed by the instrumental methods of analysis is very much useful to connect the crime with the criminal. The presence and comparison gives the information about the presence of accused in scene of crime.

In our study red chilly of three varieties, commercially available chilly powder from three different brands, chilly powder preparation namely sambar masala powder from two different brands and turmeric powder were taken and their ASTA units (total carotenoids) were measured by using Spectrophotometer.

They are marked as RC-A, RC-B & RC-C: CP-A, CP-B & CP-C: CMP-A & CMP-B : TP-A for identification and comparison purposes. This study was very much useful not only for crime cases but also for cheating cases namely trademark violation cases to compare the questioned and standard.

Analytical Investigations

Reagents

1. Methyl alcohol
2. Acetone
3. Hexane
4. Chloroform
5. Ethyl acetate
6. Potassium dichromate
7. Cobaltous ammonium sulphate
8. Sulphuric acid
9. Phosphomolybdic acid (1%) in Ethyl alcohol
10. Fast Blue B in Ethyl alcohol
11. Nin-hydrin (1%) in n-butyl alcohol
12. Sodium hydroxide (5%) aqueous solution (All reagents and chemicals are analytical grade).
13. Red chilly purchased from different regions of Chennai (RC-A, RC-B & RC-C).
14. Commercial chilly powder purchased from the market (CP-A, CP-B & CP-C).
15. Commercial chilly masala powder purchased from the market (CMP-A & CMP-B).
16. Turmeric powder purchased from Chennai (TP-A).
17. Synthetic red dye

Methods

Extraction

At first, five numbers of clean and dried conical flask (100ml capacity) were taken. 100mg of unground dried red chilli must be ground to pass 1mm opening sieve was taken in the first conical flask and to this 50 ml of methanol was added and homogenized well and allow the extraction to proceed for at least 15 minutes with occasional shaking and the homogenate was separated and transferred into a clean and dried 100 ml standard volumetric flask carefully and dilute to the mark with methanol. (extract 01) & kept in dark for 1hour. The above extraction procedure was carried out with acetone, hexane, chloroform and ethyl acetate separately in the respective conical flasks containing weighed, ground & dried red chilly and each one of them was subjected for examination using Spectrophotometer. (extract 02, 03, 04 & 05)

Next, 75mg of different brands of red chilly, chilly powder & chilly masala powder were taken in a separate conical flask (100ml capacity) and to this 50ml of methanol & acetone (1:1) mixture was added to each sample and homogenized well and allow the extraction to proceed for at least 15 minutes with occasional shaking and the corresponding homogenates were separated and transferred into their corresponding dried 100ml standard flask carefully and dilute to the mark with methanol and acetone mixture & kept in dark for 1hour and each of them was subjected for examination using Spectrophotometer.

A suitable portion of the marked samples were extracted using methanol and acetone (1:1) mixture for the determination of carotenoids by routine method. The extract was evaporated to dryness. The dried residue was dissolved in methanol & acetone mixture and subjected to Thin Layer chromatography.

Systematic & Routine Chemical Analysis

Spot test

- a. To the dried substance or methanolic extract in a depression tile, a drop of chloroform was added followed by concentrated sulphuric acid in dropwise. Appearance of blue colour indicates the positive test for the presence of chilly powder.
- b. To the dried substance or methanolic extract in a depression tile, a drop of 5% sodium hydroxide was added. Appearance of red colour indicates the positive test for the presence of turmeric.

Table 1: Results obtained from Spot Test.

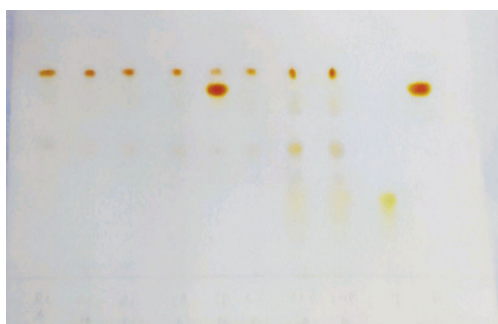
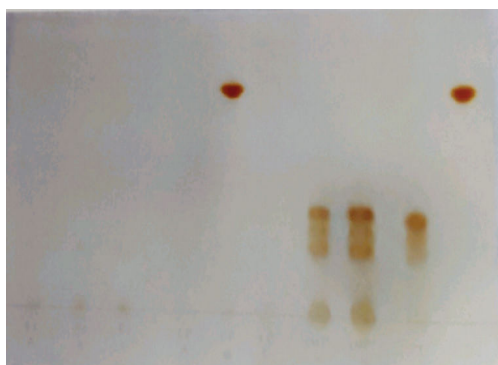
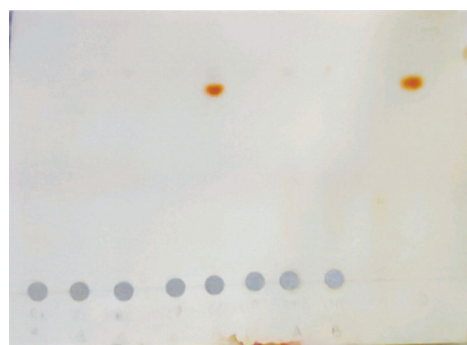
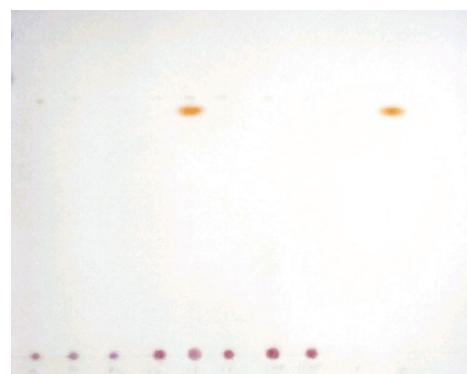
Sample Number	Chilli	Turmeric
RC-A	Present	Absent
RC-B	Present	Absent
RC-C	Present	Absent
CP-A	Present	Absent
CP-B	Present	Absent
CP-C	Present	Absent
CMP-A	Present	Present
CMP-B	Present	Present
TP-A	Absent	Present

Thin Layer Chromatography

Thin Layer chromatographic technique was carried out using precoated Silica Gel GF-254 Plates (adsorbent) and different solvent systems were tried. Phosphomolybdic acid (1%) in ethanol, Ninhydrin (1%) in n-butanol and Fast Blue B in alcohol was used as visualizing agents. The results are summarized in Table 2 and the Thin Layer chromatograms are shown in (Fig 5, 6, 7 & 8).

THIN LAYER CHROMATOGRAM

(RC-A, RC-B, RC-C, CP-A, CP-B, CP-C, CMP-A, CMP-B, TP-A & RED DYE).

**Fig. 5:** Separation of carotenoids – visual examination before spray.**Fig. 6:** Chromatogram sprayed with 1% Fast Blue B in alcohol.**Fig. 7:** Chromatogram sprayed with 1% phosphomolybdic acid in ethanol.**Fig. 8:** Chromatogram sprayed with 1% Ninhydrin in n-butanol.**Table 2:** Identification of Capsanthin, Curcumin & Ascorbic acid.

Solvent Systems	Compound	hRf	Spot
Benzene: Ethyl acetate (80:20)	Capsanthin	55	Orange
	Curcumin	37	Yellow
	Ascorbic acid	00	Blue-grey & purple
	Red dye	92	Red
n-butanol:acetic acid: water (65:15:20)	Capsanthin	90	Orange
	Curcumin	75	Yellow
	Ascorbic acid	52	Blue-grey & purple
	Red dye	88	Red

UV-Vis Spectrometry

Selection and choice of Solvent & λ_{max}

Perkin Elmer Lambda25 model UV- Vis spectrophotometer along with the computer integrated system was used for the analysis.

The experimental conditions are:

Model : Perkin Elmer Lambda25 model UV- Vis Spectrophotometer

Wavelength : 500 – 400nm

Scan Speed : 60nm/mt

The extract 01 to 05 obtained from 4.2.1 was analysed in the visible range in the scan mode to find out the maximum absorbance. Then the same extracts were analysed in the wavelength programme mode and the absorbances were measured at 420nm, 440nm, 460nm, 480nm and 500nm. The results are summarized in Table:3 The UV -spectrum of red chilly was shown in Fig (9) & turmeric in Fig (10).

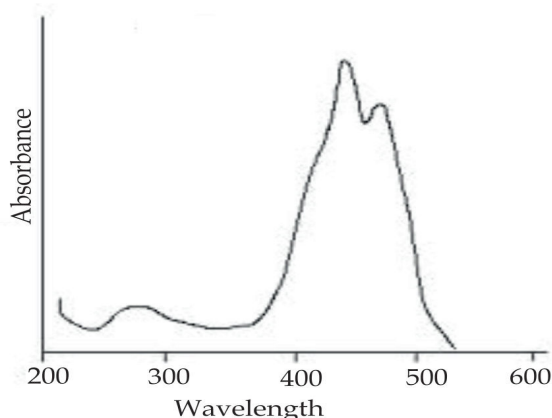


Fig. 9: UV-Spectrum of Red Chilly.

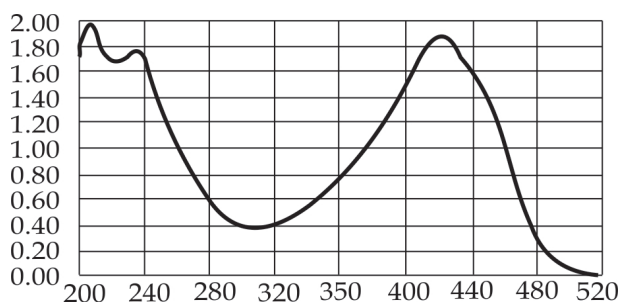


Fig. 10: UV-Spectrum of turmeric.

Table 3: Absorbances of Red chilly in different solvents and at different wavelengths.

Solvents/ Extract No	420 nm	440 nm	460 nm	480 nm	500 nm
Methanol 01	0.2053	0.1944	0.3327	0.1877	0.1253
Acetone 02	0.2013	0.1940	0.3312	0.1846	0.1238
Ethyl acetate 03	0.2008	0.1897	0.3122	0.1656	0.1132
Hexane 04	0.1955	0.1800	0.2877	0.1434	0.0955
Chloroform 05	0.1933	0.1756	0.2822	0.1388	0.0933

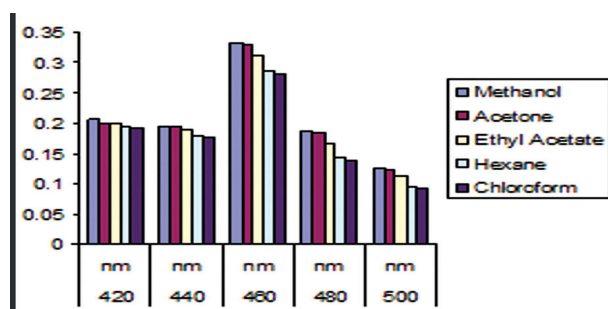


Fig. 11: Graphical Representation.

Total Extractable Colour (ASTA Units)

Standard colour Solution

0.3005gm/litre potassium dichromate and 34.96gm/litre cobaltous ammonium sulphate crystals in 1.8 M sulphuric acid was prepared. The absorbance of this solution was measured at 460nm. (As) using 1.8 M sulphuric acid as blank. The absorbance of this solution in a 1-cm path length cell at 460nm should be about 0.600.

Measurement of Samples

The absorbance (A) of the extracts of the samples obtained from 4.2.1 were measured at 460nm & 420nm using methanol and acetone (1:1) mixture as blank.

Calculation

Calculate instrument correction factor I_f from

$$I_f = 0.600 / A_s \text{ measured value (0.98).}$$

Calculate extractable colour (ASTA Colour Units).

$$ASTA = \frac{A \times 16.4 \times I_f}{\text{Sample wt in gm}}$$

Where

A = absorbance of sample

16.4 = factor for carotenoids

I_f = Instrument correction factor

The results are summarized in Table: 4

Table 4: Measurement of ASTA Units in Methanol:acetone (1:1) mixture.

S.No	Samples	Absorbances at		ASTA units determined at 460nm
		420nm	460nm	
01	RC-A	0.0772	0.2987	60.00
02	RC-B	0.0625	0.2324	45.55
03	RC-C	0.0877	0.3049	70.00
04	CP-A	0.0670	0.2566	55.00
05	CP-B	0.4778	0.7422	149.10
06	CP-C	0.1060	0.3330	76.46
07	CMP-A	0.2588	0.1633	35.00
08	CMP-B	0.2877	0.1754	40.27
09	Turmeric	0.3865	0.0015	0.3444

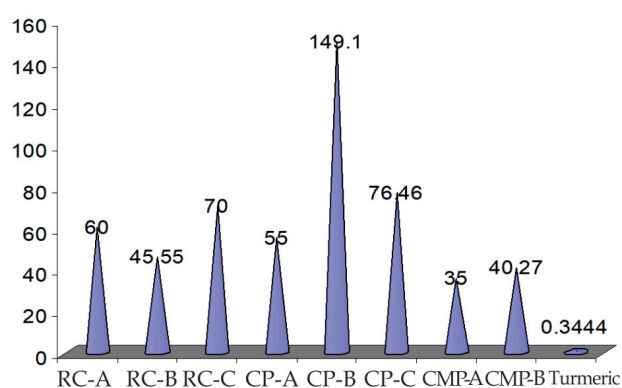


Fig. 12: Graphical Representation.

Results and Discussion

Fig. 1-4 showed the photo image of Red chilly, Chilly powder, Chilly masala powder (sambar powder) and Turmeric powder respectively. Systematic and routine chemical analysis was carried out and the spot test results are summarized in Table 1 which showed that chemical characteristics of chilly were detected in samples in S. no. 1 to 8 but not detected in sample in S. no. 9 and similarly the chemical characteristics of turmeric were detected in samples in S. no. 7 to 9 but not detected in samples in S.no. 1 to 6. This examination showed that samples marked CMP-A & CMP-B contains turmeric in addition to chilly (indication of masala powder preparation not pure chilly powder).

Thin layer chromatographic technique was used as a separation and identification technique. Various solvent systems were tried and good separation of carotenoids was achieved using Benzene:Ethyl acetate (80:20) Fig. 5. The first chromatogram was sprayed with 1% Fast Blue B in Ethanol turmeric appeared as multiple brownish red spots Fig. 6. The second chromatogram was sprayed with 1% Phosphomolybdic acid in Ethanol and dried at 60°C. Paprika characteristics of chilly appeared as blue-grey spots at the point of application but turmeric did not produce any coloured spots Fig. 7. The third chromatogram was sprayed with 1% Nin-hydrin in n-butanol and dried at 60°C. Again Paprika characteristics of chilly appeared as purple coloured spots at the point of application but turmeric did not react with it Fig. 8. Separation was carried out using n-butanol:acetic acid:water (85:15:20) as solvent system but carotenoids did not separate at all and appeared as single spot. But ascorbic acid present in paprika separated well and reacted with the % Phosphomolybdic acid in Ethanol and 1% Nin-hydrin in Ethanol. Table 2 showed the adsorption behavior of capsanthin, curcumin and ascorbic acid and red dye.

Thin layer chromatographic technique was used to detect capsanthin in RC-A, RC-B, RC-C, CP-A, CP-B, CP-C, CMP-A & CMP-B but not in turmeric and commercially available red dye (food colours) and Curcumin in CMP-A, CMP-B & turmeric but not in RC-A, RC-B, RC-C, CP-A, CP-B & CP-C. In addition to natural carotenoids, commercial red dye was detected in CP-B but not in others which showed that chilly powder is adulterated with synthetic colouring matter.

UV-Visible spectrometry was quite useful and effective in the determination of total carotenoid content. Fig. 9 & 10 showed the Spectral characteristics of chilly and turmeric. This analysis was carried out in three ways. At first different solvents were tried and the solvent which extracted the total carotenoids based on their absorbance were shown in Table 3. Next, wavelength maximum for maximum absorbance was fixed at 460nm for paprika and 420nm for turmeric. Fig. 11 Illustrated the graphical representation of absorbance of red chilli in various solvents. It was determined that the solvents used were very important in the pigment concentration and methanol & acetone were found equally good and hence mixture of methanol & acetone (1:1) were used for further determination.

ASTA units are the extractable colour in paprika and paprika preparations which corresponds to total carotenoid content. The ASTA units show the total pigment concentration synthesized in the fruit and the relation between the red and yellow carotenoids. In the present study lowest ASTA units were found in turmeric variety (0.3444) and the total yellow pigment concentration was higher in turmeric due to the presence of curcumin. Similarly the total red pigment concentration was higher in CP-B sample (149.10 ASTA units) and this abnormal increase suggested the presence of any extraneous colouring matter apart from natural carotenoids. The presence of dye is already confirmed by TLC. Considerable increase in total yellow pigment concentration in CMP-A & CMP-B samples and decrease in total red pigment concentration due to the contribution of curcumin from turmeric to capsanthin in chilly.

These experimental measurements in Table 4 was useful to find out the sample whether they are chilly powder alone or other chilly powder preparations like sambar powder and if there is any adulteration using commercially available dye. The results obtained in this work are quite consistent. Fig. 12. Illustrated the graphical representation of total extractable colour. (ASTA) of samples taken for this work.

It must be remembered that carotenoids are sensitive to light, heat, air and active surfaces; therefore, precautions must be taken during preparation and the solutions should be kept in dark before spectral measurement.

Conclusions

Capsaicin (characteristic flavanoid) and Capsanthin (characteristic carotenoid) are the active constituents of Chilly. Curcumin (characteristic carotenoid) is the active constituent of turmeric. The solvents used in the pigment extraction have an important effect and it was determined that the best solvent used for chilly and turmeric was the mixture of methanol & acetone (1:1). ASTA measurements were observed at 460nm and 420nm for identification and comparison.

Earlier work suggested that the determination of capsaicin by HPLC was useful in establishing the origin of chilly in crime cases. The most advantage of this present study was the sample quantity (even 10 mg sample is sufficient), the simple and speedy extraction procedure and experimental time & calculation. This ASTA determination was very unique and easier parameter for comparison. In general, the chilly powder encountered in crime cases are in trace levels the determination and comparison to connect the crime with the criminal plays a great and difficult task in the hands of Forensic Expert.

Good results of total carotenoid by ASTA were obtained after the analysis of chilli powder present in the victims clothes and from the suspect. Though the colour and pungency of paprika depends on many factors but Forensic examination

normally involves inclusion or exclusion among two samples.

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