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Characterisation of illicit ecstasy and diazepam tablets by colorant identification.

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Ecstasy and diazepam are two commonly abused drugs with a high potential for dependence. They are typically available as oral tablets that contain both the drug and other bulk components (excipients). Compositional knowledge of individual tablets offers a method of identification that can be used for profiling. Established analytical methods such as high performance liquid chromatography (HPLC) and liquid chromatography – mass spectrometry (LC-MS) are used to verify the nature and quantity of active components while differential scanning calorimetry (DSC) has proved valuable in providing unique 'thermal signatures' for the bulk composition.[1] Analysis of the colorants commonly used in tablet aesthetics, however, has received very little attention so the aim of this work was to develop and validate such a method. HPLC-DAD was successfully used to characterise thirteen common colorants from five key classes of dyestuffs. Calibration data ($R^2 \geq 0.999$) was used to identify and quantify specific colorants in 63 out of 64 individual tablet cases with >98% success.

1 Introduction

1.1 Drug abuse and the identification of seized/illicit tablets. Drug abuse is a major social problem that receives much public attention. Drugs of abuse are many and varied but the widespread problems associated with the illegal Class A drug, 'ecstasy' and the prescription-only Class C drug, diazepam, are of particular currency. According to Police Scotland, over 13,000 ecstasy type tablets and over 1.2 million diazepam tablets were seized in 2015-16.[2] Characterisation of seized tablets is normally restricted to drug type and content for legal and logistical reasons, however, recent efforts to exploit the unique thermal properties of unknown tablets, using differential scanning calorimetry (DSC) as a bulk characterisation method, has been documented.[1] By this method, it was possible to quickly identify key excipients such as lactose or dibasic calcium phosphate (bulking agents or diluents) and the presence of other tablet excipients such as stearic acid (tablet lubricant). Tablets from individual batches had a common 'thermal signature' that could be used with high confidence to differentiate them from unrelated tablets. Principal component analysis (PCA) of the raw thermal data produced clear groupings and even pairings of disparate diazepam tablets. Other physical attributes such as size, shape, colour, identifying marks (manufacturers' logos) and hardness were used to further corroborate similarities amongst the different types of seized diazepam tablets with varying success. Colour analysis in particular was purely subjective and it was therefore of interest to

objectify this parameter by identifying the specific colorant/s present. Such information would contribute to the forensic identification of tablets.

1.2 Tablet colorants. Tablets are generally coloured to make them identifiable, aesthetically pleasing and consequentially to improve patient compliance.[3] The colorant can be added to the tablet core or to an external sugar or polymer coating.[4,5] The term 'colorant' is commonly used to describe the entire range of colouring materials that includes dyes and pigments. Dyes and pigments are both sources of colour but the distinction is of fundamental importance based on different chemical and physical characteristics. Pigments are completely insoluble in the material into which they are incorporated and require a binder or dispersing agent to spread the colour. On the contrary, dyes are soluble and the colour is dependent on the chemical properties of the molecular structure in solution. In some cases, organic soluble dyes can be precipitated with the help of a metallic salt, for example aluminium, to form an insoluble form of the dye referred to as a lake.[6,7] Colour lakes are pigments that colour by dispersion in the absence of a solvent, unlike soluble dyes.

All colorants can be further classified according to their chemical composition (organic or inorganic) and origin (synthetic or natural). Tartrazine, Allura Red AC and Brilliant Blue FCF are examples of synthetic colorants which are the products of chemically modified precursor compounds. They represent the largest group of colorants used in the food and pharmaceutical industry and display several advantages over natural colorants in terms of colour intensity and sensitivity to light and other chemicals. Natural food colorants are derived from natural sources by solvent extraction

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and examples include Curcumin and Caramel.[6,7] Synthetic dyes and their aluminium lakes, however, are more commonly added to tablets as they are often more suitable compared to natural colorants. Insoluble aluminium lakes can reduce the risk of 'over-colouring' and mottling of tablets in addition to decreasing production times by giving full colour development in a shorter drying stage for colour-coated tablets. Many of the colorants added to medications are commonly used and approved food colours that are readily and commercially available. They can be divided into five common dye classes, namely, azo, triarylmethane, xanthene, quinophthalone and indigo. Figures 1 and 2 highlight the synthetic organic dyes and their lakes that are most commonly used in the food and pharmaceutical industries.
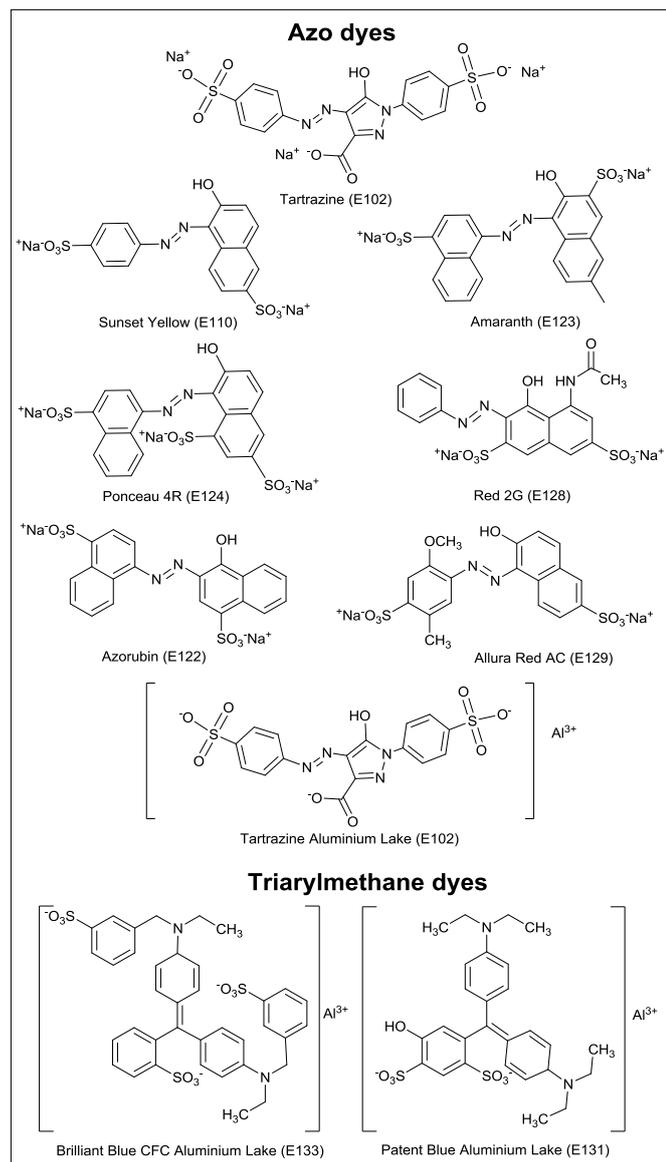

Fig.1 Chemical structures of the azo and triarylmethane dyes of interest in this study indicating the use of Al^{3+} instead of Na^{+} to form the lake.

1.3 Identification of colorants. Several methods for the separation, identification and quantification of colorants in food products have been developed and these include voltammetry, polarography, spectrophotometry, capillary electrophoresis, ion chromatography,

high performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS).[8] Ultra-violet/visible spectrophotometry (UV-vis) has limited use for the analysis of colorants in food samples as samples often contain more than one colorant resulting in overlapping bands. Derivative spectrophotometry (DS), based on normal absorption UV-vis, offers greater selectivity and sensitivity and has been successfully applied for the simultaneous determination of food colorants in different samples.[9-11] HPLC, however, is a rapid, highly selective and efficient analytical technique for the separation and detection of different compounds in a sample. Several studies describe accurate and precise HPLC methods for the separation and identification of dyes and their lakes in different foods.[8, 12-16] Most of these studies are based on reverse phase chromatography with diode array detection (DAD) and can analyse up to 40 different dyes within 20 minutes.[12] The bulk of the reported methods for the analysis of food colorants use a gradient mobile phase and suitable buffering as some colorants are pH-sensitive resulting in altered spectra and variable selectivity and sensitivity. Only one study would appear to have considered identification of the colorants in ecstasy 'pills' using capillary zone electrophoresis (CZE) with DAD, concluding that such information may play an important role in drug profiling[17].

With these considerations in mind, the proposed analysis of the colorants present in a large number of seized/illicit ecstasy and diazepam tablets is presented. UV-vis and scanning electron microscopy (SEM) with an energy dispersive X-ray analyser (EDXA) were also used to differentiate individual colorants and dyes from lakes where possible.
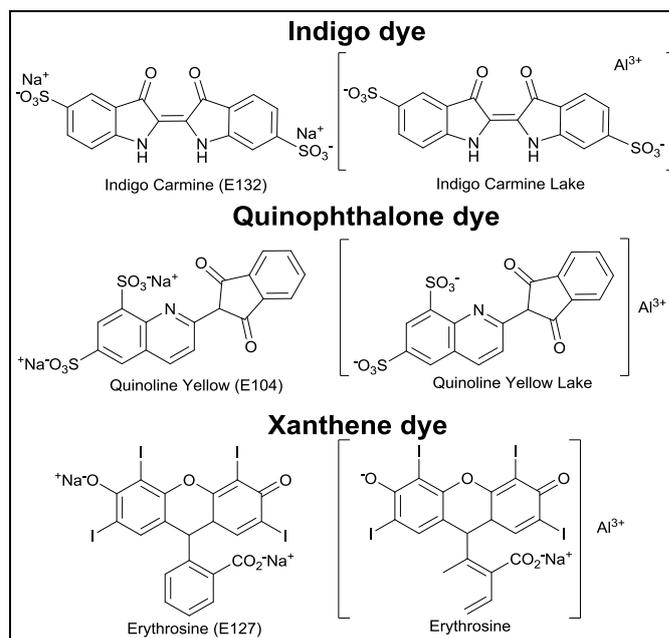
Fig.2 Chemical structures of the indigo, quinophthalone and xanthene dyes of interest in this study indicating the use of Al^{3+} instead of Na^{+} to form the lake.

2 Materials and methods

2.1 Seized ecstasy and diazepam tablets. Six cases of seized ecstasy tablets and 58 cases of seized diazepam tablets were kindly donated by Police Scotland. Tablets were grouped according to individual cases (seizures) with an associated case number and individual tablets in each case were numbered D1 to D58 for

diazepam tablets and E1-E6 for ecstasy. It should be noted that the drug type and content was already known from previously published[1] and unpublished work in our laboratories but bears little relevance to this study which considered the colourant only.

2.2 Colorants, reagents, solvents and licensed diazepam tablets.
Samples of the colorants Tartrazine (dye content ≥ 85%), Sunset Yellow FCF (dye content 90%), Quinoline Yellow (mixture of mono- and disulfonic acids), Allura Red AC (dye content 80%), Ponceau 4R (analytical standard, dye content ≥ 99%), Indigo Carmine (dye content 85%), Patent Blue V sodium salt and Brilliant Blue FCF (analytical standard) were purchased from Sigma Aldrich (Gillingham, UK). Quinoline Yellow Granular GS, Amaranth (dye content 85%), Durant Carmine WSP, Erythrosine Granular GS, Durant Red Beet WSP, Durant Anthocyanins WSP, Tartrazine Lake, Sunset Yellow Lake, Quinoline Yellow Lake, Allura Red Lake, Erythrosine Lake, Ponceau Lake 4R, Indigotine Lake, Patent Blue V Lake and Brilliant Blue Lake FCF were purchased from Fast Colours LLP (Huddersfield, UK). CI Acid Red 001 (Red 2G) was purchased from Kemtex Colours (Chorley, UK).

Analytical reagent grade sodium hydroxide, ammonium formate and 37% hydrochloric acid as well as HPLC grade methanol and acetonitrile were supplied by Fisher Scientific (Loughborough, UK).

Commercially licensed diazepam tablets (10 mg) were obtained from Actavis (Devon, UK) and Teva (Runcorn, UK) and used for reference with the seized diazepam tablets. No licensed ecstasy tablets exist.

2.3 UV-visible analysis of colourants

2.3.1   UV-vis spectrophotometry. Spectral measurements were performed using an Agilent 8453 UV visible diode array spectrometer equipped with a 10 mm cuvette holder (Agilent Technologies Inc., Santa Clara, USA). Absorption spectra were recorded between 350-700 nm in plastic cells (pathlength 10 mm).

2.3.2   Preparation of standard dye solutions. Individual standard stock solutions containing 1000 μg/mL of Tartrazine, Quinoline Yellow, Sunset Yellow FCF, Amaranth, Allura Red AC, Red 2G, Azorubin, Erythrosine, Indigo Carmine and Patent Blue V were prepared in deionised water. Another set of stock solutions were prepared separately in 0.02M NaOH to investigate the effect of the solvent on the absorption spectra. In addition, two sets of 200 μg/mL standard stock solutions of Brilliant Blue FCF and Ponceau 4R and 2000 μg/mL stock solutions of Carmine were prepared in both deionised water and NaOH (0.02M) respectively. Each stock solution was diluted to 20 μg/mL with deionised water prior to analysis.

2.3.3   Preparation of standard solutions from lakes. Lake standard stock solutions of Tartrazine Lake, Sunset Yellow Lake, Quinoline Yellow Lake, Allura Red Lake, Erythrosine Lake, Ponceau Lake 4R, Indigotine Lake, Patent Blue V Lake and Brilliant Blue Lake FCF were individually prepared by dissolving the lakes in 0.02M

NaOH with the aid of sonication for 5 minutes. Working solutions containing 20 μg/mL of the individual lakes were obtained by further dilution (50x) with 0.02M NaOH. A mixture containing 30 μg/mL of each lake was also prepared from the stock solutions.

2.3.4   Calibration of dyes and lakes. Calibration curves for all individual solutions of colorants (both dyes and lakes) were prepared by dissolution in 0.02M NaOH to an initial concentration of 500 μg/mL and neutralised immediately by the addition of 1M HCl (100 μL). It was appreciated that the stability of colorants to alkalis and acids varies considerably, with azo dyes having the best resistance.[18] Indigo Carmine, however, changes colour from blue to yellow in the range pH 11.4-13.0 and fades rapidly in acid media[18] whereas Erythrosine is stable from pH 6-9 in aqueous solution[19]; both are known to be unstable to prolonged exposure to light.[18,19] As a result of these instabilities, the Indigo Carmine stock solution was prepared at the much higher concentration of 2000 μg/mL, based on a noticeably weaker colour intensity than all of the other colorants thus calibration solutions of both were given special treatment and prepared freshly three-times a day and analysed immediately. All mixtures were analysed in order of increasing concentration to minimise the risk of carry over in the HPLC column.

Seven calibration solutions in the range 0.5-30 μg/mL were made from the stock solutions and analyses made in triplicate. The method was validated by consideration of the linearity and range of the results obtained. Limits of detection (LOD) and quantification (LOQ) were estimated from the signal to noise (S/N) ratio. The height of the smallest peak that was not known to be associated with the colorants was considered noise. A S/N ratio of 3:1 was considered the LOD and 10:1 as the LOQ. Precision was evaluated using inter- and intra-day repeatability. A mixture containing all of the dyes, except Indigo Carmine and Erythrosine, at a concentration of 10 μg/mL was analysed in triplicate and the relative standard deviation (%RSD) based on peak area and retention times calculated. The same analyses were also undertaken on three consecutive days to study intra-day precision.

Further runs were undertaken to assess accuracy using another analyst to prepare the samples and the robustness of the method determined by alterations to the methanol:acetonitrile ratio (±20%) and variations in the injection volume (5 x 20 μL, 3 x 15 μL and 3 x 10 μL). Robustness was determined by calculating %RSD and percentage change for the various solvent ratios and injection volumes (Table 1) in comparison to the conditions of Li et al (2015). A further experiment to determine the stability of Indigo Carmine and Quinoline Yellow solutions in 0.02M NaOH$_{(aq)}$ was conducted every day for one week by making a fresh dilution (20 μg/mL) from the stock solution (2 mg/mL of a 50:50 mix of both dyes) which was stored in a cupboard *i.e.* 18-23°C in the dark.

2.3.5   Preparation of colorant solutions from tablets. A tablet from each individual case was crushed in a mortar and pestle and a sample (100 mg) was accurately weighed into a small volumetric flask (5 mL) and 0.02 M NaOH was added to dissolve the colorant/s present. The extraction process was aided with sonication (5 mins). Following sonication, an aliquot (1 mL) was transferred to an Eppendorf tube and 20 μL of 1M HCl added to neutralise the

solution. The sample was then centrifuged (2 min, 10,000 r.p.m) to remove the supernatant and transfer it into an HPLC vial for analysis. A total of six ecstasy (E1-E6) and 58 diazepam (D1-D58) tablets, as specified in Section 2.1, were analysed.

2.4 HPLC analysis of colorants. Chromatography was performed using a Shimadzu HPLC system consisting of a LC-20AD prominence liquid chromatograph, a DGU 20A5 prominence degasser, a SIL 20A prominence auto sampler and a SPD-M20A prominence diode array detector (Kyoto, Japan). A C_{18} ODS Hypersil-Keystone analytical column (250mm x 4.6mm i.d., 5 μm particle size) supplied by Thermo Fisher Scientific Inc. (Waltham, USA) was used to achieve separation. The Shimadzu LC solution software (Kyoto, Japan) was used for instrument control, data acquisition and data analysis.

The mobile phase consisted of solvent A and solvent B where A was prepared by dissolving ammonium formate (1.26 g) in deionised water (1000 mL) to obtain a 20 mM ammonium formate buffer. Solvent B consisted of a 50:50 mix of equivalent volumes of methanol and acetonitrile. The precise gradients used, at a flow rate of 1 mL/min, are detailed in Table 1.

Samples (20, 15 and 10 μL respectively) of each solution were injected individually. The total time between injections was 20 minutes including a 5 minute column equilibration period. Detection was undertaken at 420, 520 and 620 nm for the yellow, red and blue colorants respectively. Absorption spectra of the analytes were recorded in the range 350 – 700 nm.

2.5 SEM-EDXA to distinguish the difference between dyes and lakes. Scanning electron microscopy (SEM) was conducted using an EVO LS10 series scanning electron microscope in combination with a variable pressure detector and ScartSEM software (Zeiss, Cambridge, UK). An Inca version 4.09 EDXA detector with microanalysis suite issue 17B+SP1 software (Oxford Instruments, Abingdon, UK) was used for EDX analysis. Samples were prepared on aluminium stubs with self-adhesive carbon pads (Agar Scientific, Essex, UK).

Small amounts of the pure Indigo Carmine dye, lake, Actavis and Teva tablets were fixed individually on the stubs. The elemental composition of the two versions of the same colorant and of the commercial diazepam tablets was investigated using the EDXA function of the SEM. Chamber pressure was maintained at 100 Pa, the working distance was 8.5 mm and the accelerating voltage of the electron gun (EHT) set at 25 kV.

3 Results and discussion

3.1 UV-vis spectroscopy of colourants in deionised water and NaOH. UV-vis spectroscopy was performed prior to the chromatographic analysis as described in Section 2.3.1 to determine the optimum detection wavelength (λ_{max}) for the various colorants. As sodium hydroxide was necessary to dissolve the lakes while the dyes were water soluble, spectra for the dyes in both deionised

water and sodium hydroxide were obtained to gauge any anticipated differences in λ_{max}. Table 2 summarises the results from the analyses of λ_{max} undertaken and includes the literature values.[20] It is clear that there are three distinct groupings based on whether the reflected colour is yellow, red or blue. Precise values for λ_{max} are distinctly different and vary from $3 < \lambda_{max} < 127$ nm in deionised water and NaOH for all but five of the colorants tested. Differences <3 nm were measured for Amaranth, Ponceau 4R, Allura Red AC, Erythrosine and Indigo Carmine. Of the eight lakes that were available for analysis, the maximum difference in λ_{max} compared with the dye in NaOH was 4 nm for Patent Blue, the others being essentially equivalent. Small differences were attributed to the highly alkaline pH which was subsequently neutralised with HCl for chromatographic analyses. Neutralisation of the colorant solutions, while maintaining solubility following dissolution in NaOH, was beneficial for both the HPLC column and the reduction of stability issues observed with Indigo Carmine.

3.2 HPLC analysis of colourants and tablets

3.2.1 Analysis of dyes. The starting point for chromatographic analysis was the method described by Li *et al* (2015) who correctly identified and quantified 34 dyes in foodstuffs.[8] Individual dye solutions were analysed separately to determine specific retention times (t_R) and corresponding absorption spectra. This data was successfully used as a reference to identify the different dyes in the standard mixture (Section 2.3.3). To improve resolution and decrease analysis time, Solvent B (methanol/acetonitrile, 50:50) was altered from 54% at 8 mins[8] to 50 and then 45%, Table 1. Minimum resolution was 1.5 and the total analysis time, including column equilibration, was reduced from 53 mins to 20 mins. These conditions were applied for the further analysis of all standards and tablet samples.

Figure 3 shows a typical chromatogram of the standard mixture containing 10 μg/mL of all 13 dyes recorded at 420 nm for quantification of the yellow components. The identity, specific retention time, minimum resolution and λ_{max} are summarised in Table 3. All yellow dyes were successfully identified using retention time data. The remaining peaks in Figure 3 are associated with the red and blue dyes also present in the standard mixture. Clearly, based on UV-vis evidence of λ_{max} values for all dyes, Table 2, quantification of red and blue dyes was more aptly undertaken at the higher wavelengths of 520 and 620 nm respectively.

Figure 4 is the chromatogram displayed at 520 nm (red) for the dye mixture and highlights the peaks associated with the stated dyes and Figure 5 concerns the blue dyes at 620 nm. Retention times, minimum resolution and λ_{max} are summarised in Tables 4 and 5, respectively.

3.2.2 Analysis of lakes. The diluted working solutions of the individual lakes and the mixture (Section 2.3.3) were analysed under the same conditions as the dyes. The specific retention times and individual analyses were successfully used to identify the lakes in the mixture. Individual peaks were well separated with a minimum resolution of 1.9 for the second Quinoline peak. No differences between the retention times and absorption spectra of the lakes compared to the dyes were observed.

Table 1. Optimised mobile phase gradient initially based on the method of Li *et al* (2015).[8]

| Time (mins)	Solvent A* (%)	Solvent B**
0	95	5
8	55	45
10	25	75
15	25	75
15.1	95	5
20	95	5

*Solvent A – 20 mM ammonium formate buffer.

**Solvent B - methanol/acetonitrile (50:50) (%w/v)

Table 2. Maximum absorbance wavelengths (λ_{max}) of the different colorants with equivalent lakes where possible.

Colourant	λ_{max} A (nm)	λ_{max} B (nm)	λ_{max} C (nm)	λ_{max} D (nm)
Tartrazine	426	426	398	398
Quinoline Yellow	411	411	384	383
Sunset Yellow FCF	485	481	446	445
Carmine	518	517	532	-
Amaranth	520	521	520	-
Ponceau 4R	505	506	507	-
Allura Red AC	504	501	504	502
Red 2G	532	531	505	-
Azorubin	516	516	505	-
Erythrosine	526	526	526	526
Indigo Carmine	610	611	612	611
Brilliant Blue FCF	630	625	630	633
Patent Blue V	638	636	625	629
Anthocyanins	525	526	NS	-
Beet Root Red	535	533	406	-

A - Literature value; B – Dye/H_2O; C – Dye/NaOH; D – Lake/NaOH; NS – not stable.

Table 3. Retention times, minimum resolution and λ_{max} of red dyes. Note that resolution data represent the minimum resolution to the next eluting peak.

Dye	t_R (mins)	Resolution	λ_{max} (nm)
Tartrazine	5.723	0	426
Quinoline Yellow	7.664	3.289	414
	8.287	1.914	
	11.947	3.169	
Sunset Yellow FCF	8.045	2.927	483
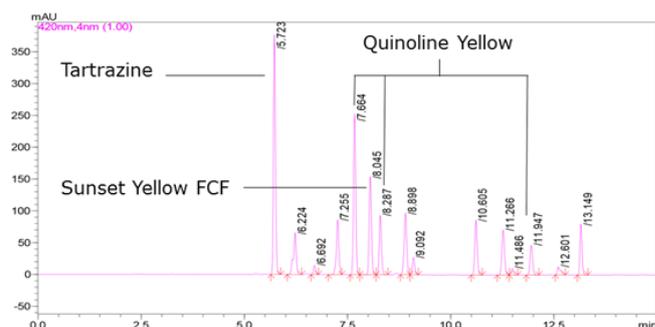
Fig.3 Chromatogram of dye mixture at 420 nm for yellow dyes. Note that red and blue dyes also show absorbance at this wavenumber *i.e.* Amaranth (6.224), Indigo carmine (6.692); Ponceau 4R (7.256); Allura Red AC (8.898); Red 2G (9.092); Azorubin (10.605); Brilliant Blue FCF (11.266); Erythrosine (12.601) and Patent Blue V (13.149 mins).
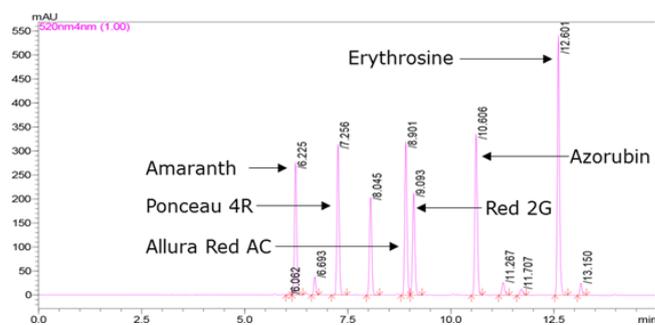
Fig.4 Chromatogram of dye mixture at 520 nm for red dyes. Note the peak at t_R = 8.045 mins is associated with Sunset Yellow FCF and the other weaker peaks are associated with Indigo Carmine (6.693), Brilliant Blue FCF (11.267) and Patent Blue V (13.150).
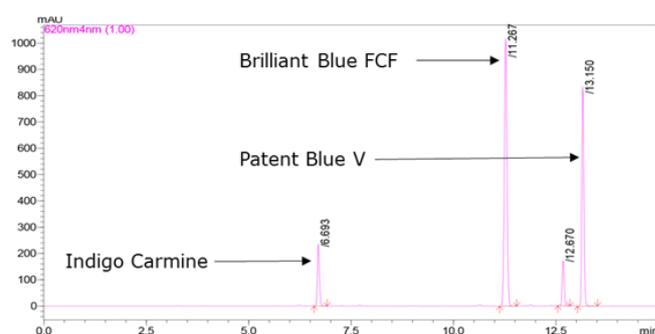
Fig.5 Chromatogram of dye mixture at 620 nm for blue dyes. Note the peak at t_R = 12.670 min is also associated with Patent Blue V from consideration of the individual dye analysis (not shown).

Chromatographic analysis was consequently not able to distinguish between lakes and dyes. Neutralisation of the caustic solution of lakes with HCl did not appear to precipitate the insoluble (aluminium) salts, maintaining solubility.

Table 5. Retention times, minimum resolution and λ_{max} of blue dyes. Note that resolution data represent the minimum resolution to the next eluting peak.

Dye	t_R (mins)	Resolution	λ_{max} (nm)
Indigo Carmine	6.693	0	611
Brilliant Blue FCF	11.267	36.932	628
Patent Blue V	13.150	4.516	634

Prior to the method described for solubilising lakes, extraction of the colourant in methanol was only partially successful with much of the lake remaining insoluble. A previous study suggested a lake extraction with a mixture of H_2O:methanol:37% HCl (1:1:2 by volume) but the Brilliant Blue FCF lake and Patent Blue V lake used in this particular study changed colour from blue to yellow and orange respectively after addition of HCl.[21] Best results were obtained by using sodium hydroxide although stability issues were noted after a while. Sodium hydroxide was successfully used to extract aluminium lakes in another study but no stability issues were reported.[22]

3.2.3   Calibration of dyes and lakes. For quantitative analysis, a calibration curve for each dye and lake was performed in the concentration range 0.5-30 µg/mL following the procedure described in Section 2.3.4. Twelve of the 13 dyes analysed gave linear correlations for 'absorbance as a function of concentration' with correlation coefficients (R^2) of ≥0.999. One of the dyes, Carmine, could not be calibrated due to its low absorptivity while Erythrosine and Indigo Carmine showed notably higher %RSDs of

4.21 and 6.24% respectively, indicating probable instability in solution during the course of the calibration measurements. The remainder of the dyes gave a %RSD which varied from 0.05% for Brilliant Blue FCF to 0.52% for Patent Blue V, the remainder all falling within the range 0.08-0.24%, Table 6.

Table 6. Summary of calibration data for all of the dyes tested, including slopes of calibration lines, mAU/t_R (R^2≥0.999, n=3), percentage relative standard deviation (%RSD), signal to noise ratio (S/N), limit of detection (LOD) and limit of quantification (LOQ). Note the gradual decrease in the response of the detector to Erythrosine and Indigo Carmine between measurements.

Dye	mAU/t_R Run 1	mAU/t_R Run 2	mAU/t_R Run 3	%RSD	S/N	LOD (µg/mL)	LOQ (µg/mL)
Tartrazine	141371	141194	141153	0.08	20.74	0.07	0.24
Quinoline Yellow	95977	95706	95603	0.20	13.91	0.11	0.36
Sunset Yellow FCF	61947	61759	61669	0.23	0.17	0.17	0.57
Carmine	-	-	-	-	-	-	-
Amaranth	106470	106164	106055	0.20	15.09	0.10	0.33
Ponceau 4R	117419	117055	116954	0.21	16.96	0.09	0.29
Allura Red AC	130011	129665	129689	0.15	17.00	0.09	0.29
Red 2G	88554	88303	88210	0.20	11.87	0.13	0.42
Azorubin	147979	147647	147568	0.15	18.24	0.08	0.27
Brilliant Blue FCF	440019	439688	439595	0.05	52.65	0.03	0.09
Patent Blue	292743	292038	294945	0.52	44.14	0.03	0.11
Erythrosine	197549	187079	181934	4.21	27.21	0.06	0.18
Indigo Carmine	83829	76261	74689	6.24	17.13	0.09	0.29

3.2.4   Analysis of tablets. The developed method of analysing colorants by HPLC was successfully applied to tablet samples. Seized tablets were analysed according to the procedure described in Section 2.3.5. Retention times and absorbance of the peaks detected for the ecstasy tablets (E1-E6) were compared with those of the standards. The colorant was identified based on retention time and the content was calculated with the aid of the appropriate calibration data, Table 7.

Table 7. Identification and quantification of colorants in 100 mg of crushed ecstasy tablets (E1- E6). Note that three distinct colorants were detected in E4.

Tablet	t_R (mins)	Colourant	Quantity (µg)
E1	5.519	Tartrazine	572.2
E2	-	(Not identified)	-
E3	11.044	Brilliant Blue FCF	448.1
E4	7.052	Ponceau 4R	29.8
	8.636	Allura Red AC	316.0
	10.261	Azorubin	587.5
E5	7.799	Sunset Yellow	192.0
E6	11.036	Brilliant Blue FCF	150.2

All of the tablets analysed were significantly different based on the colorants identified. Both E3 and E6 contained Brilliant Blue FCF but in greatly different quantities of 448.1 and 150.2 µg respectively. This would suggest that they were most probably not from the same batch. The other tablets were completely different in terms of colour and E4, in particular, contained three different red colorants in differing quantities, Azorubin being the most prevalent at 587.5 µg compared with Allura Red AC and Ponceau 4R at 316.0 and 29.8 µg respectively. One colorant in E2 was not identified in this study.

Of all the diazepam tablets analysed, 16 contained Indigo Carmine and 40 contained Brilliant Blue FCF, Table 8. According to the manufacturers of the two licit batches of diazepam tablets analysed (Teva and Actavis), both types of licit diazepam (10 mg) tablets contain Indigo Carmine (Patient Information Leaflet contained within consumer packaging). Analysis of these two types of diazepam tablet indicated that both did indeed contain Indigo Carmine in quantities of 12.96 ± 0.95 µg for the Actavis tablets and 11.28 ± 0.15 µg for Teva (n=3 for each of five samples of both batches of licit tablets). These results would suggest that with the exception of D9, D28 and possibly D43, 81.25% of the diazepam tablets tested and found to contain Indigo Carmine were consistent with those levels detected in the licit reference tablets.

It would also be realistic to assume that these measurements slightly underestimate the absolute quantities of this colorant considering its known degradation in solution (see Section 3.1 and Table 6). Such concerns were absent, however, for 70% of all the diazepam tablets that contained Brilliant Blue FCF as opposed to Indigo Carmine. The quantities of this colorant detected were highly variable and ranged from 1.1 – 179.2 µg (Table 8). This variance could be separated into groups of tablets with quantities of colorant <2 µg (17.5%), between 2 and 5 µg (15%), 5 and 12 µg (32.5%) and 17 and 35% (32.5%).

Table 8. Identification and quantification of colorants in 100 mg of crushed diazepam tablets (D1-D57).

Tablet	Indigo Carmine	Brilliant Blue FCF	Quantities (μg)
D1, D9, D12, D13, D17, D18, D25-D30, D33, D36, D43, D53	✓	–	Range 4.3 – 38.3 Mean (±SD) 12.74 ± 8.75
D2-D7, D10-11, D14-16, D19-24, D32, D34-35, D37-42, D44-52, D54-57.	–	✓	Range 1.1 – 179.2 Mean (±SD) 19.01 ± 31.21
D31	✓	✓	102.65 Indigo Carmine 7.03 Brilliant Blue FCF

Two further tablets returned values of 97.2 and 179.2 μg respectively. This high variability in colorant content between different tablets could be used in conjunction with other forensic analysis methods to aid in the identification and grouping of specific tablets. For example, D31 contained both Indigo Carmine and Brilliant Blue FCF making it quite unique and not related to any of the others tested.

3.2.5 Difference between lakes and dyes using SEM-EDXA.

HPLC-DAD was unable to differentiate the difference between dyes and lakes as would be expected considering that lakes solubilised in NaOH are essentially the same as the soluble dyes (Section 1.2 and Table 2). SEM-EDXA, however, gave a clear signal for aluminium in the solid lake at 1.5 keV that was absent in the dye, indicating the usefulness of this technique to distinguish between dye and lake. A simple qualitative test involved adding deionised water to the crushed tablets and agitating with a vortex mixer. On removal of the water, the crushed tablet should maintain its coloured hue if a lake is present, the converse being true if a water soluble dye has been used.

4 Conclusions

An accurate, precise and sensitive HPLC-DAD method for the separation of thirteen colorants was developed and validated. Calibration showed a linear relationship with a minimum correlation coefficient $R^2 \geq 0.999$ for all thirteen colorants over a range of 0.5-30 μg/mL. The limits of detection were between 0.03-0.17 μg/mL and recoveries for Indigo Carmine and Tartrazine were 106.6% and 93.4% respectively. The method was successfully applied for the identification and quantification of colorants in seized ecstasy and diazepam tablets. A total of six ecstasy and 58

diazepam cases were successfully analysed with a single inconclusive result (E2), this equates with a success rate of >98%.

The developed method outlined can provide important additional drug intelligence about the colorant/s used in unknown tablets and this information can be used in conjunction with other analytical data that identifies the drug and drug content (chromatographic); bulk excipients (thermoanalytical) and other established forensic imaging methods (physical attributes); to reveal connections between disparately seized tablets. Such intelligence would greatly assist in the continuing war on the uncontrolled availability of illicit medicines.

Conflicts of interest

There are no conflicts to declare.

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