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Chromatographic analysis of asymmetric sulphophthalocyanines using a diode-array detector

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ABSTRACT

Hydroxyaluminium naphthosulphobenzoporphyrazines (AlOH-NSBP) are novel dyes for photodynamic therapy. These asymmetric molecules can be considered as hybrids between the tetrasulphophthalocyanine and the unsubstituted naphthalocyanine. The molecular symmetry evolution from the trisulphonated AlOH-NSB$_3$P to the monosulphonated AlOH-N$_3$SBP affects their absorption spectrum, which becomes a fingerprint for the degree of sulphonation. The diode-array HPLC detector uses the spectral properties of AlOH-NSBP for spectral monitoring of the analytical HPLC traces of synthetic fractions, thus facilitating the preparation of derivatives with a selected degree of sulphonation.

INTRODUCTION

Photodynamic therapy (PDT) is an alternative cancer treatment currently in phase III clinical trials (for a recent review, see ref. 1). The protocol involves systemic administration of a photosensitizer, which after preferential uptake in neoplastic lesions is locally activated with red light, resulting in tumor necrosis. The photosensitizer most commonly used consists of a mixture of haematoporphyrin derivatives with a weak absorption band at 630 nm and a preparation enriched in the active components is commercially available as Photofrin II (Quadralogic Technologies, Vancouver, Canada). Photofrin II consists of a mixture of poorly characterized haematoporphyrin dimers and oligomers and their skin retention results in prolonged skin sensitivity, obliging patients to refrain from exposure to sunlight for periods of up to 6 weeks [2]. Further, the outcome of PDT could be improved through the use of sensitizers that absorb light of longer wavelengths (>650 nm), where tissue is more transparent, permitting deeper penetration of therapeutic light [3,4]. It should also be noted that at these higher wavelengths more economical and reliable diode laser light sources are available. Hence it is generally recognized that Photofrin II is not the

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ideal drug for PDT and several second-generation photosensitizers have been advanced over recent years [5]. Among these second-generation sensitizers, phthalocyanines (Pc) and particularly the sulphonated metallo derivatives (M-PcS) have received considerable attention in view of their advantageous photophysical properties (for recent reviews, see refs. 6-8). Structure-activity relationship studies of M-PcS revealed that the degree of sulphonation (i.e., the number of sulphonate groups per Pc) strongly affects their biological behaviour with the amphiphilic disulphonated analogues providing the most promising activities in both *in vitro* and *in vivo* models [9,10]. Preparation of M-PcS either via direct sulphonation of unsubstituted M-Pc or via the condensation of phthalic and sulphophthalic precursors gives complex mixtures of differently sulphonated M-PcS which can be fractionated by reversed-phase (RP) HPLC [11]. As all fractions

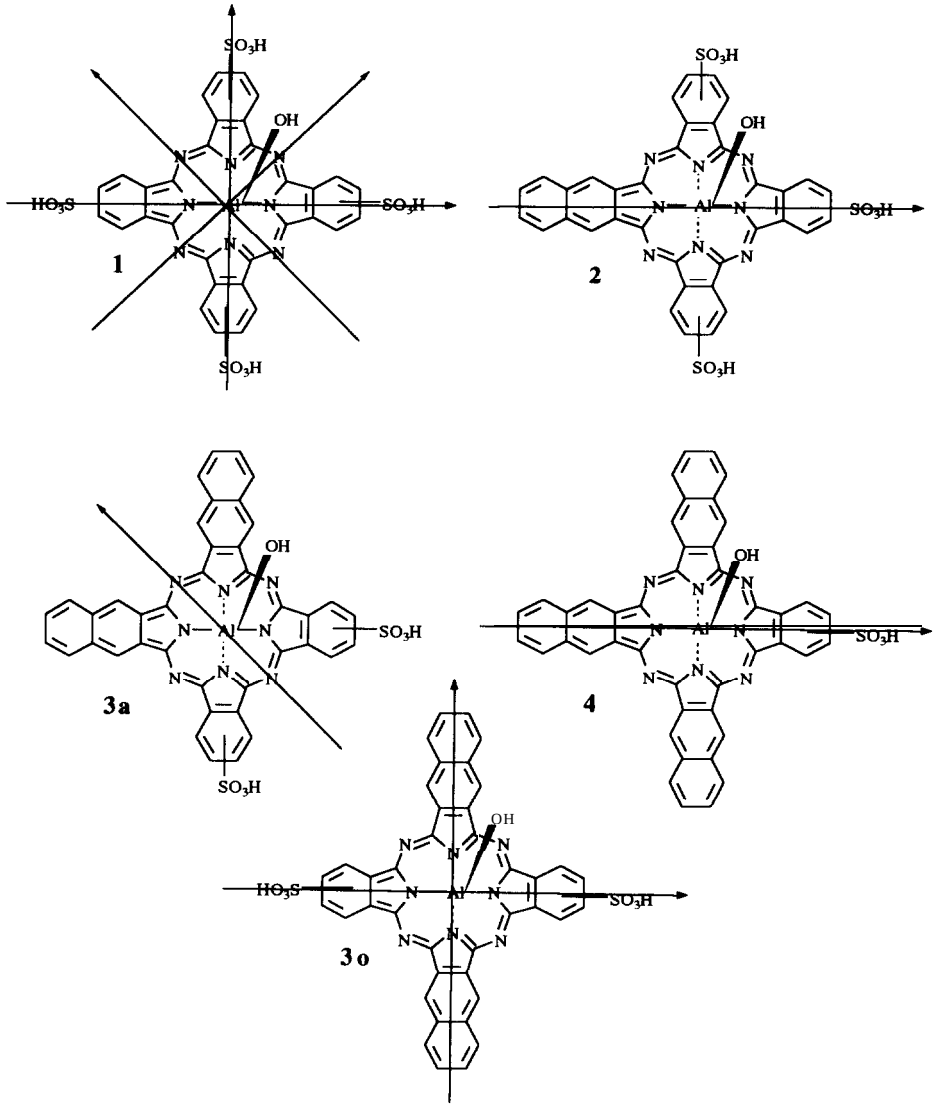

Fig. 1. Structures of AlOH-PcS$_4$ (1), AlOH-NSB$_3$P (2), AlOH-N$_2$SB$_2$P-*trans* (3o), AlOH-N$_2$SB$_2$P-*cis* (3a) and AlOH-N$_3$SBP-*trans* (4). Arrows represent macrocycle symmetry axis: 1, D_{4h}; 3o, D_{2h}; 2, 3a and 4, C_{2v}.

exhibit similar spectral properties, the degree of sulphonation can only be established from HPLC retention times and oxidative degradation assays [11]. It is evident that associating the degree of sulphonation with characteristic spectral shifts would greatly facilitate the chromatographic purification step.

Whereas the sulphonate substituents on the benzene rings of PC only slightly influence the conjugated π electrons which are responsible for the spectral properties of the PC [12], replacement of the four benzene rings by naphthalene units results in a strong bathochromic shift of 100 nm [13]. Metallo naphthosulphobenzoporphyrazines (M-NSBP) [14] can be perceived as hybrid molecules of tetrasulphophthalocyanine (M-PcS,, or tetrasulphobenzoporphyrazine, M-SB_4P) and unsubstituted naphthalocyanine (Nc, or tetranaphthoporphyrazine, M-N_4P) (Fig. 1). They feature spectral characteristics of both parent molecules resulting in composite spectra that vary in shift according to the number of naphthalene or sulphobenzene units, thus providing a fingerprint for the degree of sulphonation. We have recently shown that these asymmetric and amphiphilic M-NSBP exhibit good photodynamic properties under both *in vitro* and *in vivo* conditions [14,15], and that their activities correlate well with earlier findings on the corresponding M-PcS [16].

This paper reports on the use of a diode-array detector in conjunction with analytical HPLC for the monitoring of AlOH-NSBP to assign the degree of sulphonation and chemical composition of selected fractions.

EXPERIMENTAL

Reagents and chemicals

HPLC-grade methanol was obtained from SDS (Paris, France) and used as received. Water was purified with a Milli-Q water purification system from Millipore (Guyancourt, France). UV–visible absorption spectra were recorded on a Perkin-Elmer (Norwalk, CT, USA) Lambda 5 spectrophotometer.

The synthesis and purification of hydroxyaluminium naphthosulphobenzoporphyrazines (AlOH-NSBP) have been described previously [14,15]. Briefly, the monosodium salt of 4-sulphophthalic acid and naphthalene-2,3-dicarboxylic acid (Aldrich, St. Quentin Fallavier, France) were condensed in the presence of urea, aluminium chloride and ammonium molybdate in sulpholane. The AlOH-NSBP were washed with acetone and a mixture of alkaline methanol-dimethylformamide (MeOH–DMF) and purified on a 30 cm x 4 cm I.D. glass column packed with RP C_{18}, particle size 25-40 μm (Macherey-Nagel, Düren, Germany), fitted with a 10 cm x 2 cm I.D. precolumn packed with the same material. Elution was carried out by a stepwise gradient from 10 m*M* sodium acetate (pH 5)–DMF (9:1) to MeOH–DMF (9:1) at a flow-rate of 2-3 ml/min. Eluting dyes were monitored via their visible absorption spectra and their analytical HPLC profiles. In this manner we obtained homogeneous fractions consisting of isomers with the same degree of sulphonation, *i.e.*, from the tetrasulphophthalocyanine (AlOH-PcS_4 or AlOH-SB_4P, 1) to the trinaphthomonosulphobenzoporphyrazine (AlOH-N_3SBP, 4). The isomeric AlOH-NSBP preparations are identified by their HPLC profiles and retention times, identical and characteristic absorption maxima of the individual isomers in each preparation with the same degree of sulphonation and characteristic mass spectral ions [15].

Instrumentation

A Waters-Millipore (Guyancourt, France) HPLC system, consisting of a Model 600 multisolvent delivery system, a Model 712 WISP autosampler, a Model 990 diode-array detector, a 15 cm x 0.39 cm I.D. stainless-steel column packed with Nova Pak RP C_{18} (4 μm) and an RP C_{18} precolumn, was used. The Waters 990 program pilots this HPLC system from a microcomputer.

Chromatographic conditions

All solvents were filtered through a 0.45-μm nylon filter (Millipore) and degassed in an ultrasonic bath prior to use. Helium purging (20 ml/min) was performed throughout the HPLC experiments, which were carried out at ambient

temperature. A 50-μl aliquot of AlOH-NSBP solution was injected. HPLC was conducted at a flow-rate of 1 ml/min with a linear gradient (50 min) from 0 to 100% MeOH in 10 m*M* sodium acetate buffer (pH 5), followed by a 10-min isocratic step.

RESULTS

An aliquot of the crude AlOH-NSBP reaction mixture was loaded on the analytical column before fractionation by medium-pressure RP column chromatography. The resulting chromatographic contour (Fig. 2) reveals five typical spectra with single or doubled Q bands, which red shifted progressively during elution.

Only four separate fractions were obtained by medium-pressure RP chromatography. The hydrophilic blue AlOH-PcS_4 (1) eluted first and was characterized by a single Q band at 678 nm (spectrum I). The subsequent fractions were identified by mass spectrometry as AlOH-NSB_3P (2), AlOH-N_2SB_2P (3o and 3a) and AlOH-N_2SBP (4), with the colour shifting from blue to green.

The three-dimensional chromatogram of fraction 2 exhibits identical spectra (II) for each peak with two absorption maxima at 692 and 714 nm (Figs. 2 and 3). The following three-dimensional chromatogram corresponds to AlOH-N_2SB_2P with two types of visible spectra (1110 and IIIa, Figs. 2 and 4). Spectrum 1110 of the early-eluting minor components was composed of two well separated maxima at 692 and 760 nm

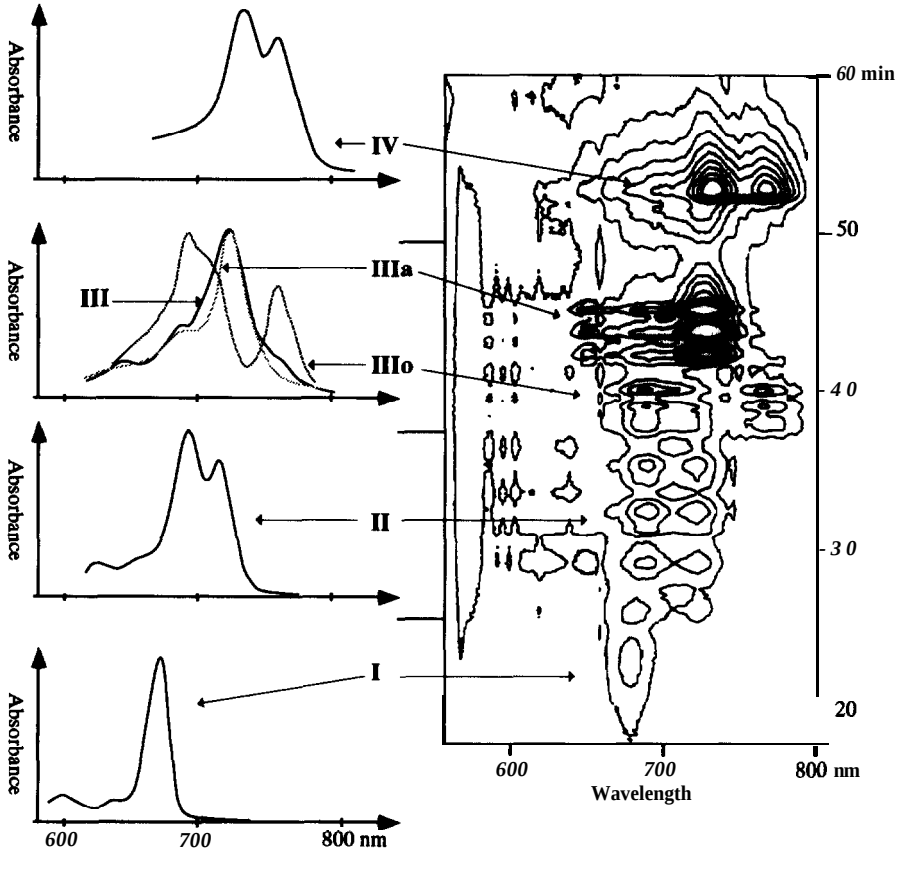

Fig. 2. Reversed-phase analytical HPLC contour of the AIOH-NSBP mixture before medium -pressure RP chromatography. Absorption spectra were established from the three-dimensional chromatographic data.

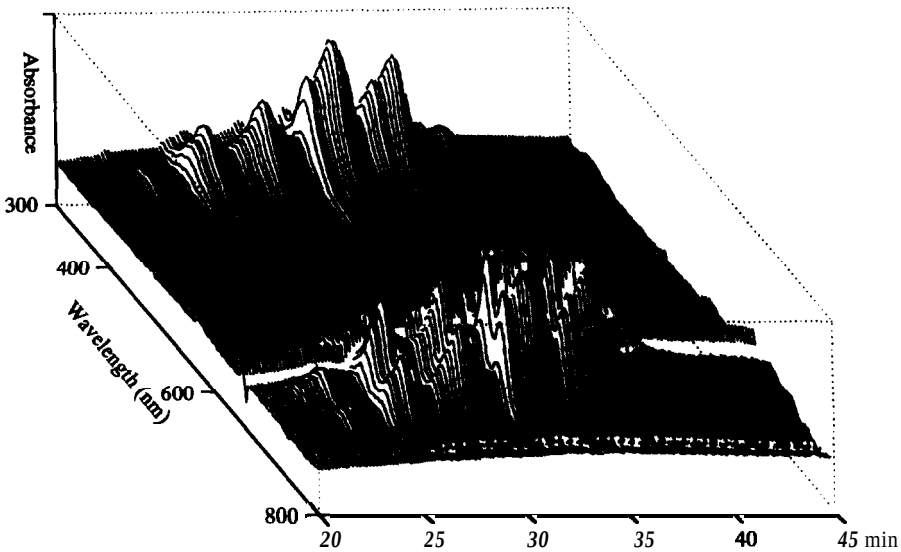

Fig. 3. Three-dimensional RP-HPLC of the **AlOH-NSB$_3$P** (2) fraction.

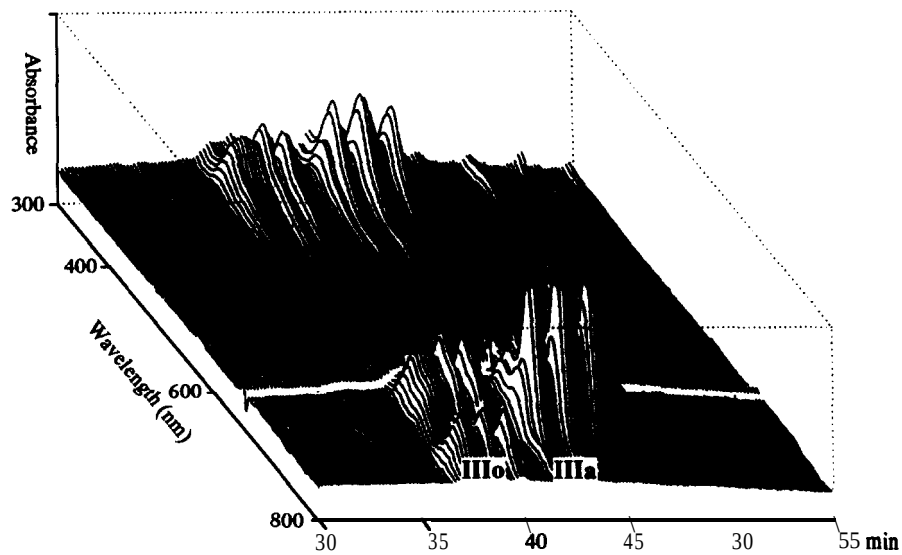

Fig. 4. Three-dimensional RP-HPLC of the **AlOH-N$_2$SB$_2$P** (3) fraction. The isomers **AlOH-N$_2$SB$_2$P-*trans*** (30) and **AlOH-N$_2$SB$_2$P-*cis*** (3a) are characterized by their absorption spectra **IIIo** and **IIIa**.

while the later-eluting major components were characterized by a single broadened absorption peak at 724 nm (spectrum **IIIa).** The spectrum of the total fraction (spectrum III) is dominated by this major spectral component, with on both sides a shoulder contributed by the absorption maxima for the minor components. Finally, the last fraction, which contains one major peak, was assigned to **AlOH-N$_3$SBP** (4). Its absorption spectrum (IV) is a bathochromic transposition of spectrum II with double Q bands absorbing at 735 and 760 nm (Figs. 2 and 5). The contour in

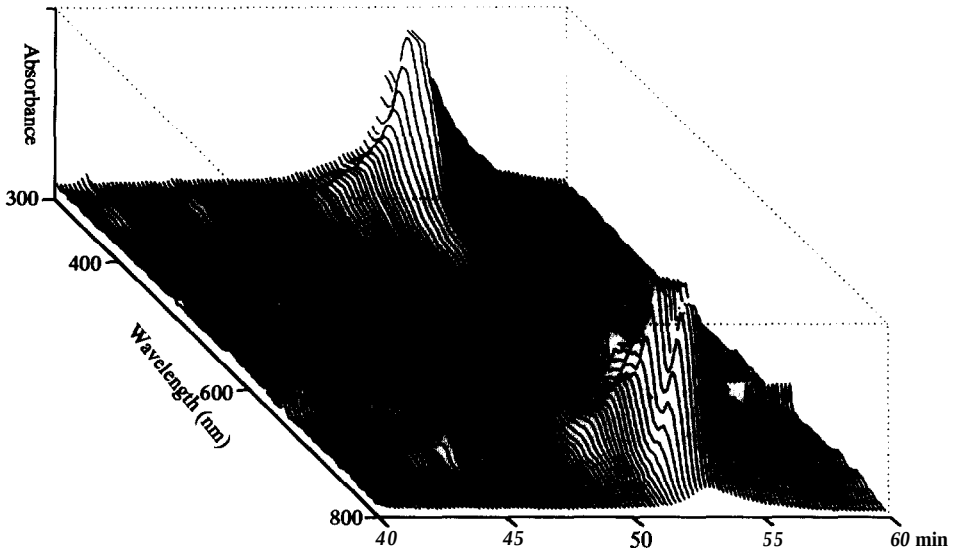

Fig. 5. Three-dimensional RP-HPLC of the AlOH-N_3SBP (4) fraction.

Fig. 2 reveals that spectra 1110 and II share the same lower absorption maximum at 692 nm while 1110 and IV share the higher absorption maximum at 760 nm.

DISCUSSION

The order of elution from the RP column of the various AlOH-NSBP provides a first clue to the chemical structure. Components elute according to the increase in hydrophobicity, which results from the progressive naphtho substitution of the sulphobenzo groups. For the M-PcS series, empirical solvent conditions were determined to separate the differently sulphonated products by medium-pressure RP column chromatography using a stepwise gradient. Each elution step provides a fraction enriched in compounds sulphonated to the same degree, with the level of sulphonation assigned via a routine degradation test [11].

Spectral profiles (Fig. 2) of the eluting components indicate that the various structures possess different absorption spectra. Solovev *et al.* [17] explained the spectral evolution during the substitution of methine by aza bridges in metallo tetrabenzoporphyrin to yield metallo phthalocyanine, by the method of molecular orbitals. Replacement of a single methine group results in a symmetry change from D_{4h} to C_{2v} and a spectral change from a single red absorption band to a doublet. A similar spectral change occurs when three methine bridges are substituted, likewise representing C_{2v} symmetry. Complete aza substitution gives the single Q band spectrum of the phthalocyanine. Solovev *et d's* calculations and experiments showed that substitution of two opposite methines lead to a large split of the Q band (D_{2h} symmetry) while the adjacent bisubstitution gave a large single band. The symmetry in the latter instance was C_{2v} but the axis of symmetry did not go through the modified atoms. Thus, subsequent aza substitution in tetrabenzoporphyrin results in a progressive red shift of the absorption maxima to yield the final characteristic phthalocyanine spectrum. The same phenomenon was noticed by Gaspard *et al.* [18,19] during the complexation of the aza bridges of Cu-PC by $AlCl_3$.

We observed similar changes in the absorbance spectra during the chromatographic separation of differently sulphonated AlOH-NSBP, which we assign to the symmetry modifications from structures 2-4. Sulphonation and the addition of an axial ligand do not substantially

change the spectra of M-Pc or M-Nc [12]. Thus symmetry axes (Fig. 1) were drawn without taking into account the sulpho substituents and axial ligands. The substitution of a sulphobenzo by a naphtho group decreased the symmetry of 2 from D_{4h} to C_{2v} (Fig. 1). In analogy with Solovev *et al.*'s work, spectrum II, which features a doublet (Figs. 2 and 3), can be attributed to AlOH-NSB$_3$P (2). This compound elutes directly after the very hydrophilic tetrasulphonate **1**, for which spectrum I on the contour in Fig. 2 is characterized by a single strong Q band at 678 nm. A similar reasoning suggests that spectrum IV of the slowest eluting AlOH-NSBP fraction represents AlOH-N$_3$SBP (4). Continuing the analogy with Solovev ef *al.*'s study, spectrum 1110 (Fig. 4) can be assigned to AlOH-N$_2$SB$_2$P-*trans* (**3o**), of symmetry D_{2h}, whereas spectrum IIIa is assigned to the *cis* analogue (**3a**) as the C_{2v} symmetry axis does not fall across the benzo/naphtho groups. We emphasize the parallel between our observations on the AlOH-NSBP and those reported on the analogous aza-substituted tetrabenzoporphyrins [17]. Solovev et *al.*'s calculations, in fact, can predict the common maxima between spectra 1110 and II, and 1110 and IV Thus the expansion of the aromatic ring system of the AlOH-NSBP induces optical changes such that the different peaks in each fraction which exhibit identical spectra correspond to positional isomers sharing the same macrocycle and the same number of sulphonate groups.

Although **3a** and **3o** were not resolved in fraction 3, the three-dimensional profile (Fig. 4) reveals that the amphiphilic **3a** is the main component. We confirm that the adjacently disulphonated isomers elute after the opposite sulphonated products during RP chromatography, as suggested by Ah *et al.* [11] for the analogous M-PcS and shown by Kessel et *al.* [20] for differently sulphonated tetraphenylporphines.

Finally, contaminations by related chromophores which may elute during the AlOH-NSBP fractionation process are readily detected on the three-dimensional elution profiles. This verification is more reliable than the retention times provided by monochromatic monitoring only.

CONCLUSION

The HPLC diode-array detector allowed us to fractionate AlOH-NSBP according to spectral variations inherent to the degree of sulphonation. This technique can substantially improve the fractionation and analysis of chromophores in dye mixtures from chemical synthesis or natural sources.

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