

## An alternative tracking dye for gel electrophoresis

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**Bromophenol blue (BPB) is regularly used as a dye marker to find out an ion front in the electrophoretic techniques. However, all the chemical, physical and toxicological properties of BPB have not yet been thoroughly investigated. Material safety data sheet of all standard companies providing BPB are advised that due care be exercised when handling this material as it may cause irritation with redness and pain. It is a well known fact that colorants from synthetic sources can be harmful and cause allergies. Thus, the present study is aimed in a preliminary manner, to find an additional/alternative tracking dye from natural sources to replace the synthetic BPB dye. *Bixa orellana* L., commonly known as annatto, yielding orange to red colour dye from its pericarp, was tested for its potential as a tracking dye. This dye has characteristics similar to those of BPB and shows no interference with any of the test proteins. The utility of this dye was tested using proteins that exhibit different physico-chemical properties and compared with other commonly used staining methods as well as Western blot methods. Our studies show that the pigment from *B. orellana* L. can be used as a tracking dye in place of BPB. The procedure is found to be easy, practical and reliable.**

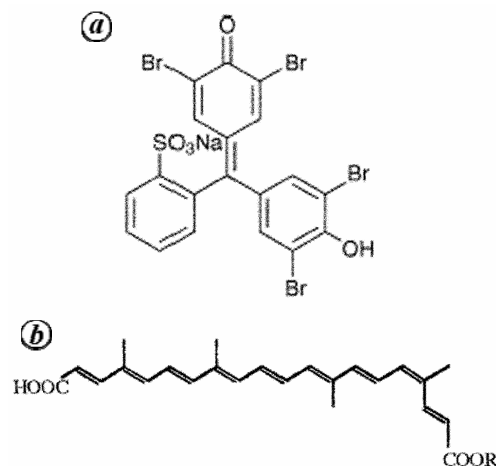
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POLYACRYLAMIDE gel electrophoresis (PAGE) is one of the most reliable methods available for the separation of proteins in complex mixtures and for assessing protein purity<sup>1</sup>. Cross-linked chains of polyacrylamide were introduced as matrices for electrophoresis by Raymond and Weintraub<sup>2</sup>. Bromophenol blue (BPB; Figure 1 a) is a tetrabromophenol sulfonaphthalein, widely used as a 'tracking dye' by the scientific community. It is an acid-base indicator whose useful range lies between pH 3.0 and 4.6. Since BPB carries a slight negative charge at moderate pH, it will migrate in the same direction as DNA and protein in a gel and thus can be used as a marker ion front<sup>3</sup>. However, to the best of our knowledge all the chemical, physical and toxicological properties of BPB have not yet been thoroughly investigated and Material Safety Data Sheet (MSDS) of all standard companies providing BPB are advised that due care must be exercised when handling this material. BPB may cause irritation with redness and

pain when it comes in contact with the skin. In case of accidental inhalation, it may cause irritation to the respiratory tract. Symptoms may include coughing and shortness of breath. Furthermore, it may cause pain and irritation in the cornea when it comes in contact with the eye. It is well known that colorants from synthetic sources can be harmful and cause allergies in humans<sup>4</sup>. Therefore, interest in natural dyes has increased considerably during the last few years. Nowadays, fortunately, there is an increasing awareness among people towards the use of natural products<sup>5</sup> as a substitute for synthetic dyes. Due to their non-toxic property, low pollution and less side effects, natural dyes are used more often in food products as well as for other important regular uses. Natural dyes are considered to have fewer side effects, are less toxic, less polluting, less hazardous to health, non-carcinogenic and non-poisonous<sup>6</sup>. Of importance is the fact that they are environment-friendly and can be recycled after use<sup>7</sup>.

In India alone, nearly 450 plants are known to yield dyes<sup>8</sup>, among which 50 are important<sup>9</sup>: ten of these are from the roots, four from barks, five from leaves, seven from flowers, seven from fruits, three from seeds, eight from wood and three from gums and resins. The present study is aimed in a preliminary manner, to find an additional and attractive suitable tracking dye for SDS-PAGE, as well as native PAGE from natural sources. We have employed a wide range of proteins, staining techniques and also Western blot analysis to show that the novel tracking dye works well under various conditions tested and to confirm that there is no interference with the protein.

Annatto extract is a natural food colour, which is obtained from the outer coatings of the seeds of the annatto tree, *Bixa orellana* L., belonging to the family Bixaceae. Although the plant species originated in northern South



**Figure 1.** a. Structure of bromophenol blue; b. Structures of bixin and norbixin cis-isomers. Bixin: R = CH<sub>3</sub>; Norbixin, R = H.

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America, it is now widely cultivated in tropical areas for commercial production<sup>10,11</sup>. Annatto extract, a natural carotenoid, has been employed in the food industry as an important colorant, mainly in dairy products such as cheese and butter. It has been considered safe for human consumption, since it has been used as a food colorant in Latin America for many centuries. Indeed it has been reported that the annatto extract does not exert any genotoxicity, subacute and chronic toxicity, reproductive toxicity or carcinogenicity<sup>12–16</sup>. The two major components of the extracts, oil-soluble bixin (methyl hydrogen 90-*cis*-6,6'-diapocarotene-6,60-dioate, C<sub>25</sub>H<sub>30</sub>O<sub>4</sub>) and water-soluble norbixin, the corresponding dicarboxylic acid C<sub>24</sub>H<sub>28</sub>O<sub>4</sub> (Figure 2), are widely used for the colouring of food, pharmaceutical and cosmetic products, to give yellow to red hues<sup>17</sup>.

One gram of matured seed from a single annatto plant was taken in a container with 10 ml of water. It was further mixed well for 2–3 min using a cyclomixer. Orange–red supernatant dye was collected in a separate container after filtration using Whatman No. 3 filter paper. The above step was repeated once more for complete extraction of the pigment. Finally, the extract was centrifuged for 10 min at 12,500 rpm. The resulting supernatant solution was considered as the annatto tracking dye stock. The 1X sample loading buffer contained: 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1%  $\beta$ -mercaptoethanol and 1% annatto dye.

Different percentages of separating gels ranging from 6 to 12 (w/v) of SDS–PAGE were prepared with a discontinuous buffer system<sup>18</sup>. Protein molecular weight markers (Bangalore Genei, India) for SDS–PAGE ranging from 3.5 to 20.5 kDa with concentration 4.5 mg/ml (cat #PMW–B; broad range), 14.3 to 97.4 kDa, with concentration 2.5 mg/ml (cat #PMW–M; medium range) and 3.5 to 4.34 kDa

with concentration 3.6 mg/ml (cat #PMW–L; lower range) were used. We have chosen a wide range of protein to find out the efficiency of the tracking dye on different molecular weights of the proteins. Apart from the commercially available protein molecular weight markers, the dye was also tested against proteins extracted from different sources such as plants, microbes and plasma. To each gel proteins treated with two different dye markers were added, viz. Laemmli tracking dye or BPB in one lane, considered as control and annatto dye in the other lane for comparison. Gel electrophoresis was performed at 50 V for 3–4 h or until the tracking dyes migrated to the bottom of the gel<sup>18</sup>.

Electroblotting or Western blotting was carried out according to the protocol given by Bangalore Genei (KT21/21A/21B). Bacterial lysate having glutathione-S-transferase (GST) fusion protein was electrophoresed in duplicates along with a standard protein marker. Annatto tracking dye was used in one lane along with the BPB control. The electroblotted sample was then detected using anti-GST IgG as primary antibody and secondary antibody labelled with horse radish peroxidase (HRP). HRP was then detected using hydrogen peroxide as a substrate and tetramethylbenzidine (TMB) as a chromogen.

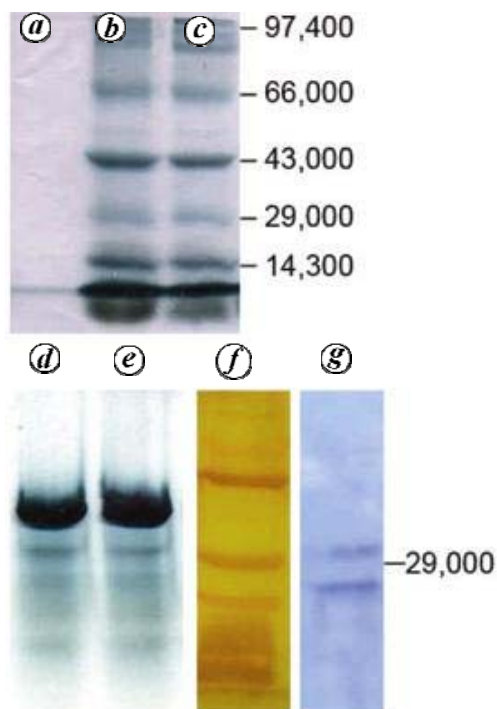
The migration of annatto dye extract was found to be similar to that of BPB (Figure 2). Bright yellow-orange colour ion front was observed in the lane where annatto dye was used. Figure 2 depicts the unstained gel and clearly indicates that the relative mobility of the dyes is similar. It was observed that no other molecules were present which could interfere with the electrophoretic migration of the sample.

Different staining methods like coomassie brilliant blue, silver nitrate staining and eze staining given by Bangalore Genei were carried out. Staining and destaining times required for plant-derived dyes were similarly compared to BPB. Upon staining the gels with coomassie brilliant blue<sup>19</sup>, all the lanes displayed identical proteins (Figure 3 *b, c*). This clearly indicates that our dyes did not form a complex with the sample proteins and were free from proteinaceous substances. Silver nitrate staining of different protein samples also shows similar results as that of BPB. This again indicates that our tracking dye does not inhibit any protein sample, either qualitatively or quantitatively (Figure 3 *f*). Apart from standard proteins used as molecular weight markers here, the annatto tracking dye also works well for proteins of different sources. Figure 3 *d* and *e* shows the migration pattern of proteins extracted from a plant sample using annatto extract as tracking dye.

To further demonstrate that the annatto extract does not chemically alter the proteins, Western blot test was carried out. As GST protein was analysed using HRP as secondary antibody, TMB was used as a chromogen for the present study. HRP acts on hydrogen peroxide to release oxygen, which oxidizes TMB to TMB oxide. The TMB oxide is



**Figure 2.** Comparison of relative mobility of annatto dye with bromophenol blue in SDS–PAGE before staining (*a* and *b*) and after staining (*c*).



**Figure 3.** Coomassie brilliant blue staining of protein marker sample where annatto dye and bromophenol blue (BPB) are compared; *a*, Annatto dye alone after staining (without loading protein); *b*, Annatto dye with protein marker; *c*, BPB with protein marker; *d*, Plant protein sample with annatto dye; *e*, Same plant protein sample with BPB; *f*, Silver nitrate staining of plant protein sample with annatto extract as the tracking dye; *g*, Western blot of GST protein with annatto dye. Proteins in all the lanes show equal mobility characteristics indicating similarity in the property of BPB and the dyes used in the present study.

deposited wherever the enzyme is present and appears as a blue band on the nitrocellulose membrane (Figure 3 *g*).

It is interesting to compare the chemical properties of bixin and norbixin with those of BPB. All these compounds are acidic in nature. However, there are differences in the overall structure of BPB and these components of the annatto dye. BPB is aromatic in nature, whereas bixin and norbixin are unsaturated aliphatic acids. However, differences in chemical structure do not affect the utility as a tracking dye in both these cases.

The present investigation advocates the use of natural dyes instead of BPB as tracking ion front in electrophoretic techniques. To conclude, we have shown that annatto dye, a yellow-coloured pigment from the tropical plant *B. orellana* L., can be used as a tracking dye for routine SDS-PAGE of proteins instead of BPB. This procedure is easy, practical and reliable in case of SDS-PAGE and native PAGE. Additionally, this dye-yielding plant is commonly available in tropical regions and is not listed in the endangered or endemic Red data list<sup>20</sup>. The utility of the extracts from *B. orellana* as reported in the present study suggests that there could be many more compounds from dye-yielding plants that could be used effectively for similar applications.

1. Simpson, R. J., *Proteins and Proteomics – A Laboratory Manual*, I.K. International Pvt Ltd, New Delhi, 2003, pp. 39–91.
2. Raymond, S. and Weintraub, L., Acrylamide gel as a supporting medium for zone electrophoresis. *Science*, 1959, **130**, 711–712.
3. Burford, G. D. and Pickering, B. T., Influence of the concentration of bromophenol blue, used as a tracking dye, on the resolution of proteins by polyacrylamide gel electrophoresis. *Biochem. J.*, 1972, **128**, 941–944.
4. Lea, A. G. H., HPLC of natural pigments in foodstuffs. In *HPLC in Food Analysis* (ed. Macrae, R.), Academic Press, London, 1988, pp. 277–333.
5. Siva, R., Assessment of genetic variation in some dye yielding plants using isozyme data. PhD thesis, Bharathidasan University, Tiruchirappalli, 2003.
6. Siva, R. and Krishnamurthy, K. V., Genetic diversity studies on *Bixa orellana* Linn. using isozyme data. In *Plant Taxonomy: Advances and Relevance* (eds Pandey, A. K., Wen, J. and Dorga, J. V. V.), CBS Publishers, 2005, pp. 411–422.
7. Siva, R., Status of natural dyes and dye yielding plants in India. *Curr. Sci.*, 2007, **92**, 916–925.
8. Chandramouli, K. V., *Sources of Natural Dyes in India – A Compendium with Regional Names*, PPST Foundation, Chennai, 1995.
9. Siva, R. and Krishnamurthy, K. V., Isozyme diversity on *Cassia auriculata*. *Afr. J. Biotechnol.*, 2005, **4**, 772–775.
10. Collins, P., The role of annatto in food colouring. *Food Ingredients Process.*, 1992, **23**, 23–27.
11. Evans, W. C., Annatto: A natural choice. *Biologist*, 2000, **47**, 181–184.
12. Hagiwara, A. *et al.*, A thirteen-week oral toxicity study of annatto extract (norbixin), a natural food color and chemical extracted from the seed coat of annatto (*Bixa orellana* L.), in Sprague-Dawley rats. *Food Toxicol.*, 2003, **41**, 1157–1164.
13. Haveland-Smith, R. B., Evaluation of the genotoxicity of some natural food colours using bacterial assays. *Mutat. Res.*, 1981, **91**, 285–290.
14. JECFA, Toxicological evaluation of certain food additives. Twenty-sixth Report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series, 1982, vol. 17, pp. 22–27.
15. Hallagan, J. B., Allen, D. C. and Borzelleca, J. F., The safety and regulatory status of food, drug and cosmetics color additives exempt from certification. *Food Chem. Toxicol.*, 1995, **33**, 515–528.
16. Fernandes, A. C. *et al.*, Norbixin ingestion did not induce any detectable DNA breakage in liver and kidney but caused a considerable impairment in plasma glucose levels of rats and mice. *J. Nutr. Biochem.*, 2002, **13**, 411–420.
17. Paumgarten, F., De-Carvalho, R., Araujo, I., Pinto, F., Borges, O., Souza, C. and Kuriyama, S., Evaluation of the developmental toxicity of annatto in the rat. *Food Chem. Toxicol.*, 2002, **40**, 1595–1601.
18. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, **227**, 680–685.
19. de Moreno, M. R., Smith, J. F. and Smith, R. V., Mechanism studies of coomassie blue and silver staining of proteins. *J. Pharm. Sci.*, 1986, **75**, 907–911.
20. *The Wealth of India – A Dictionary of Indian Raw Materials and Industrial Products*, Council of Scientific Research and Industrial Research, National Institute of Science Communication and Information Resources, New Delhi, 2003.

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