



UNIVERSITY OF LEEDS

This is a repository copy of *At least four distinct blue cationic phthalocyanine dyes sold as "alcian blue" raises the question: what is alcian blue?*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/182755/>

Version: Accepted Version

Article:

Ali, S, Dapson, RW, Horobin, RW et al. (2 more authors) (2022) At least four distinct blue cationic phthalocyanine dyes sold as "alcian blue" raises the question: what is alcian blue? *Biotechnic and Histochemistry*, 97 (1). pp. 11-20. ISSN 1052-0295

<https://doi.org/10.1080/10520295.2021.2018497>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

Contact:

Dr A Kazlauciusas — Colour Science Analytical, School of Chemistry, University of Leeds, Leeds LS2 9JT, UK.

T: UK-0113-343 2939

Email: A.Kazlauciusas@leeds.ac.uk

**At least four distinct blue cationic phthalocyanine dyes are sold as
“alcian blue”. So, what *is* alcian blue?**

Ali S ¹, Dapson RW ², Horobin RW ³, Kiernan JA ⁴ and Kazlauciusas A ¹

¹ Colour Science Analytical, School of Chemistry, University of Leeds, Leeds LS2 9JT,
UK.

Orcid ID: [http:// orcid.org/0000-0002-5859-0811](http://orcid.org/0000-0002-5859-0811)

² Dapson & Dapson, LLC, 6951 East AB Avenue, Richland, MI, 49083, USA.

Orcid ID: [http:// orcid.org/0000-0002-9432-7113](http://orcid.org/0000-0002-9432-7113)

³ Chemical Biology and Precision Synthesis, School of Chemistry, University of
Glasgow, Glasgow, Scotland, UK.

Orcid ID: [http:// orcid.org/0000-0002-6612-6664](http://orcid.org/0000-0002-6612-6664)

⁴ Department of Anatomy and Cell Biology, University of Western Ontario, London,
ON, Canada.

Orcid ID: [http:// orcid.org/0000-0002-3324-1092](http://orcid.org/0000-0002-3324-1092)

Abstract

Physicochemical characteristics of dye lots sold as “alcian blue” were investigated using the Biological Stain Commission (BSC) precipitation test, differential scanning calorimetry, high performance liquid chromatography, thin layer chromatography, and ultra violet/visible spectroscopy. Four different blue phthalocyanine dyes were detected in 11 commercial dye lots. These four included the original Ingrain blue 1/CI 74000 dye and the dye sold with the name “alcian blue pyridine variant.” The possible identities of the additional two dyes are discussed. A proposed extension to the BSC analytic scheme is presented which could distinguish three categories of commercial alcian blue dyes from each other and from the original alcian blue 8G.

Keywords: alcian blue, alcec blue, alcian blue pyridine variant, astra blue 6GLL, Biological Stain Commission, certification, fast blue 8XM, labeling, propyl astra blue.

Dyes labeled alcian blue are currently sold and used as biological stains for glycosaminoglycans and other mucosubstances, and such applications have a considerable history (Steedman 1950; and see Pearse 1960; Lillie & Fulmer 1976; and Kiernan 2015) and are certified by the Biological Stain Commission (BSC) for such applications (Penney et al. 2002). Commercial lots of alcian blue — although probably all phthalocyanines that are cationic under conditions of use — are not all chemically identical to the original dye of that name (i.e., alcian blue 8G [GS or GX], CI 74240, Ingrain blue 1), as described in a previous publication from the BSC concerning alcian blue certification (Fagan et al. 2020).

Unfortunately, histotechnically relevant properties, as well as identities, of commercially available alcian blue dyes can differ from those of the original alcian blue 8G. As an organization concerned with the quality of dyes used in biology and medicine, the BSC has to date addressed this problem by modifying their criteria for certification, including the introduction of an additional analytical procedure, the BSC precipitation test, as previously described by Fagan et al. (2020). This test divides alcian blue dyes on the basis of the occurrence, or not, of two phenomena:

1. Whether dye precipitates from aqueous solution when alkali is added.
2. If precipitation occurs, whether the precipitate dissolves on acidification.

Alcian blue samples are thus assigned to one of two categories, reflecting end user needs. A dye lot which is precipitated and which does not redissolve is categorized as “alcian blue 8G or equivalent”. Such a dye is required if the alcian blue is used in a stain amplification procedure (Scott 1996), and can also be used both for routine low pH staining of mucosubstances as well as in the critical electrolyte concentration (CEC) technique (Scott and Dorling 1965). A dye lot which either does not precipitate from alkaline solution, or which does precipitate but then redissolves when acidified, is categorized as an alcian blue variant. Such samples may be used in both routine low

pH staining of mucosubstances and in the CEC technique. Indeed, they may be preferred for the latter procedure since problems due to poor solubility and unwanted precipitation are reduced with this category of alcian blue, see Churukian et al. (2000) and also the Discussion, below.

Compounds with varied chemistries may be included in both of these BSC categories. Consequently, in an effort to better understand the problems experienced with the alcian blue dyes, a variety of analytical methods — the BSC precipitation test, differential scanning calorimetry (DSC), reverse-phase high performance liquid chromatography (HPLC), thin layer chromatography (TLC) and ultra violet/visible (UV/Vis) spectroscopy — were applied to a set of commercial samples of alcian blue dyes. This has led to the discovery of two chemically different alcian blue dyes whose identities are not currently known.

Materials & methods

Dyes lots investigated

These are listed in Table 1, and were obtained from the archives of the BSC Certification Laboratory and one of the present authors (JAK). Samples of dyes were obtained from six different vendors but, as they include dyes submitted to the BSC for certification, vendors are not named and each lot is identified by an individual code.

Analytical and other procedures used

Assessment of aqueous solubility

Dye solutions were prepared by adding 0.25 g of dye powder to 25 mL of 3% v/v aqueous acetic acid, and sonicating for one h. If undissolved dye remained at this time, a further one-hour sonication period was adopted. The resulting solutions were filtered through grade 4 Whatman filter papers. Note that a 1% w/v dye concentration is the highest used in staining solutions.

BSC precipitation test

This was carried out as described by Fagan et al. (2020). Briefly, drops of dye solution were placed at three separate positions on a clean microscope slide. One drop of distilled water was added to the negative control drop of dye. One drop of 1.0 M sodium hydroxide was added to each of the other two drops of dye. After one minute, all three drops were examined against a white background. A blue precipitate may form on addition of alkali. If this occurred, a drop of 2 M acetic acid was added to

one of the drops containing the precipitate. If no precipitate was produced by addition of alkali, the dye was categorized as “alcian blue variant 1”; and if the precipitate dissolved after acidification the dye was categorized as “alcian blue variant 2”. If the precipitate did not dissolve, the dye was categorized as “alcian blue 8G or equivalent”.

Differential scanning calorimetry (DSC)

DSC analysis was carried out on four dye lots (L2, L7, L9, L10). Aliquots of 1–3 mg were weighed into standard DSC aluminum pans, which were hermetically sealed for analysis. Samples were then exposed to a temperature range of 30–300°C at a heating rate of 10°C per min, with an inert dry nitrogen flow rate of 50 mL per min in a Q20 DSC device (TA Instruments/Waters Ltd., Leatherhead, UK). Calibration of the temperature and enthalpy scale was performed using an indium standard.

High performance liquid chromatography (HPLC)

This was carried out on a single dye lot (L7). A solution was prepared by dissolving 35 mg of dye in 10 mL 3% v/v aqueous acetic acid, which was diluted 50-fold with the same solvent. This was applied to a reverse-phase Kinetex 2.6 μ M peptide C18 100A column (Phenomenex, Macclesfield, UK). These columns were used in a Dionex 3000 UBPLS chromatograph (ThermoFisher, Loughborough, UK). Eluant was monitored at two wavelengths, 610 nm and 331 nm.

Thin layer chromatography (TLC)

Solvents were obtained from Sigma-Aldrich (Gillingham, UK) and used as purchased. Silica gel 60 F254 20×20 cm 250 μm TLC chromatography plates were from Merck (Darmstadt, Germany). Samples were applied to the plates in aqueous acetic acid solution using micropipettes, with spots being less than 1 mm diameter. Development was carried out using n-butanol-acetone-water-28% w/w aqueous ammonia at 5:5:1:2 by volume in a closed tank. Dyes of the BSC's "alcian blue 8G or equivalent" category and those in our "alcian blue variant 2" category will be precipitated by this alkaline solvent, and so were expected to be chromatographically immobile in this system.

Ultra violet/visible (UV/Vis) spectroscopy

Solutions were prepared by dissolving 35 mg of dye in 100 mL of 3% v/v aqueous acetic acid, using ultrasonic agitation when necessary, and then diluting 5 mL of this solution to 50 mL with the same solvent. Spectra were measured over the range 200–800 nm in 1 cm quartz cells using a Varian-Cary 50 UV/Vis spectrophotometer (Varian/Agilent Technologies, Cheadle, UK).

Assessment of hydrophilic/hydrophobic character of various alcian blue dyes

The logarithm of the octanol-water partition coefficient, termed $\log P_{\text{oct}}$, was adopted as an indicator of the hydrophilic/hydrophobic character of the dyes. $\log P_{\text{oct}}$ values for the various chemical structures discussed in the current paper were estimated computationally using the procedures of Hansch and Leo (1979). This widely used semi-empirical approach can derive a $\log P_{\text{oct}}$ estimate of any structure for which the chemical fragments are available in their data set, and whose intramolecular interactions can be modelled by the available factors provided by these authors.

Results

Assessment of aqueous solubility — After filtration no substantial residue was found on the filter paper for alcian blue samples L1, L4, L7, L8, L9, L10 and L11. Substantial residues of undissolved blue material were found for dye samples L2, L3, L5 and L6. After sonication, foam and bubbles were noted for dye solutions of L3, L5, L7 and L10, indicating that these lots probably contained added surfactant.

The outcomes of the *BSC precipitation test* and of *thin layer chromatography* are summarized in Table 2.

DSC measurements — Three different patterns of heat flow occurred. Two dye lots (L7 and L10) had glass transition temperature (T_g) values of ca. 65°C (66.7 and 60.4°C respectively) and ca. 270°C (269.5 and 269.8°C respectively). One dye lot (L2) had T_g values of 107.9°C and 259.5°C. One dye lot (L9) had T_g values of 86.4 and 187.1°C. Note that T_g values are thermal transitions rather like melting points, and as such are indications of identity, so that similar values suggest similar identities, and vice versa.

HPLC measurements — The results concern a single dye lot, L7, which falls into the BSC category of alcian blue variant. At both wavelengths monitored (331 and 610 nm), two well-resolved major peaks occurred, of markedly unequal size. Both peaks comprised at least four poorly resolved sub-peaks. Because a reverse-phase column was used, the two major peaks probably represent dyes with two different numbers of amino-substituted side-chains. If this were so, the sub-peaks could correspond to

stereochemically different patterns of substitution of the side-chains into the phthalocyanine core.

Thin layer chromatography — The R_f values are summarized in Table 2.

UV/Vis spectroscopy — The results are summarized in Table 2.

Hydrophilicity/hydrophobicity assessments of the various dyes and dye species — Calculated $\log P_{\text{oct}}$ values for a number of dyes and various of their ionic species are given in Table 3.

Discussion

Identities of commercial products sold as alcian blue

The UV/Vis and BSC precipitation test data, summarized in Table 2 for the 11 alcian blue dyes investigated in the present study, demonstrate that even in this small sample there were four chemically different dyes. Thus, dye lots fell into Group A, which included L1-L6; Group B, which included L7, and L10-11; Group C, which was represented by L8; and Group D which was represented by L9. This conclusion is supported by the TLC chromatograms, see Table 2. For a subset of dye samples (L2, L7, L9 and L10) the DSC findings are also in agreement with this conclusion.

What can be said regarding the chemical identities of these four alcian blue dyes? Two dyes investigated in the present study are of known structure (Groups A and B), whilst the structures of the other two were not known, or at least were not disclosed by the vendors. There are in addition alcian blue-related dyes with known or partly known structures (see below) but none of these were found in the present investigation. This complex situation will now be discussed, starting with dyes which fall into the BSC “alcian blue 8G or equivalent” category.

The two dyes of this type found in the current study (Groups A and B) both have published structures. Thus, alcian blue 8G/CI 74240 is the original member of this BSC category, whose structure was established due to the efforts of J. E. Scott's group (1972a, 1973). For the structure of a major component of commercial samples of this dye see Fig. 1, structure **a**. Alcian blue 8G was present in six of the lots studied in the current investigation (L1–L6). Most of these lots were obtained when alcian blue was still manufactured as a textile dye in the UK, at which time there was no commercial motive to seek alternative dyes for histological or histochemical

applications. One sample, however, was purchased long after that period. The other dye falling into the BSC “alcian blue 8G or equivalent” category was alcian blue-tetrakis(methylpyridinium) chloride (unfortunately, see comments below, marketed as alcian blue pyridine variant), lot L8, for whose structure see Fig. 1, structure **b**. This structure has been given by vendors for many years (PubChem 2021). This dye did not respond to the BSC precipitation test in precisely the same way as did the samples of CI 74240, for an explanation of this see below.

Fig. 1 and 2 near here

Cuproinic blue is an alcian blue-related but non-phthalocyanine dye of known structure (see Fig. 2). This product of J. E. Scott’s group (1972b) is available commercially. As, however, cuproinic blue has a strong visible absorption peak at 640 nm, and since no dye investigated by us had such a peak, it can be concluded that this compound was not present in any of the dye lots of the present study.

Fig. 3 near here

Next, consider the dyes which fall into the BSC “alcian blue variant” category. The structures of the two dyes of this type found in the current study are not known. Three examples of one dye (Group B), hereafter termed unidentified alcian blue 1, were found (namely L7, L10 and L11). The second unknown structure (Group D), hereafter termed unidentified alcian blue 2, occurred in only one dye sample (L9). Although current data does not allow assured identification, possible identities will now be considered. It may be noted that, whatever their precise structures, these two unidentified dyes will be seen to fall into the proposed BSC categories of “alcian blue variant 1” and “alcian blue variant 2”, respectively.

The BSC precipitation test provides some indication of the chemical properties of these two unidentified dyes. Aqueous solutions of both gave precipitates when made alkaline, which precipitates re-dissolved upon acidification. Such behavior could

arise if the side-chains of these dyes included amino groups. Such groups would be present as uncharged free bases under alkaline conditions, potentially resulting in a dye having a hydrophobic character which would therefore be of low solubility in water. However, acidification and consequent protonation of the amino groups would yield hydrophilic cations, which could result in water solubility. Two questions arise. As we are here concerned with commercially available materials, are phthalocyanine dyes which carry amino groups available commercially? And, if so, are the free base species of such dyes likely to be insoluble in water, whilst their amine salts are likely to be soluble?

First consider dye availability. An on-line search for commercially available phthalocyanines whose substituents carry amino groups turned up two such compounds. One was astra blue 6GLL, which carries three tertiary aliphatic amino substituents, as shown in Fig. 1, structure **c**. The structure of this dye was first reported by Scott (1980). This dye is currently (2021) available from a number of suppliers. The other dye offered for sale was propyl astra blue (shown in Fig. 1, structure **d**), perhaps derived from astra blue 6GLL by propylation of two of its three amino groups. This dye was listed, and the structure specified, by a single supplier — although it has in the past been sold by additional suppliers. Note: the dye name “propyl astra blue”, like alcian blue, is problematic, as mentioned below.

Next consider the aqueous solubilities of the salt and free base species of these two amino phthalocyanines. Astra blue 6GLL has in fact been observed to precipitate from alkaline solution, whilst being soluble in acidic or neutral aqueous solution (Scott 1980). The pH dependent nature of the side-chains of this dye is illustrated in Fig. 4 **a**. This finding not only answers the second question for the case of astra blue 6GLL but also allows us to check if $\log P_{\text{oct}}$ (the logarithm of the octanol-water partition coefficient) is an indicator of the solubility of such dyes. To this end we estimated the

values of $\log P_{\text{oct}}$ of the salt and of the free base species of astra blue 6GLL, see Table 3. Whilst the salt has a $\log P_{\text{oct}}$ value of -6.3 , the free base has a value of 6.3 . As a further check on the validity of $\log P_{\text{oct}}$ as a proxy measure, $\log P_{\text{oct}}$ values were estimated for a major alcyan blue 8G species, and for the pigment derived from it. Inspection of the resulting values, listed in Table 3, shows that the water-soluble salt has a negative $\log P_{\text{oct}}$ value whilst the non-ionic pigment has a positive value. The pH dependent nature of the different side-chains involved in these processes in both compounds is illustrated in Fig. 2.

Fig. 4 near here.

As a result of these findings, we now know that astra blue 6GLL does precipitate at high pH and redissolves at low pH. More generally, it seems likely that $\log P_{\text{oct}}$ values can be used to indicate aqueous solubility or insolubility of such phthalocyanine dyes. Consequently, we will now consider the possible solubility properties of the second commercially available amino phthalocyanine, propyl astra blue.

This dye is stated by the vendor to have two of its three amino groups present as propylated quaternary salts (Riedel-De Haën AG, 2021). This compound could be synthesized by propylating astra blue 6GLL. However, it is quite improbable that such propylation would always occur at precisely two of the three amino groups of that dye, although it is possible that a two-phase synthetic system might achieve such selectivity. Another factor to consider is that a quaternizing reaction giving rise to a di-propylated product will necessarily first give rise to mono-propylated material and, potentially, also produce some tri-propylated material. The latter reaction step would be favored by use of an excess of propylating agent and extended reaction times. Since use of either a two-phase system or extended quaternization would increase

costs, a commercial product may well mostly comprise the di-propylated compound plus some of the precursor mono-propylated form. Our findings from reverse-phase HPLC of the sample of unidentified alcian blue 2 (lot L7) is compatible with this idea, as it shows that unidentified alcian blue 2 is a mixture of two compounds, differing significantly in their hydrophilicity/hydrophobicity. This could represent a di-propylated compound plus a mono-propylated component. We therefore estimated the $\log P_{\text{oct}}$ values for the salt and free base species of both di- and mono-propylated compounds. The nature of the pH dependency is shown in Fig. 4 **b**. As summarized in Table 3, the di-propylated compound, which contains a single $-\text{N}(\text{CH}_3)_2$ group, is hydrophilic whether or not this tertiary amino group is protonated. However, the mono-propylated compound (which would also carry two $\text{N}(\text{CH}_3)_2$ groups) has a hydrophilic salt (with an estimated $\log P_{\text{oct}}$ value of -6.9) but a hydrophobic free base ($\log P_{\text{oct}}$ value of 1.5).

It is thus possible that unidentified alcian blue 1 and unidentified alcian blue 2 correspond to two commercially available dyes. Unfortunately, we currently lack confirmatory information, and further experimental investigation is required to confirm or disconfirm these suggestions.

Finally, one more category of alcian blue dyes must be considered, since various dyes which would probably fall into this category are available commercially. This category comprises those alcian blue dyes which do not precipitate from an aqueous solution when this is made alkaline. Dyes expected to behave in this way include those in which all cationic substituents are stable hydrophilic quaternary ammonium or hydrazinium salts. No dye of this type was found among the samples of alcian blue dyes investigated by us. However, there are at least three probable examples of this category with published structures, at least one of which is available commercially. These are: alcec blue (see Fig. 1, structure **e**); a similar dye but with only three cationic side-chains, unfortunately also sold at this time as propyl astra blue (see

Fig. 1, structure **f**); and National fast blue 8 XM (see Fig. 1, structure **g**). Any improved analytic scheme for alcian blues must be able to take such compounds into account.

The behavior of alcian blue pyridine variant in the BSC precipitation test

As already mentioned, and see also Table 2, the compound sold as “alcian blue pyridine variant” behaved somewhat differently in the BSC precipitation test compared to alcian blues of the CI 74340/Ingrain blue 1 type. Whilst a precipitate was formed from the pyridine variant lot at high pH, and whilst this precipitate did not completely redissolve after acidification, nevertheless some dye did redissolve. This more complex behavior can be understood on the basis of the known chemistry of N-alkyl pyridinium salts (Moracci et al. 1976). As shown in Fig. 4 **c** and Table 3, treatment with alkali produces two hydrophobic species, a pyridone and a dihydropyridine free base, via a pseudobase intermediate. Hence precipitation from alkaline solution is expected. Moreover, these reactions are irreversible. However, whilst the pyridone remains unchanged by subsequent acidification, the dihydropyridine free base will form a hydrophilic salt, leading to the partial dissolving of the precipitate.

Labeling errors seen with alcian blue dyes

At this point it can be mentioned that, regardless of the chemical identities of commercial samples of alcian blues discussed above, the labeling of such dyes by vendors can constitute an independent source of confusion. Thus, the sale of four different compounds under a single dye name (alcian blue) is certainly unfortunate. As is selling two different compounds under the name propyl astra blue, whether or not

one of these compounds is also sold by another vendor as alcian blue. In the case of the alcian blues, all dyes which give insoluble precipitates after addition of alkali are being labeled in the same way whether or not they redissolve at low pH, an issue currently addressed by the BSC's distinction between the "alcian blue 8G or equivalent" and the "alcian blue variant" categories. In addition, two further labeling problems were seen with the dye samples investigated. First is the potential confusion resulting from a commercial product being labeled "alcian blue pyridine variant" by a vendor, whilst the BSC describes this dye as falling into the "alcian blue 8G or equivalent" category. Second is the application of the Colour Index number CI 74240 to a dye lot (unidentified alcian blue 1) whose structure certainly does not correspond to that CI number, a particularly unfortunate inaccuracy.

Dye solubility and dye identity

The histological and histochemical applications of alcian blue require that it is completely soluble in neutral or acidic aqueous solutions. However, dyes of the alcian blue 8G or equivalent category dyes must give precipitates in alkaline solutions, which do not redissolve when acidified. Moreover, even solid samples of the original alcian blue 8GS or 8GX type can decompose if stored for extended periods, especially if the amount of boric acid stabilizer present is low (Churukian et al. 2000). Consequently, the possibility arises that alcian blues of this type will contain significant amounts of insoluble pigment, which is technically inconvenient. The behavior of the present 11 alcian blue lots supports this generalization. All four dye lots containing significant amounts of undissolved colorant (L2–3, L5–6) fell into the alcian blue 8G or equivalent category. Moreover, they were all lots dating from the twentieth century, so were most likely alcian blue 8GS/8GX dyes, which had been stored for at least 20 years (Scott 1996). However, one twentieth century lot in the "alcian blue 8G or

equivalent” category (L4) did not contain significant amounts of insoluble blue material. Moreover, the “alcian blue pyridine variant” lot (L8), which also fell into the alcian blue 8G or equivalent category, was completely soluble in water after at least a decade of storage. Finally, note that all lots of unidentified alcian blue variants 1 and 2 were also stable and soluble. Although this is a small set of samples, the implication is that if a laboratory only occasionally uses alcian blue, they might be advised to purchase either an “alcian blue variant”, or the product sold under the name “alcian blue pyridine variant” with the structure shown in Figure 4 b.

What has the BSC done about this and what else might be done?

In response to the various confusions involving alcian blues, the BSC has made changes to its certification procedures, as described in a previous publication (Fagan et al. 2020). In the light of the additional information in the present paper, further changes are being considered, as indicated in the draft analytical scheme for alcian blues outlined as a flowchart in Fig. 5. However, to validate this approach we need additional UV/Vis and TLC data on alcian blue and related dye samples. Once this information is obtained the new analytical scheme, or some variant thereof, will be trialed on the alcian blue lots submitted to the BSC for certification. Determination of the actual structures of the currently unidentified dyes is also a priority.

Fig. 5 near here.

Conclusions

Investigation of 11 commercial alcian blue samples found that four different dyes were present. The structures of two (alcian blue 8G and “alcian blue pyridine variant”) are known, whereas the other two are unknown, but may be astra blue 6GLL and

propyl astra blue. An improved analytical scheme has been devised which, after further analytical investigations, should allow the reliable classification of alcian blue dye lots as either the BSC's "alcian blue 8G or equivalent" or as one of two chemically distinct BSC "alcian blue variant" categories, with resulting benefit to the end users of alcian blue dyes.

Acknowledgements: We thank Mr. Chad Fagan, Head of Laboratory Operations at the BSC Assay Laboratory, Rochester, for access to archived dye samples. RWH thanks Dr. Justin Hargreaves, Head of School of Chemistry, University of Glasgow, Scotland, for provision of facilities.

Conflict of interest: The authors declare they have no conflicts of interest.

References

Churukian CJ, Frank M, Horobin RW. 2000. Alcian blue pyridine variant – a superior alternative to alcian blue 6GX: staining performance and stability. *Biotech Histochem.* 75:147–150.

Fagan C, Dapson RW, Horobin RW, Kiernan JA. 2020. Revised tests and standards for certification of alcian blue dyes by the Biological Stain Commission. *Biotech Histochem.* 95:333–340.

Hansch C, Leo A. 1979. *Substituent constants for correlation analysis in chemistry and biology.* Wiley, New York. pp 18–47.

Kiernan JA. 2015. *Histological and histochemical methods, theory and practice*, 5th ed. Scion, Banbury. pp 269–271.

Lillie RD & Fulmer HM. 1976. *Histopathologic technic and practical histochemistry*, 4th ed. McGraw-Hill, New York. pp 638 & 642.

Merck. 2021. Propyl astra blue iodide. www.sigma-aldrich.com/product/sial/363375?lang=en®ion=GB. Last inspected online 2nd August 2021.

Moracci MF, Casini A, Liberatore F, Carelli V. 1976. Dihydropyridines and pyridones from 3-cyano-1-methylpyridinium iodide in aqueous NaOH. *Tetrahedron Lett.* No. 41:3723–3724.

Pearse AGE. 1960. *Histochemistry theoretical and applied*, 2nd ed. Churchill, London. pp 259 & 838.

Penney DP, Powers JM, Frank M, Willis C, Churukian C. 2002. Analysis and testing of biological stains.—The Biological Stain Commission procedures. *Biotech Histochem.* 77:237–275.

PubChem. 2021. Alcian-blue-pyridine-variant.

<https://pubchem.ncbi.nlm.nih.gov/compound/22836391>. Last inspected online 2nd August 2021.

Riedel-De Haën AG. 2021. Propyl astra blue.

[ChemicalBook.com/ChemicalProductProperty_EN_CB7115839](https://www.chemicalbook.com/ChemicalProductProperty_EN_CB7115839). Last inspected online 2nd August 2021.

Scott JE. 1972a. Histochemistry of alcian blue. II. The structure of alcian blue 8GX. *Histochemie.* 30:215–234.

Scott JE. 1972b. Histochemistry of alcian blue. III. The molecular biological basis of staining by alcian blue 8GX and analogous phthalocyanins. *Histochemie.* 32:191–212.

Scott JE. 1973. Alcian dyes: I.C.I. cease manufacture and release details of composition. *Histochemie.* 37:379–380.

Scott JE. 1980. The molecular biology of histochemical staining by cationic phthalocyanin dyes: the design of replacements for alcian blue. *J Microsc.* 119:373–381.

Scott JE. 1996. Alcian blue. Now you see it, now you don't. *Eur J Oral Sci.* 104:2–9.

Steedman HF. 1950. Alcian blue 8GS; a new stain for mucin. *Q J Microsc Sci.* 91:471–479.

Figure captions

Fig. 1. Structures of alcian blue and related dyes discussed in the current paper. Identities of structures, with their sources, are as follows. **a.** Alcian blue/CI 74240/Ingrain blue 1 (Scott 1973 1980). **b.** Alcian blue pyridine variant (PubChem 2021). **c.** Astra blue 6GLL (Scott 1980). **d.** Propyl astra blue (Riedel-De Haën AG, 2021). **e.** Alcec blue (Scott 1980). **f.** Propyl astra blue, see text for comments on confusing nomenclature (Merck 2021). **g.** Fast blue 8XM (Mowry & Emmel 1966, Scott 1980).

Fig. 2. Identities of six side-chains of alcian blue dyes (S1–S6) seen in Fig. 1.

Fig. 3. Structure of cuprolinic blue (Scott 1972b, 1980).

Fig. 4. The pH-sensitivity of the various amino and aromatic nitrogen moieties present in certain alcian blue and related dyes. See Fig. 1-2 for complete structures of these dyes. **a.** The hydrophobic free base and hydrophilic salt species occurring in astra blue 6GLL and propyl astra blue at, respectively, high and low pH. **b.** Formation of a hydrophobic non-ionic thiol from the hydrophilic thiouronium substituent of alcian blue 8GS/8GX at high pH. **c.** Formation of hydrophobic pyridone and dihydropyridine derivatives from alcian blue pyridine variant at high pH, plus the hydrophilic salt formed from the latter at low pH. For simplicity the unstable pseudobase intermediate is not shown.

Fig. 5. Flowchart illustrating the bases of a possible analytical scheme for alcian blue dyes. This would distinguish between the two types of alcian blue dyes of concern to end users, namely alcian blue 8G and alcian blue variant. The scheme would also differentiate two sub-types of chemically different alcian blue variant dyes.