2. INTRODUCTION

This thesis deals with the research carried out by the writer for the past three years on structural characterization of degradation products in various food dyes by HPLC-DAD-MS/MS and evaluating their *in vitro* genotoxic potential by bacterial reverse mutation assay. Before discussing the experimental procedures adopted and results obtained, a brief introduction on food dyes, permitted azo dyes in food, analytical methods, validation, need for the identification and toxicity evaluation of degradation products of food dyes would be discussed in detail.

a. Food dyes

Colour is one of the important properties by which food is generally evaluated. It influences the perception of food quality and stimulates the appetite, since it is perceived to relate to flavor (1). The use of colour additives dates back to the Ancient Egyptian period (1500 BC) when colorants were added to wine and confectionaries (2). Since then numerous naturally-derived colours (such as indigo, alkanet (borage root), turmeric etc.) have been used to improve the appearance of foods. The use of mineral and metal based compounds for this purpose was also reported following the industrial revolution. However, the ingestion of such toxic colouring chemicals as red lead (Pb₃O₄), vermilion (HgS) and copper arsenate in food could cause illness and death (1). In 1856 the British chemist Sir William Henry Perkin created the first synthetic dye ‘mauveine’ by oxidizing aniline (2). As a result of the “colour revolution” thousands of new colouring compounds were synthesised for industrial applications.

A wide variety of food colorants of both natural and synthetic origin are added to food products in order to make them more visually aesthetic to consumers and to reinstate their original appearance which was lost during production process. However, most of the dyes obtained from natural sources are unstable and can undergo degradation easily during the processing of food. Therefore, dyes of synthetic origin are widely used, not only because of their stability but also the cost of production is very low when compared to dyes of natural origin (3). These synthetic dyes are divided into five major classes: azo compounds (Tartrazine, Carmoisine and ponceau 4R etc.), the triaryl methane group (Fast Green FCF), the chinophthalon derivatives of Quinoline Yellow, Xanthenes (such as Erythrosine), and the indigo dyes (4).

Azo-dyes are synthetic food colours and do not occur naturally. These colours are manufactured by chemical synthesis that allows them to be produced in high purity, constant quality, and large quantities. Within the synthetic food colours, azo dyes are the
largest group and are able to provide colouring shades from yellow, red, blue, and black (5). The chemical group classifying colours as azo-dyes is the presence of azo groups (-N=N-) in the molecular structure, typically linking two aromatic systems. Depending on the number of azo groups in the molecular structure, azo colours can be classified into mono-, di-, tri-, tetra-, and poly-azo compounds (6).

Azo dyes have many applications in the food and beverage industries, such as being used to make food more appealing, hiding defects or to strengthen consumer perception of the association between colour and flavor. For example, lime flavor is associated with the colour green and, thus, lime soft drinks are often coloured with green food dye. However, some azo dyes have potential toxicity because these compounds are mainly metabolized in the intestinal wall and liver, producing free aromatic amines that are potentially neurotoxic, carcinogenic and mutagenic (7-9). Because of such problems many countries in the world have banned the usage of most of the azo dyes in food products. Over the last few decades, the use of azo dyes in food has been highly regulated by each country's domestic and export food supplies. Most of the countries generally follow the regulations of those of seven major world markets (10). The detailed information on approved azo dyes for coloring food by different countries are given in Table 1 and 2.

Table 1. List of azo food dyes approved and banned in different countries

<table>
<thead>
<tr>
<th>Country</th>
<th>E102</th>
<th>E110</th>
<th>E121</th>
<th>E122</th>
<th>E123</th>
<th>E124</th>
<th>E129</th>
<th>E151</th>
<th>E154</th>
<th>E155</th>
<th>E180</th>
<th>Orange B</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td>x</td>
<td>X</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<tr>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<td>x</td>
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<td>x</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
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<td>✓</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
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<td>✓</td>
<td>x</td>
<td>x</td>
<td>✓</td>
<td>X</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Australia and New Zealand</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

(✓) approved; (x) banned azo dyes.

USA, United States of America; EU, European Union.
Table 2. Names, codes, chemical formulae, structure, colour, solubility and average molecular weight of azo dyes used in food matrices

<table>
<thead>
<tr>
<th>Names, codes and chemical formula</th>
<th>Structure</th>
<th>Colour</th>
<th>Solubility</th>
<th>MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tartrazine (E102), C.I. 19140, FD&amp;C Yellow 5, Acid Yellow 23/Trisodium (4E)-5-oxo-1-(4-sulfonatophenyl)-4-[(4-sulfonatophenyl)hydrazono]-3-pyrazolecarboxylate C_{16}H_{9}N_{4}Na_{3}O_{9}S_{2}</td>
<td><img src="image" alt="Structure of Tartrazine" /></td>
<td>Lemon yellow</td>
<td>Water</td>
<td>534.36</td>
</tr>
<tr>
<td>Sunset yellow FCF (E110), C.I. 15985, Orange Yellow S, FD&amp;C Yellow 6/ Disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate C_{16}H_{10}N_{2}Na_{2}O_{7}S_{2}</td>
<td><img src="image" alt="Structure of Sunset yellow FCF" /></td>
<td>Yellow</td>
<td>Water and Ethanol</td>
<td>452.37</td>
</tr>
<tr>
<td>Citrus Red 2 (E121), C.I. Solvent Red 80, C.I. 12156/1-(2,5-Dimethoxy-phenylazo)-naphthalen-2-ol C_{18}H_{16}N_{2}O_{3}</td>
<td><img src="image" alt="Structure of Citrus Red 2" /></td>
<td>Orange to yellow</td>
<td>Insoluble in water but readily soluble or dark in many organic solvents</td>
<td>308.33</td>
</tr>
</tbody>
</table>
Carmoisine (E122), Azorubine, Food Red 3, Azorubin S, Brilliant carmoisin O, Acid Red 14, or C.I. 14720/disodium 4-hydroxy-2-[(E)-(4-sulfonato-1- naphthyl)diazenyl]naphthalene-1-sulfonate C_{20}H_{12}N_{2}Na_{2}O_{7}S_{2}

Amaranth (E123), FD&C Red No. 2, C.I. Food Red 9, Acid Red 27, Azorubin S, or C.I. 16185/trisodium (4E)-3-oxo-4-[(4-sulfonato-1-naphthyl)hydrazono]naphthalene-2,7-disulfonate C_{20}H_{11}N_{2}Na_{3}O_{10}S_{3}

Ponceau 4R (E124), C.I. 16255, Cochineal Red A, C.I. Acid Red 18, Brilliant Scarlet 3R, Brilliant Scarlet 4R/trisodium (8Z)-7-oxo-8-[(4-sulfonatonaphthalen-1-yl)hydrazinylidene]naphthalene-1,3-disulfonate C_{20}H_{11}N_{2}Na_{3}O_{10}S_{3}

Red to Water maroon

Reddish-brown, dark red to purple

Red Water and Ethanol 604.47
Allura Red (E129), Food Red 17, C.I. 16035, FD&C Red 40/ disodium 6-hydroxy-5-((2-methoxy-5-methyl-4-sulfophenyl)azo)-2-naphthalenesulfonate
C\textsubscript{18}H\textsubscript{14}N\textsubscript{2}Na\textsubscript{2}O\textsubscript{8}S\textsubscript{2}
Brilliant Black BN (E151), Brilliant Black PN, Brilliant Black A, Black PN, Food Black 1, Naphthol Black, C.I. Food Brown 1, or C.I. 28440/Tetrasodium (6Z)-4-acetamido-5-oxo-6-[[7-sulfonato-4-(4-sulfonatophenyl)azo-1-naphthyl]hydrazono]naphthalene-1,7-disulfonate C\textsubscript{28}H\textsubscript{17}N\textsubscript{5}Na\textsubscript{4}O\textsubscript{14}S\textsubscript{4}
Brown FK (E154)*, Kipper Brown, Chocolate Brown FK, and C.I. Food Brown 1
Brown HT (E155), Chocolate Brown HT, Food Brown 3 and C.I. 20285/Disodium 4-[(2E)-2-[(5Z)-3-(hydroxymethyl)-2,6-dioxo-5-[(4-sulfonatophenalen-1-yl)hydrazinylidene]-1-cyclohex-3-enylidene]hydrazinyl]naphthalene-1-sulfonate C\textsubscript{27}H\textsubscript{18}N\textsubscript{4}Na\textsubscript{2}O\textsubscript{9}S\textsubscript{2}

Brown Water 652.56

LitholRubine BK (E180), Pigment Rubine, Carmine 6B, Brilliant Carmine 6B, Permanent Rubin L6B, Litholrubine, Latolrubine, C.I. Pigment Red 57, C.I. Pigment Red 57:1, D&C Red No. 7, or C.I. 15850:1/Calcium (4Z)-4-[(4-methyl-2-sulfonatophenyl)hydrazono]-3-oxo-2-naphthalenecarboxylate C\textsubscript{19}H\textsubscript{12}CaN\textsubscript{2}O\textsubscript{6}S

Red Dimethylformamide 424.44
Orange B (C.I. Acid Orange 137)/ Disodium 4-[N'-[3-ethoxycarbonyl-5-oxo-1-(4-sulfonatophenyl)-4-pyrazolylidene]hydrazino]-1-naphthalenesulfonate

\[ \text{C}_{22}\text{H}_{16}\text{N}_{4}\text{Na}_{2}\text{O}_{9}\text{S}_{2} \]

* A multi component mixture of azo dyes
b. Regulation on food colours in India, United States, European Union, Japan and China

The legislation concerning food colours varies widely across different countries. In India the use of colours in food is regulated by the Food Safety and Standards Authority of India (10). The regulation in India permits eight synthetic colours (Tartrazine, Carmoisine, Ponceau 4R, Erythrosine, Sunset Yellow FCF, Indigo Carmine, Brilliant blue FCF and Fast Green FCF with a maximum permissible level of 100 ppm) and 11 natural or nature identical additives, no inorganic dyes are permitted. In the United States the use of colours in food is regulated by the U.S. Food & Drug Administration (F&DA). The regulations concerning food additives are contained in the Title 21 of the Code of Federal Regulations. There are only nine synthetic food colours allowed (that are subject to certification) of which seven are for general use. Thirty five derived from vegetable, animal, mineral sources or synthetic duplicates of naturally occurring colouring compounds are exempt from certification and eight of them are for use in animal feed only. The colours subject to certification must have their identity and purity checked by F&DA.

The first regulation within the European Economic Community countries concerning colouring matters was introduced in 1962. This was replaced by European Parliament and Council Directive 94/36/EC on colours for use in foodstuffs. Most recently Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 harmonizing the use of all food additives was introduced within the EU. Only additives included in EU regulations can be used in food. There are currently 25 colours of natural (or nature identical) origin and 15 synthetic on the list. Japanese legislation allows use of 12 synthetic colours in food. Chinese legislation permits 11 synthetic colours in food, of which one (new red) is illegal in the EU, US, Japan and India (11).

c. Photodegradation studies on food dyes

Recent interests devoted to the protection of the human health are addressed to study the degradation processes that some chemicals, contained in different matrices, can naturally undergo often for the action of sun light. Most of these studies concern the degradation of herbicides in the environment and only some consider the degradation of dyes. Great attention must be devoted to study the degradation pathway that takes place in wastes of dye industries, when undergone to decolourisation treatments before the final disposal. Ecological alarms denounce that photodegradation processes lead to the
decolourisation of the dye, but not to a complete mineralisation, since potentially toxic organic species are formed. A particular aspect of this phenomenon regards the natural degradation, for the action of sun light, of some food dyes largely used to colour soft drinks. These dyes are not toxic and allowed by the European Directives and FSSAI but, likely due to the co-presence of other ingredients, are unstable to the effect of sun light and naturally undergo degradation reactions. The current legislations lists the colourants permitted in food products and gives the maximum permissible amount, but it does not contain information on dye stability towards sunlight action, and possible side reactions with other compounds present in food and drinks (12).

d. Chromatographic methods

This is a method of separation, where the individual components are separated and analyzed. In this technique two or more components are separated by a dynamic differential migrational process, in a system consisting of two phases, one of which moves continuously in a given direction in which the individual components exhibit different mobilities due to the difference in their adsorption or partition or molecular size etc. Most reliable and widely used chromatographic techniques for the separation and estimation of dyes and their degradation products are liquid chromatography-mass spectrometry (LC-MS) and ultra high performance liquid chromatography coupled with high resolution tandem mass spectrometry (UHPLC-HR-MS/MS) (13-15).

(I) Liquid chromatography – mass spectrometry

This is one of the most commonly used hyphenated techniques. This creates an ideal analytical tool for the laboratory. An HPLC column can separate almost any mixture that can be dissolved and a mass spectrometer can ionize the separated peak solution and provide a molecular weight for each peak component. One of the most widely used liquid insertion interfaces for HPLC – MS is the thermo spray, which works best in reverse phase chromatography. HPLC – MS with thermo spray interface represents one of the ultimate analytical hyphenated procedures for the detection and identification of metabolites and drugs in the body fluids.

Sometimes two similar instruments were used in the analysis such as TANDEM Mass spectrometry, which involves coupling of one mass spectrometer to a second one, in which the first one isolates the molecular ions of various components of a mixture and the second one used for fragmentation of the molecular ions produced by the first one. The latest and recent methods involves, potentially more powerful combination of GC – FTIR – MS, in which these three instruments are operated in series. The GC effluent is
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usually split, so that about 2% goes directly to the more sensitive MS and the remaining 98% goes into the FTIR. The MS& IR spectra obtained is used, each for its separate library search (16).

(II) **Ultra high performance liquid chromatography coupled with high resolution tandem mass spectrometry (UHPLC-HR-MS/MS)**

The ultra-high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) technique is a new approach in the chromatographic separations and has been successfully employed for fast, high resolution separations with required sensitivity. The TOF-MS is helpful in the structure elucidation and identification of fragmentation patterns of the compounds. UHPLC is becoming an alternative technique to HPLC in the field of pharmaceutical and food analysis. In many aspects, it is a better technique as compared to HPLC; it shows better chromatographic separation, resolution, speed of analysis, and less solvent consumption (17).

In the TOF-MS, analytes are charged in the capillary tube which causes a coulomb explosion making smaller droplets of the analyte. These charged particles enter into evacuated flight tube and detected according to their mass values. Q-TOF is an ideal mass spectrometer for the analysis of large biomolecules such as peptides, proteins, amino acids, carbohydrates, fats and lipids. Hence, the technique must be employed for every area of analytical chemistry where the small amounts of analytes are determined with accepted accuracy and precision. The technique has been used recently in the identification of metabolites, degradation products, and harmful trace materials in drinking water, pesticide residues in foods, synthetic compounds, toxicants, organic pollutants and doping agents.

The analytical technique mostly used for degradation studies is HPLC-UV and/or HPLC-MS, but the main drawback is that, these techniques are time consuming and do not provide adequate information regarding resolution of all the impurities and degradation products. The UHPLC/Q-TOF-MS system provides changeable collision energy values and allows the generation of mass information with acceptable accuracy and precision, which is ultimately helpful in structure elucidation, identification of fragmentation patterns of the drugs, identification of degradation products and establishment of degradation mechanisms.

The QqTOF tandem mass spectrometer can be described in the simplest way as a triple quadrupole with the last quadrupole section replaced by a TOF analyzer. In the usual QqTOF configuration, an additional r.f quadrupole Q0 is added to provide
collisional damping, so the instrument (Figure. 1) consists of three quadrupoles, Q0, Q1 and Q2, followed by a reflecting TOF mass analyzer with orthogonal injection of ions (in one of the commercial instruments, the quadrupoles Q0 and Q2 are replaced by hexapoles; however, the basic operating principles are the same) (18).

Figure 1. Schematic diagram of the tandem QqTOF mass spectrometer.

For single MS (or TOFMS) measurements, the mass measurements, the mass filter Q1 is operated in the r.f. only mode so that it serves merely as a transmission element, while the TOF analyzer is used to record spectra. The resulting spectra have a benefit from the high resolution and mass accuracy of the TOF instruments, and also from their ability to record all ions in parallel, without scanning. In principle, it is still possible to perform Q1 scans for single MS analysis by using the TOF section as a total ion current detector only. However, owing to the described advantages of the TOF spectra, this operational mode is used for Q1 calibration and tuning only. One of the main advantages of QqTOF instruments over triple quadrupoles is the high mass resolution of TOF, typically around 40,000 (m/Δm, where Δm is the full peak width at half-maximum (FWHM). As a result of this, interfering peaks of ions having the same
nominal mass can be resolved partially or completely, the charge state of multiply charged ions in many cases can be determined from their isotopic spacing, and signal-to-noise ratio is improved owing to grouping of ions into narrower peaks (increasing the peak height). In general, higher the resolution the better will be the mass accuracy, if it does not compromise sensitivity (18).

e) Validation

Validation is the process of evaluating the performance of a measuring procedure and checking that the performance meets the present criteria. In simple words, validation of analytical procedure is to demonstrate that the procedure developed is suitable for its intended purpose and it works in a reproducible manner when carried out by the same or different persons, in the same or different laboratories, using different brands of chemicals, reagents and equipments etc.

Typical validation parameters as per Food and Agriculture Organization (FAO) guidelines (19), which should be considered, are,

(I) Accuracy
(II) Precision
(III) Specificity
(IV) Linearity and range
(V) Limit of detection and
(VI) Limit of quantitation

(I) Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals. Accuracy is calculated from the test results as the percentage of analyte recovered by the experiment.

(II) Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (Coefficient of variation) of a series of
measurements. The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimate of standard deviation or relative standard deviation. The precision determinations permit an estimate of the reliability of single determination and are commonly in the range of 0.3 to 3% for assays.

(III) Specificity

The Food and Agriculture Organization (FAO) documents define specificity as the ability to assess unequivocally the analyte into the presence of components that may be expected to be present, such as impurities, degradation products and matrix components.

In case of assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, it can be done by spiking the substance or product with appropriate levels of impurity or recipients and demonstrating that the assay results are unaffected by the presence of this extraneous material.

If impurity or degradation product standards are unavailable specificity may be demonstrated by comparing test results of samples containing impurities or degradation products to a well characterized procedure. This comparison should include sample stored under relevant degradation conditions.

(IV) Linearity and range

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. It should be established across the range of the analytical procedure. Linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentration of analyte.

The range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to determine with a suitable level of precision, accuracy and linearity using the method written. The range is normally expressed in the same unit as test result. (eg. percent/ppm).

(V) Limit of detection and

Limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitative, under the stated experimental conditions. The
detection limits is usually expressed as the concentration of analyte (eg. percent/ppm) in the sample.

(VI) Limit of quantitation

Limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. It is expressed as the concentration of analyte (eg. percentage, ppb) in the sample.

f) In vitro mutagenicity assay (Bacterial reverse mutation assay)

Mutagenicity refers to the induction of permanent transmissible changes in the structure of the genetic material of cells or organisms. These changes (mutations) may involve a single gene or a block of genes.

In vitro mutagenicity assay is also known as Ames assay. The test uses amino-acid requiring strains of Salmonella typhimurium and Escherichia coli to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (20-22). The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain.

Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds (23).