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Development of New Extraction Methods for Analysis of Natural and Synthetic Organic Colourants from Historical and Artistic Matrices

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Chimica
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Kathryn Raeburn McClure
Matricola 1885666

Relatore
Professor Roberta Curini

Correlatore
Dr. Ilaria Serafini
Dr. Alessandro Ciccola

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Abstract

In this thesis, an innovative approach for the extraction and clean-up of natural and synthetic textile dyes based on a recently developed ammonia extraction protocol and a novel Dispersive Liquid-Liquid Microextraction (DLLME) is presented. The thesis builds upon recent research highlighting the benefits of the ammonia-based extraction protocol for efficient extraction and preservation of the glycosyl moieties present in some types of natural dyes. This state-of-the-art extraction technique requires the use of a clean-up step to purify and pre-concentrate the dye molecules for analysis. This clean-up step has never before been investigated or developed, and current methods rely upon traditional Liquid-Liquid Extractions (LLE), which are not well suited to the very small quantities of materials available for the analysis of artefacts of cultural heritage. The novel DLLME protocol presented by this thesis was developed in order to improve the recovery of natural dyes for analysis, and is also the first clean-up protocol to be developed for the analysis of synthetic textile dyes from cultural heritage matrices. Whilst a clean-up system has never before been applied to synthetic dyes within cultural heritage, pre-concentration and pre-treatment protocols are frequently reported for analysis of the same type of dyes used in food colourants. For this reason, this research adapts a DLLME method from the analysis of edible products, and combines this with the state-of-the-art ammonia extraction method reported in literature. DLLME protocols for both natural and synthetic dyes were developed and optimised first on known analytical standards, considered representative of the possible structures of natural dyes and synthetic azoic acid dyes respectively. The extraction recoveries of a variety of disperser and extraction solvents were analysed using HPLC coupled with targeted mass spectrometry. The optimised conditions were then coupled with the ammonia based extraction to ensure coherence of the methods. The results showed significant improvements in the recovery of natural dye analytes compared to current methods, as well as increased precision and efficiency. For synthetic dyes, results showed adequate recovery of analytes and allowed the ammonia-based extraction method to be applied successfully for the first time. After optimisation, the protocol for synthetic dyes was applied successfully to 15 samples (11 fibres, 4 powders) of suspected azo dyes from the Azienda Coloranti Nazionali e Affini (ACNA) synthetic dye collection housed at Sapienza University of Rome's Museum of Chemistry. The novel protocol was performed after preliminary Raman screening to obtain some introductory information about the unknown samples in the collection. After application of the novel protocol, the samples were identified through untargeted analysis by HPLC-HRMS.

Keywords: *cultural heritage, textile dyes, natural dyes, azo dyes, DLLME, Raman spectroscopy, HPLC-MS*

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1. Introduction

1.1 Introduction to Historical Textile Dyes

Fabric dyeing has been part of human culture since antiquity [1] and has been carried out in many contexts: from primarily functional products (such as clothing and carpeting) to works of art (such as tapestry and embroidery). Through museology and archaeology, we are left today with the remains of these dyed household objects and artistic works from a wide range of historical periods. The scientific study of these artefacts can provide information about past societies and artistic styles, as well as key data to inform the conservation and preservation of the artefacts themselves. This analysis is a complex process which relies on not just advanced analytical procedures, but also an in-depth understanding of the historical, artistic and human context from which the object being studied originates.

In general, textile artefacts are complex matrices consisting of fibres, dyes, and often mordants to fix the dye to the fibre – which are very common for most classes of natural dyes (except in specific cases such as indigoids), and unnecessary for most synthetic dyes due to their chemical structures which are specifically designed to have affinity for the functional groups present on fibres. All components are highly sensitive to degradative mechanisms, which means that they are rarely preserved in archaeological contexts. [2] This factor means that those artefacts that do survive are extremely precious to our understanding of the use of dyes and textiles by past human societies. It is therefore essential that the care and preservation of dyed artefacts in heritage collections is considered paramount by the institutions they are stored and displayed in. This is made difficult by their sensitivities to exposure to open-air environments (such as museums), which can lead to photo-oxidation and subsequent mechanical degradation over time. [3] The complexity of their make-up means that all the different degradation mechanisms of each individual component of the dyed textile must be studied as well as considering how the object is affected as a whole by degradative effects.

These matters of degradation, despite being beyond the scope of the research presented in this thesis, are the reason why this research is important. The identification of the chemical compositions of all the components of dyed fibres is necessary to understand their possible sensitivities to environmental conditions. This understanding is an essential requirement for the development of effective conservation strategies for specific objects. Currently, the analysis of dye composition is one of the most interesting analytical challenges facing the study of dyed textile artefacts. Specifically, the basis of understanding these chemical compositions is the extraction of the dyes from the fibre, purification of the dye extracts and

their subsequent analysis by high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) - and these procedures are the foundations of the research in this thesis. The extraction procedure is a particularly important part of the analytical process, as during this step we must ensure that the maximum quantity of dye molecules are extracted from the fibre whilst still ensuring the molecules (which in many cases are easily degraded in harsh conditions) are preserved. [4]

Furthermore, the fragility of these materials, alongside their intrinsic human value and the abundance of information they can provide about human history places increased importance on the principle of non-destructiveness, whereby artefacts should undergo as little damage as possible during analysis. This means that sample sizes and the evaluation of ethical questions surrounding sampling must be placed at the forefront of all textile dye studies. [5] In recent years, these have been highlighted as reasons why research must focus on multi-technical and high-sensitivity approaches, which take into account the minimum sample size requirement for each technique.

The historical and analytical challenges surrounding the analysis of textile dyes varies depending on the type of dye being analysed. Both natural and synthetic dye studies are informative and valuable for cultural heritage, but they must each be considered in the context of their chemistries and histories, which are very different from each other.

1.2 Natural Dyes

1.2.1 History and Value of Study of Natural Dyes

Until the 19th century, natural resources were the only sources for textile dyeing, meaning that throughout the majority of human history all organic textile dyes were extracted from plants, animals and lichens. [6] The dyeing processes hence relied on the availability of specific highly coloured biological species which could be used as dyes.

Due to this historical importance, natural dyes have had the focus of almost all of the scientific research on historical textiles since the field was developed in the late 20th Century. [7] This research generally seeks to find the chemical composition of the dye by individuating possible diagnostic markers to hypothesise about the type of biological source from which it was extracted. This analysis can be highly informative, in particular contributing to the rebuilding of the histories of the object under analysis and the entire society from which the object originates. One type of information it is possible to obtain from natural dye studies is the provenance of the artefact, for example, the identification of specific compounds can distinguish whether artefacts were dyed using Armenian or American cochineal dyes. These

insect dyes had an intriguing and complex trading history, as the American cochineal dyes were highly sought after and over a very short period completely replaced the use of Armenian cochineal. Periods of change such as this are historically very interesting, and identifiying which source species cochineal dyes from this period were obtained from can provide information about the behaviours of traders and dyers during this time. [4] It is also possible to obtain information about the technological processes used in a dye's synthesis, for example, the presence of Turkey Red madder dye indicates the application of a long, complicated and advanced process involving up to fourteen operational steps. [8] Furthermore, the identification of dyes used can also point towards the history and value of objects, for example, the use of the rare and valuable Tyrian Purple can demonstrate that the artefact is likely to have belonged to a person of a high social standing. [9] In turn, these fragments of information can be compiled with other scientific and historical data to obtain a fuller picture of the society from which an object originated. As such, scientific studies of natural dyes can provide excellent historical information, and hence are an extremely valuable resource.

In addition to the historical information, a full understanding of the chemical compositions of the natural dyes on a historical artefact is an essential requirement for ensuring ethical and responsible conservation and preservation. Different dyes are affected in various ways by their external environmental conditions. Yellow flavonoid dyes, for example, are photosensitive and will lose their colour rapidly when displayed or stored under intense lighting. [10] Brazilwood red dye can also be completely faded to beige when incorrectly stored, raising significant questions for restorers when presented with an object which has undergone a significant change from its original colour. [11] Natural dye studies hold the key to ensuring that collection holders are well informed on the contents of their collection and understand issues that may arise with their maintenance and preservation.

1.2.2 Chemistry of Natural Dyes and Analytical Challenges

The reliance of natural dye production on natural resources mean that generally these dyes fit into a finite number of chemical classes. These classes are split into mordant, direct, and vat dyes. Mordant dyes require the use of a "fixative" to bond strongly to their fibre whilst direct and vat dyes are applied directly to the fibre without use of a fixing agent. The mordant dyes will be the primary focus of this thesis and mainly fall under the chemical classes of anthraquinones, flavonoids and tannins. Within each of these individual classes is a vast series of chromophoric compounds, owing to the natural biodiversity of the source species. An example of this is the madder extracts (from the roots of the *Rubia* spp. plants) which are composed of up to sixty eight distinguishable anthraquinone species [12] that vary in

percentage and composition greatly, often influenced by external factors such as the terrain and climate in which they were grown. [13] In contrast, the commercial success of synthetic dyes is due to their rapid production and reproducible hues – which are achieved precisely by limiting the number of chemical structures synthesised through the highly controllable chemical process. For example, when madder dye chromophores were recreated industrially, laboratories largely chose to only synthesise one of the anthraquinone derivatives: alizarin. [14]

For the research produced by this thesis, four analytical standards were chosen as they represent prominent compounds in some of the most significant natural dye classes. The four dyes chosen (alizarin, purpurin, carminic acid and luteolin-7-O-β-D-glucoside) can be considered representative of aglycones, C-glycosides and O-glycosides, which are the main typologies of compounds expected from natural dye sources. [15] The choice to use carminic acid and luteolin-7-O-β-D-glucoside was also specifically due to the sensitivities of the glycosidic bonds to acid conditions. [4] These standards were used to ensure that the processes developed by this research preserved these bonds, enabling the detection of these specific species.

1.2.2.1 Aglycone Standards

Alizarin and **purpurin** are two common chromophoric compounds found in the Madder dyes extracted from the root of the *Rubia* spp. plants. They are aglycone compounds, which are one of the main typologies of compounds we expect to find in natural dye sources. They belong to the anthraquinone dye class and share similar basic structures comprised of the anthraquinone structure with hydroxyl groups in the 1 and 2 positions; with purpurin also containing an extra hydroxyl group in the 4 position. The madder dyes were fixed to the fibres by mordants, most commonly the alum mordant which obtains a vibrant red colour. The mordant is used because the dye species have little affinity for the fibre, and as such the mordant forms a coordination complex between the fibre and the dye molecule, fixing them together. For alizarin and purpurin, the aluminium ion coordinates to the adjacent oxygen atoms in the 1 and 13 positions, and then also to the fibre. [12]

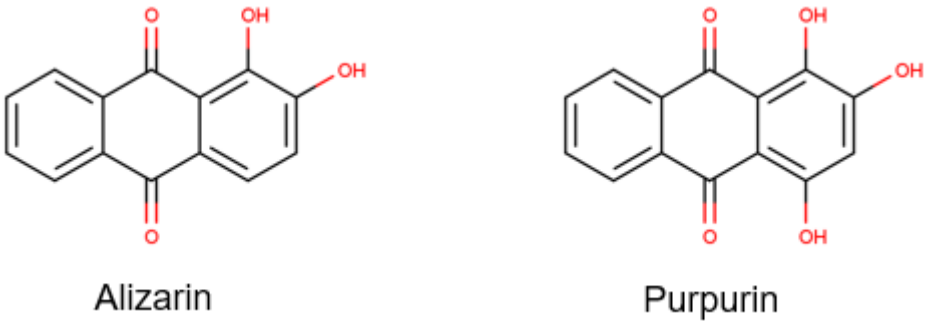

Figure 1: Structural formulae of alizarin and purpurin, two common anthraquinone derivatives present in Madder dyes. [12]

1.2.2.2 Glycosidic Standards

Carminic acid is another structure from the anthraquinone class, this time a glycosidic species and is derived from scale insects such as the American, Armenian and Polish cochineal. It is generally applied with an alum mordant, as with the madder dyes, to obtain a bright red hue. In carminic acid, the structure consists of a tetrahydroxyanthraquinone group substituted with a methyl group; carboxyl group and a 1,5-anhydro-D-glucitol moiety connected to the anthraquinone group by a C-glycosidic bond. [16] Glycosidic bonds are highly sensitive to hydrolysis by strong acids, and the preservation of this moiety must therefore be considered paramount when developing chemical techniques for the analysis of natural dyes. [4]

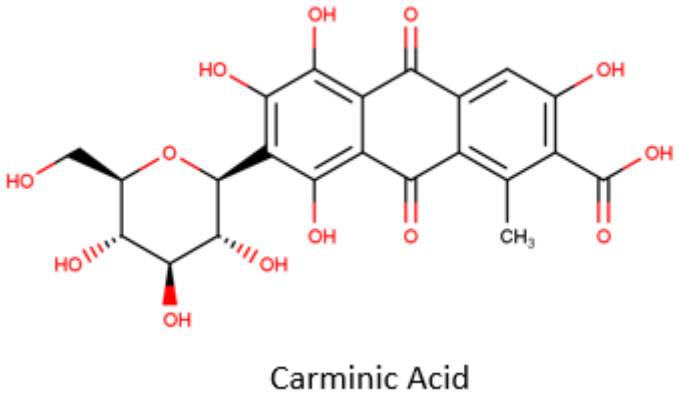

Figure 2: Structural formula of carminic acid, a natural anthraquinone derivative present in some scale insects. [16]

Luteolin-7-o-β-D-glucoside is a glycosidic compound of the flavonoid class – which is a wide ranging class derived from a variety of biological sources including fruits, bark and flowers. Due to the wide range of sources, it is an extremely prominent type of natural dye. Luteolin-7-o-β-D-glucoside is a non-coloured molecule but is obtained alongside many of the yellow flavonoid dyes. It is composed of the basic flavonoid structure with three substituted hydroxyl groups and a β-D-glucopyranosyl moiety, which is bonded to the flavonoid group by an O-glycosidic bond. [17] The sensitivity of this glycosidic bond to acid conditions must be considered, as with carminic acid. [4]

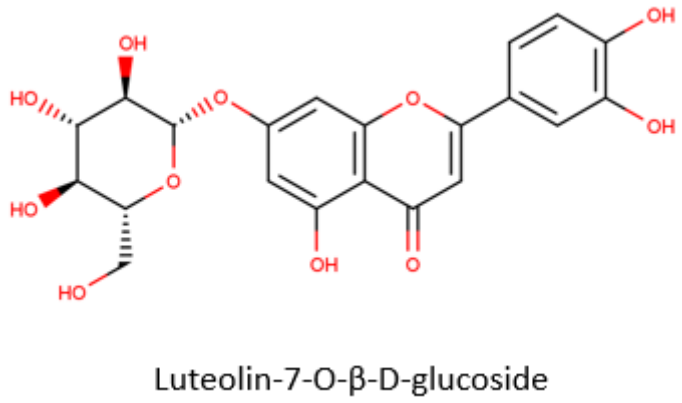

Figure 3: Structural Formula of Luteolin-7-O-β-D-glucoside, a glycosidic flavonoid compound obtained from a wide variety of natural resources. [17]

1.2.2.3 Analytical Challenges

The chemical compositions of the dye molecules present significant challenges for the identification of natural dyes from historical textiles. For example, although the limited molecular classes of natural dyes have allowed useful databases to be developed for comparison of results, the variability of composition depending on the biodiversity of the natural matrix means that several varied compounds can be combined in low concentrations within a single dye – making them difficult to detect. Even when the type of dye used is known it can be extremely difficult to predict which specific chromophore may be present in a sample.

The organic nature of the dyes, alongside their biodiversity means that their analysis is highly complex. It is evident from literature that methods for non-destructively analysing naturally dyed artefacts still need significant improvement. This issue can be attributed to specific problems that organic compounds present for the portable, non-destructive techniques that are widely used for inorganic studies in cultural heritage. For example, Raman spectra acquired in-situ are often affected by a high fluorescence background, meaning that the

signals are overwhelmed. In addition, the elemental analysis obtained through X-ray fluorescence (XRF) is not generally indicative or useful for the identification of specific organic molecules. The most common method for the analysis of natural dyes is HPLC combined with a detector, usually a mass spectrometer (MS) or diode array detector (DAD), which is an inherently destructive process.

The requirement to identify dyes using destructive processes means that utmost importance must be placed on minimising sample sizes, and analyses must therefore have efficient extractions; high preservation of molecular species; and high recovery. It is also necessary to utilise multi-technical; high-sensitivity approaches for these analyses to maximise the information obtained from the sample.

1.2.3 State-of-the-Art for Analysis of Natural Dyes

It is clear that natural dye studies provide excellent opportunities to understand the history and conservation requirements of the archaeological and artistic objects which contain them. The protocol for analysis of natural dyes has undergone significant developments in a variety of areas in recent years to move towards less destructive and more informative protocols. One area which has been significantly improved in recent years is the use of Raman studies for preliminary investigation of artefacts to make predictions about possible structural features. [18, 19, 20, 21] As mentioned previously, for organic compounds (particularly those fixed to a polymeric textile fibre) in-situ, non-destructive Raman spectra can be overwhelmed with fluorescence. In recent years however, developments in Surface Enhanced Raman Spectroscopy (SERS) – where a metal colloid is applied to the fibre to enhance the intensity of Raman signals – have meant that even in cases of high fluorescence, preliminary data can be obtained about the structure of the natural dye from even a microscopic sample. [22]

The extraction of the dyes from the fibre (henceforth referred to as the initial extraction) is another area that has received significant research in recent years. In traditional studies on naturally dyed textiles, this process was carried out by placing the fibre in a small quantity of strong acid and heating to high temperatures. These methods, such as those using HCl were evaluated in a comparative study by Zhang and Laursen in 2005 [23] who showed that strongly acidic extraction methods – whilst achieving high yields for some dye types – caused more sensitive dye analytes (such as glucoside-based flavonoids) to be completely destroyed. In these tests, results showed that milder techniques – utilising Na_2EDTA and formic acid as the extraction agents achieved higher yields and allowed the preservation of the more sensitive molecules. The area continued to be investigated in subsequent years, for example Valianou

et al. [24] trialled the use of trifluoroacetic acid for the extraction – which achieved excellent efficiency in extracting the dye from the fibre, but had significant consequences on the ionisation efficiency of the dyes by the electrospray ionisation sources used in many mass spectrometers – hindering their detection. The use of hydrofluoric acid in organic solvents was proposed by Sanyova in 2008 [25] – however the use of such a strong acid in a field which involves specialists from a wide range of academic disciplines raises concerns about safety and applicability of such a method. Kirby and White [26] proposed a method using boron trifluoride and methanol for the extraction, but further analyses found that this methodology caused molecular changes in the molecule, impacting detection. [27] It is clear from these results that the complexity of natural dye samples mean that it is very difficult to define widely applicable methodologies. Many more studies were undertaken in the years following and a comparative work by Manhita et al. in 2011 [28] concluded that the Na_2EDTA methodology, combined with using DMF as a solvent obtained the best results for natural dyes on wool fibres and this is therefore the method which has been most widely used in recent years.

In more recent research published in 2016, Lombardi et al [29] presented evidence that utilising ammonia alongside Na_2EDTA negated the need for high temperatures during the extraction – which reduced the alteration of sensitive compounds and increased the efficiency of extraction. Further research by the same group found that the basic conditions used in this methodology preserved never-before observed glycosyl compounds – which can be key in identifying species-specific differences in carminic insect dyes, allowing the differentiation of Armenian and American cochineal described earlier in this thesis. [4] This approach, using ammonia, Na_2EDTA and NaCl as the extraction solution is the state-of-the-art for natural dye extraction and provides the best results recorded in literature. This methodology, following the protocol described in the 2017 paper by Serafini et al. [4] was utilised in this thesis.

One area which has not received significant research is the isolation of the dyes from this extraction mixture and their pre-concentration and enrichment before analysis by HPLC-MS (henceforth referred to as the clean-up). Until recently, clean-up protocols were not routinely observed in natural dye extraction protocols, however they offer significant benefits. Applied after the initial extraction, clean-up protocols can be used to obtain as pure a sample as possible and involve extracting the dye molecules from the initial extraction solution into an immiscible organic solvent. This purification allows the dye signals to be enhanced on analysis as interference from unwanted molecules – which can be present as dirt or precipitation on the fibre – is minimised. As an additional benefit, whilst a relatively large quantity of extraction mixture takes a significant amount of time to dry in preparation for analysis; the addition of an effective clean-up protocol means that the sample is generally contained within a small quantity of organic solvent, which usually quickly evaporates under airflow.

The recent development of the ammonia-EDTA extraction method [4, 29] requires separation of the salts present in the extraction mixture from the analytes for injection into HPLC-MS systems. A first step towards a clean-up strategy was therefore required, and was proposed through a traditional liquid-liquid extraction (LLE) performed in a separating funnel. This method was simply used as a tool to separate the salts present in the extraction mixture from the dye analytes, and did not seek to pre-concentrate the sample or optimise recovery. The benefits of a well-suited clean-up protocol have therefore never been investigated, and it is clear that traditional LLEs are not well-suited for dye analysis. For example, the use of a separating funnel – whereby layers are mixed by manual inversion and separation is controlled through a tap at the bottom of the funnel – is not well-suited to the small volumes used within cultural heritage. Where the quantity of extract is low, even a small volume of unrecovered phase can have significant impact on the quality of the results – risking making signals undetectable. It is therefore clear that significant research must be performed to improve the clean-up methodology and develop a strategy better suited to cultural heritage. The research presented in this thesis will seek to tackle these problems through the development and application of a novel protocol based on Dispersive Liquid-Liquid Microextraction (DLLME) – which is very well-suited to the requirements of this type of analysis.

After the sample is extracted and purified in preparation for analysis, HPLC-MS or HPLC-DAD is used to separate, detect and analyse specific characteristic qualities of the molecule depending on the detector used. Chromatography is particularly effective as it means that for samples dyed with more than one type of colourant, all analytes can be detected. If a tandem mass spectrometer is used, it is also possible to obtain structural information about the analyte using information about its fragmentation pattern. To obtain sufficient information from an unknown dye sample, it is necessary however that a high resolution mass spectrometer is used to allow untargeted analysis to be performed.

1.3 Synthetic Dyes

1.3.1 History of Synthetic Dyes and Value of Study

In the mid-19th century, the unintentional development of mauveine (the first synthetic dye) by British chemist William Henry Perkin [30] signalled the beginning of a revolution in fabric dyeing. The synthesis of dyes in the laboratory meant that scientists could explore new methodologies, obtaining new hues and shades which no longer relied upon the intrinsic variability of the natural matrix. Price of production was reduced and the dependency of the textile dye industry on limited natural resources disappeared. [31] This development completely reshaped the industry and allowed the scale and market access of textile dye

production to expand rapidly, leading to the launch of new large chemical firms across the world. [32]

As a result of the development of synthetic dyes both the manufacturing industry and the art world underwent a new phase of experimentation and development. Related objects from this period are hence very useful in the study of the history of manufacture, fashion, and contemporary art. Cultural heritage studies focussing on synthetic dyes represent a relatively new area of study due to their modernity. However, as the 19th century disappears into the past, and dye collections and dyed artefacts produced in the last century begin to require conservation and raise curatorial questions – research into synthetic textile dyes is becoming increasingly important. [6] A good understanding of the early production and use of synthetic dyes can help to shape our understanding of these interesting periods of change in human behaviour, and this type of study is therefore valuable and worthwhile.

1.3.2 Chemistry of Synthetic Dyes and Analytical Challenges

In general, the synthetic procedure means that unlike natural dyes – synthetic dyes are produced under a controlled environment independent of the natural matrix. This usually means that a single synthetic dye produced in the laboratory displays a much higher molecular uniformity than a single natural dye derived from a biological source. [33] However, in contrast to natural dyes; there is a vast range of molecular classes for synthetic textile dyes. The industrial boom associated with their development and the subsequent increase in research meant that dyes quickly moved away from the limited molecular classes observed with natural dyes to a huge molecular diversity and an extensive variability. [34]

The vast chemical diversity of these dyes means that it is extremely difficult to develop analytical protocols that are applicable across the whole range of synthetic dyes. The research in this thesis hence focuses on the development of a clean-up protocol to be used in the extraction of acid dyes, and is optimised on acid dyes from the azo class specifically. This dye class was one of the earliest developed, with the first – Biskmarck Brown – commercially synthesised after 1861. The azo dyes are characterised by an N=N bond and are usually associated with red, yellow and orange hues (however other colours can be obtained by modifications to the chemical structure). [35] Their popularity in the early synthetic dye market is clearly illustrated by the fact that they are one of the most ubiquitous classes in early synthetic dye collections [35] – and this feature is one of the main reasons they have been chosen as the specific focus for this research.

Alongside their popularity in the textile dyeing industry, azo dyes have also commonly been used as food dyes – and it is for this role that they have received significant negative attention. Food scientists found evidence of carcinogenic properties linked to exposure to azo dyes – and following this, several have been banned by governmental bodies. [36] To aid regulation of these requirements, there has been a rapid improvement in the detection and identification of azo dyes in foods using analytical chemistry. [37, 38, 39] This improvement has however not yet been adapted for textile studies in cultural heritage, an approach which this research introduces.

For the research on synthetic dyes produced by this thesis, three analytical standards were chosen to optimise the method for two reasons: firstly, the structures chosen were deemed broadly representative of the azoic acid dye class; and secondly they were predicted using commercial nomenclature to be likely to exist within the ACNA dye case study performed at the end of this thesis. The standards chosen were Acid Yellow 25 (CI: 18835), Congo Red (CI: 22120), and Red 2G (CI: 18050). All three dyes are highly water soluble and are negatively charged species.

1.3.2.1 Azo Dye Standards

Acid Yellow 25 is a yellow mono-azo dye. It also belongs to the sub-class of pyrazolone dyes owing to its 5-membered ring containing two adjacent nitrogen atoms. The molecule also contains a sulfonated phenyl group, as well as a phenyl sulfamoyl moiety bound to an amino substituted benzene ring. Acid Yellow 25 has a single sulfonate group and hence exists as a singly charged anionic species under most conditions. The charge is balanced by a sodium ion when the dye is in its powder form. [40]

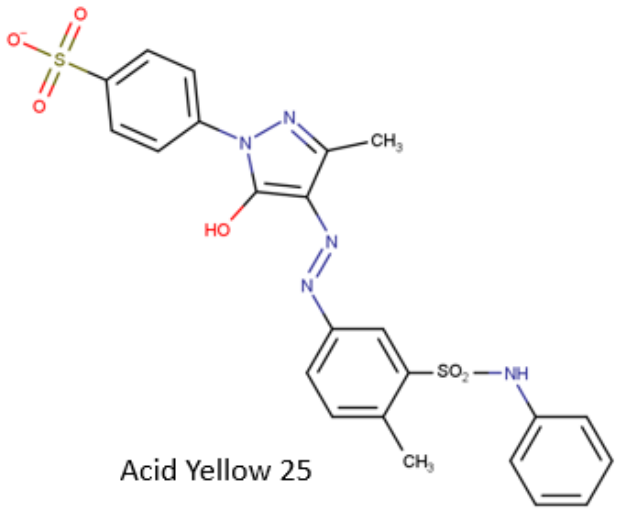

Figure 4: Structural formula of the mono-azo dye Acid Yellow 25. [40]

Congo Red is a symmetric diazo dye that appears red under alkaline and neutral conditions and blue under strongly acidic conditions. It consists of two sulphonated aminonaphthalene groups each bound to one of two azo groups which are connected by two central phenyl groups. In the dyes powder form, the double negative charge of the sulfonate groups is balanced by two sodium ions. [41] It is well documented that Congo Red has carcinogenic properties and its use has therefore declined in recent years. [10]

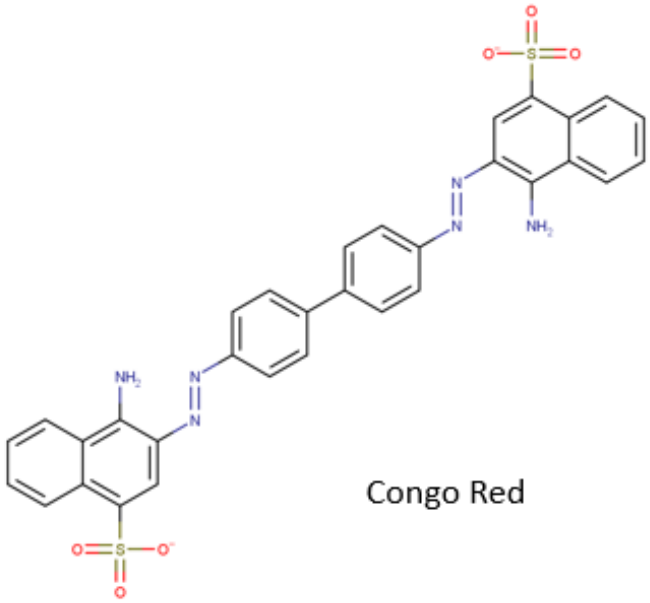

Figure 5: Structural formula for the diazo dye Congo Red. [41]

Red 2G also referred to as **Acid Red 1** is a red mono-azo dye built upon a naphthalene structure. The naphthalene group is substituted with two sulfonate groups in the 2 and 7 positions; an acetamido group in the 5 position; a hydroxyl group in the 4 position, and a phenyldiazenyl group in the 3 position. The two sulfonate groups give the molecule a double negative charge which is balanced by two sodium ions when the dye is in its powder form. [42]

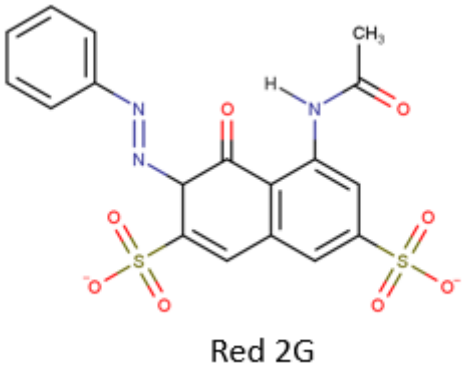

Figure 6: Structural formula for the mono-azo dye Red 2G. [42]

1.3.2.2 Analytical Challenges

The chemistries of synthetic dyes present specific problems for their analysis. Similarly to the natural dyes, the organic chemical nature of synthetic dyes means that it is extremely difficult and often not possible to identify them through non-destructive methods alone, as they are not well suited to many of the non-invasive analytical techniques commonly used in cultural heritage studies.

The aforementioned uniformity of dye molecules from a single controllable process means that synthetic dyes often produce more intense signals on spectra when studied than the natural dyes, which are more likely to be made up of several low concentration chromophores. However, the wide range of classes means that databases are particularly difficult to develop and maintain for synthetic dyes, meaning that it is often difficult or impossible to find analytical references for the less common synthetic textile dyes. The situation is further complicated by different dye companies using different (non-standardised) names for chemically identical dyes (and conversely using the same names for chemically different dyes) [43] alongside the tendency for these names to be used varyingly among scientific papers. Together, these factors mean that the identification of synthetic dyes from historical and artistic matrices is a complex matter requiring well-defined methodologies and the use of a vast range of bibliographical sources.

1.3.3 State-of-the-Art for Analysis of Synthetic Dyes

The analysis of synthetic dyes presents an excellent opportunity to study the history of manufacture, fashion and design, and also to conserve our heritage. The protocol for their analysis is a relatively new area of study and hence needs significant development, however

some interesting work has been presented in recent years. Preliminary analysis using Raman and SERS is an excellent example of this that has received attention from several groups within the last few years. [44] Whilst the organic nature of these synthetic dyes does mean that Raman spectra suffer from high fluorescence similar to natural dyes, the molecular uniformity of single dye species means that they often produce signals that are possible to observe above the fluorescence effects when low energy lasers are used. Where the fluorescence is overwhelmed and signals are masked; or when dye signals are overwhelmed by the Raman peaks from the polymeric fibres – SERS can be useful to obtain enhanced data. [45, 46]

Due to the newness of the field, extraction methods are generally adapted directly from natural dye studies and applied to synthetically dyed artefacts – however significant chemical differences mean that this is not always possible. This is clearly illustrated in literature by the initial extraction of dye molecules from fibres. Whilst natural dye extraction protocols have undergone the excellent developments in recent years reported previously – and transitioned to the use of ammonia based extractions which are well suited to acid dyes, synthetic dyes of all types continue to rely upon the use of acid extractions such as oxalic acid at high temperatures. [31, 47] The ammonia-EDTA extraction has never before been applied to synthetic dyes; despite the fact that it is likely to be well suited to synthetic acid dyes – such as those focussed on for this thesis. This means that there is currently a gulf between synthetic and natural dye studies; meaning that two separate samples must be taken from a textile artefact which is likely to contain both natural and synthetic dyes, or that one analyte type is neglected for the analysis of the other. These factors work in opposition to the cultural heritage goal of achieving minimal destructiveness.

One of the reasons that the ammonia-EDTA approach has not yet been applied to synthetic dyes is that it requires the application of the clean-up protocol, unlike the acidic extraction methods which can simply be dried in their extraction mixtures. The reason for this is that the salts used in this extraction procedure are not compatible with the use of HPLC-MS for analysis, and the dye must therefore be isolated from these species. Traditional LLE approaches cannot be used for the clean-up of most synthetic dyes, which are charged species. Azoic acid dyes, for example, are stored as a powder with a cationic counter-ion before application to textiles; to which they bind directly through their ionic group. [48] When extracted from the textiles; they revert to their anionic form and hence have a high affinity for water. This charged characteristic means they have an extremely low affinity for less polar solvents; and are hence unlikely to be recovered in the extracting solvent during a liquid-liquid extraction. This is a significant gap in research which this thesis addresses through the application of DLLME with the addition of an ion-pair reagent (IP-DLLME).

Currently, after extraction in acid conditions the extraction solution is simply evaporated to dryness before reconstitution and injection to the HPLC-MS or HPLC-DAD system for analysis. This evaporation is likely to take a significant amount of time as the extraction mixture is generally >1ml in volume. The lack of purification also means that interference from unwanted molecules is much higher and signals are less easy to detect.

1.3.4 Case Study: Collection of ACNA dyes from the Sapienza University of Rome Museum of Chemistry

The Museum of Chemistry, located within Sapienza University of Rome's Department of Chemistry, holds an extensive collection of early synthetic dyes from several different dye companies. One of the most significant groups in the collection are the dyes from the Azienda Coloranti Nazionali e Affini (ACNA) – an Italian chemical company active from 1882 until 1999. [32] The company was extremely controversial throughout the entirety of its history. It was first founded as an explosives factory which operated under the name Dinamitificio Barbieri and subsequently the Italian Society of Explosive Products. The company later moved away from the production of explosives and retooled as a colourant manufacturer after being acquired by Italgas in 1925. The ACNA received significant investment from the fascist regime in the years immediately following in a campaign to promote Italian manufacturing industries and its name was once again changed, this time to the Associated National Chemistry Companies - at which point it obtained the acronym ACNA. It was then acquired by the larger companies IG Farben and Montecatini who gave it its final name: Azienda Coloranti Nazionali e Affini. [49] Under these companies the ACNA manufactured colourants until 1999, during which time their production caused extensive pollution of the surrounding areas, inflicting severe damage to both the environment and the health of the residents. [49]

The collection of dyes analysed by this thesis contains glass jars filled with dye powders and card-backed sheets holding samples of dyed wool fibres. The collection is likely to date from the 1930s – which is the period immediately following the companies change in direction from the production of explosives.

15 samples were taken from the collection – 11 fibre samples from one card-backed sheet, and 4 powder samples from glass jars. The samples were chosen after an initial visit, during which names were recorded to allow literary research into their commercial names. This particular group of dyes was then chosen due to research indicating a high likelihood that a majority of the dyes in the group are of the azo class.

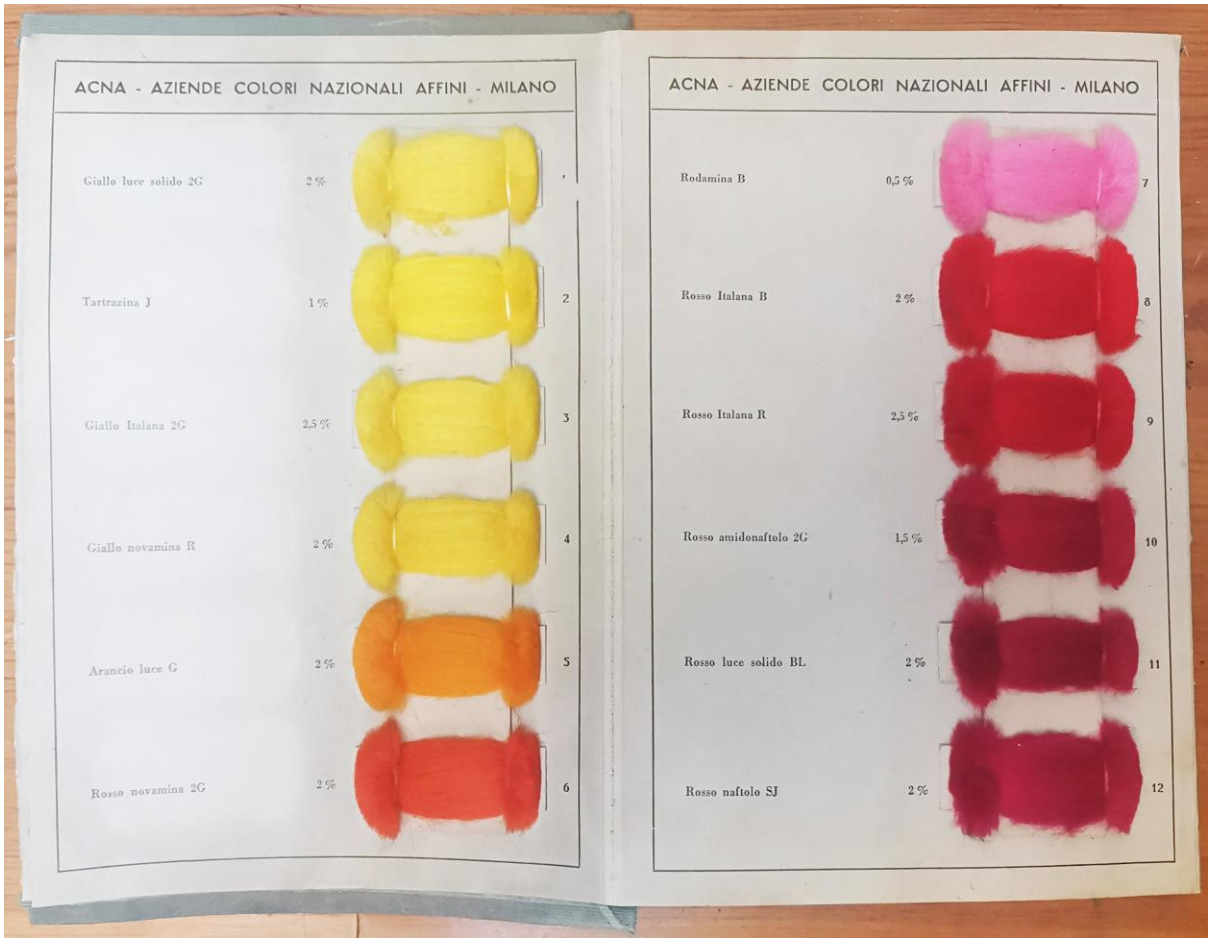

Figure 7: Photograph of the card-backed sheet containing the sampled dyes.
Note: all dyes were sampled except Rodamina B, which was excluded due to predictions from the name that it was likely to be a Rhodamine basic dye and hence would not be suitable for the new methodology - which is optimised for azo acid dyes.

This study marks a first step in understanding the full collection of synthetic dyes held by the Museum of Chemistry, and also provides insight into the naming conventions of the ACNA which could aid further studies such as this. The study of these dyes is an excellent opportunity to understand the behaviours and synthetic procedures of a company that was active throughout the entirety of the 20th century. Studying this through the lens of collections obtained directly from the manufacturer, such as the collection in Sapienza University's Museum of Chemistry, offers both the chance to obtain information about dyes that were available on the market – which have the potential to offer a synthetic dye database; and the opportunity to study dyes which may never have been placed on the market and therefore could provide insight into the internal testing and motivations of the ACNA. In particular, results from dyes such as *Rosso Amidonaftolo 2G* and *Giallo Luce Solido 2G* for which both

powders and dyed fibres were analysed provides interesting information regarding the consistency of naming.

The samples were studied first using non-destructive Raman spectroscopy before proceeding to extraction (including the novel clean-up protocol proposed by this thesis) and untargeted HPLC-HRMS analysis. These results were combined to obtain information regarding the identities of the dyes studied.

1.4 Research Aims

As briefly mentioned in the above sections, the research for this thesis seeks to trial the effectiveness of the application of DLLME to improve the recovery, efficiency and reproducibility of the clean-up process of historical textile dye extractions performed using the state-of-the-art ammonia-EDTA methodology. The optimisation of two methods – one for natural dyes and one for synthetic dyes, was evaluated using analytical standards and targeted HPLC-MS.

DLLME utilises the rapid injection of the extraction phase alongside a dispersing solvent to increase the surface area of the interface between the two phases and hence provides more opportunities for analytes to enter the extraction phase. [50] This has the effect of increasing the recovery of analytes, and also does not need to be performed in a separating funnel, which is effective for large quantities of material but not for the small volumes used in cultural heritage where the quantity of extract is very low and unrecovered phase risks making signals undetectable. The simple apparatus involved in DLLME also means that several samples can be simultaneously prepared, increasing speed and efficiency.

The development of clean up procedures **for natural dyes** utilised features of the traditional LLE methodology to make decisions on solvent quantities, and adapted these to develop a DLLME procedure. [4] The aims of this novel process were primarily to increase:

- the recovery of analysis – meaning that detection was achieved at the minimum sample size possible;
- the efficiency of extractions – meaning that samples can be analysed more rapidly;
- and the precision of analysis – meaning that variables are more controlled and results more reproducible.

To optimise the method, different extraction solvents and dispersers were tested and the results were evaluated using targeted HPLC-MS analysis and quantified by peak area integration recovery calculations.

The novel DLLME protocol for **synthetic dyes** addresses a different set of issues, as currently clean-up methodologies are not used for synthetic dyes from historical textiles – which are extracted in acid conditions instead of using the newer ammonium-EDTA method developed for natural dyes. Successes in the application of such clean-up protocols to synthetic azo dyes are reported in other fields, such as food science. [37, 38, 39] However, this has never been applied to textiles for cultural heritage research. Acid dyes – such as the kind of azo dyes focussed on for this research – are likely to be well-suited to the basic extraction protocol, and the development of this novel clean-up method allows this to be utilised. This clean-up protocol utilises an ion-pair reagent in order to facilitate the movement of the anionic dye species into the extraction phase and this protocol is referred to as ion-pair-DLLME (IP-DLLME). It utilises a methodology based on reported protocols used within synthetic dye analysis in food studies [37], and has been adapted and implemented for the first time to the field of cultural heritage. The method uses an ion-pair reagent to overcome the problems associated with traditional LLE and ionic molecules. Different dispersers were tested on three analytical standards, and the results were evaluated using targeted HPLC-MS and quantified using peak area integration recovery calculations.

Additionally, for unknown cases in which an artefact could contain both natural and synthetic dyes, as highlighted by non-destructive analyses, the initial extraction process could be performed on a single sample – hence reducing the necessary sampling on an artefact. This change in analytical pathway is illustrated in Figure 8.

After the optimisation of clean-up methods for both natural and synthetic dyes, and their insertion into the overall analytical protocols, the new synthetic dye protocol was successfully applied to a group of unknown dyed fibres and powders suspected to be azo dyes obtained from Sapienza University of Rome's Museum of Chemistry. In this case, the analyses were performed in untargeted HPLC-HRMS.

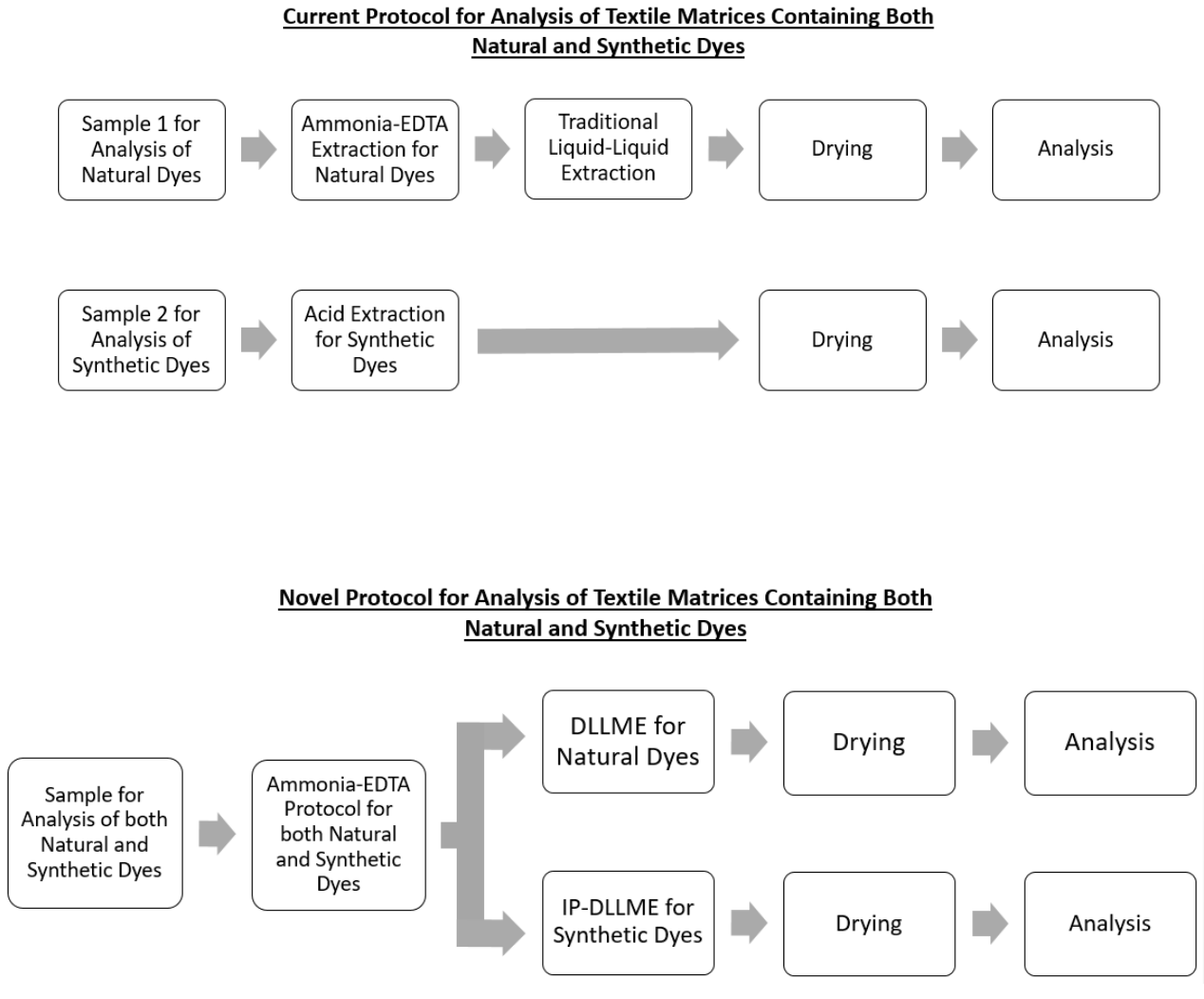

Figure 8: Comparison of the current protocol for the analysis of artefacts containing natural and synthetic dyes and the new single sample protocol allowed by the development of IP-DLLME for synthetic dyes outlined in this thesis.

2. Experimental

2.1 Reagents

High purity analytical standards of Alizarin, Purpurin, Carminic Acid, Luteolin 7-O-β-D-glucoside, Congo Red, and Red 2G were purchased from Sigma Aldrich. A 40% purity standard of Acid Yellow 25 was also purchased from Sigma Aldrich. Solvents, acids and bases were purchased from Sigma Aldrich and used without further purification. $Na_2EDTA•2H_2O$ was purchased from Carlo Erba while other salts were purchased from Sigma Aldrich.

2.2 Overview of Techniques

2.2.1 DLLME

The novel protocols proposed by this research use dispersive liquid-liquid micro-extractions in place of the traditional liquid-liquid extraction. In general, DLLME is characterised by the rapid injection of the extracting solvent alongside a disperser solvent into the aqueous phase containing the analytes – resulting in a single cloudy solution. [50] This cloudy solution is the result of very finely dispersed micro-droplets of organic solvent. This phenomenon results in a very high surface area at the interface between the phases, which leads to an increase in the number of opportunities for the analytes to move between the phases. The dispersion is further enhanced by subjecting the mixture to an ultrasonic bath [50] after which the phases are separated by centrifugation. A syringe is used to remove the lower layer, and the extraction phase is then dried.

The increased surface area provided by the DLLME gives analytes ample opportunity to move between phases. This means that under the correct chemical conditions the analytes are recovered more effectively in the organic phase. [50] The method requires a lower quantity of organic solvent than the traditional method (usually <200ul) and is hence used widely to improve the environmental implications of a liquid-liquid extraction. [51] For the research described in this thesis – although reduced environmental consequences are desirable – the main benefit of the new methodology is the increased recovery. Cultural heritage science strives to reduce the sample sizes necessary to obtain good results, and by increasing the recovery of dye analytes to the extraction phase we are able to reduce the sample size while still obtaining high quality results. Alongside this, DLLME is primarily performed in a simple disposable Eppendorf or Falcon tube – depending on the overall volume of the mixture. As such, several samples can be lined up and extracted rapidly washing only the syringe between each injection, the whole sample set can then undergo the sonication and centrifugation steps

simultaneously. This is an extremely efficient process and was applied effectively during the optimisation of the two protocols for natural and synthetic dyes and then to the case study museum samples. Using a low-volume syringe for the extraction of the lower phase also offered superior precision to that of the separating funnel, alongside the opportunity to measure the volume of organic phase recovered.

Where the researcher is dealing with small volumes – DLLME is simple to perform, precise, rapid, and increases recovery. These features make it perfectly suited to the field of historical textile dye analysis.

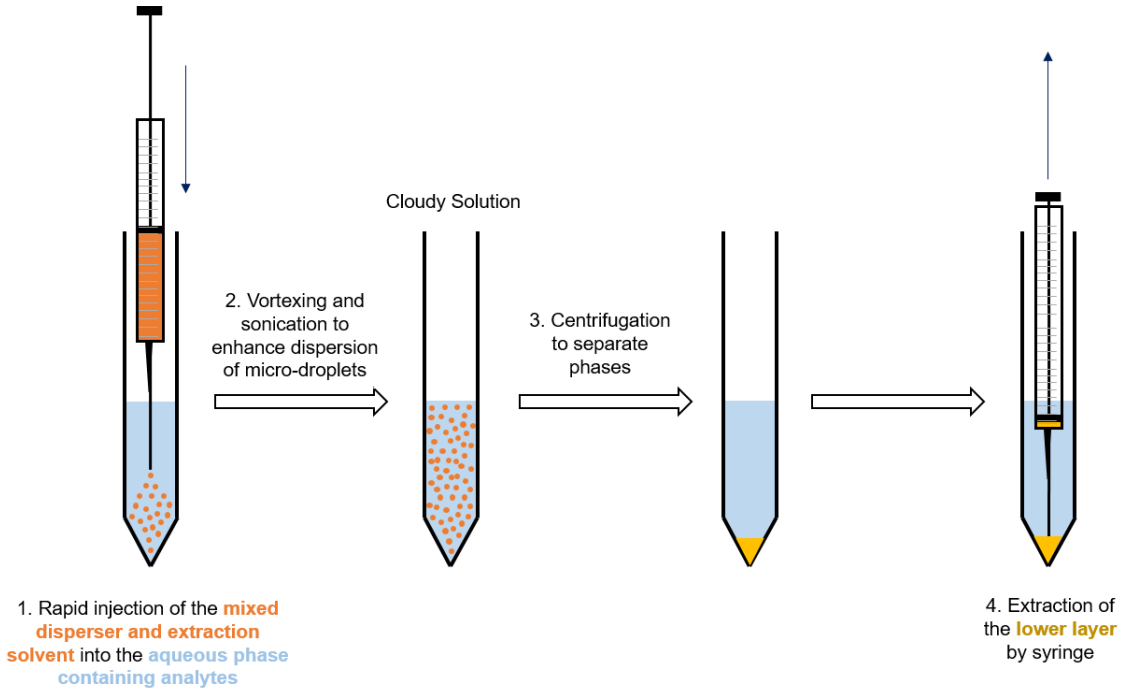

Figure 9: Schematic illustration of DLLME.

2.2.2 HPLC

High-performance liquid chromatography is one of the key instruments used for textile dye analysis. Liquid chromatography utilises a packed chromatographic column as the stationary phase, and two separate solvents as the mobile phase. The column used for this thesis is a reverse-phase C-18 column, which interacts more significantly with non-polar molecules. Under this condition, less polar analytes are slowed by their interactions with the column packing, and analytes are separated according to their polarities and resulting retention times.

In order to obtain sufficient separation of analytes – the column and mobile phases used must be chosen carefully and optimised for the specific analytes in question.

For this thesis, two different mass spectrometers were used as the detectors for chromatography. This is justified by the need for targeted analysis for quantitative evaluation of the novel methodology proposed by the research, and untargeted analysis for qualitative evaluation of the unknown museum samples in the case study.

2.2.3 Targeted Mass Spectrometry

For targeted analysis, a triple quadrupole mass analyser based in Sapienza University of Rome's Department of Chemistry was used. Targeted analysis is used when the researcher knows which specific molecules they are looking for. These values are programmed into the mass spectrometer, which then focuses only on detecting this specific range of values. [52] The triple quadrupole mass spectrometer consists of a primary quadrupole mass analyser – which filters the ions to obtain only the target parent ion; followed by a non-mass resolving quadrupole which acts as a collision cell – fragmenting the molecules in predictable patterns, followed by a second quadrupole mass analyser – which filters ions to obtain only the target fragments. [53]

This type of mass spectrometry system is particularly useful when specific known substances are being studied, as it provides excellent sensitivity for selected reaction monitoring through the filtering system for tandem mass spectrometry. [52] This meant that for the optimisation of the two new protocols – for natural and synthetic dyes – which required several tests, the triple quadrupole mass spectrometer was very effective in obtaining quantitative information which defined the conditions with the best recovery.

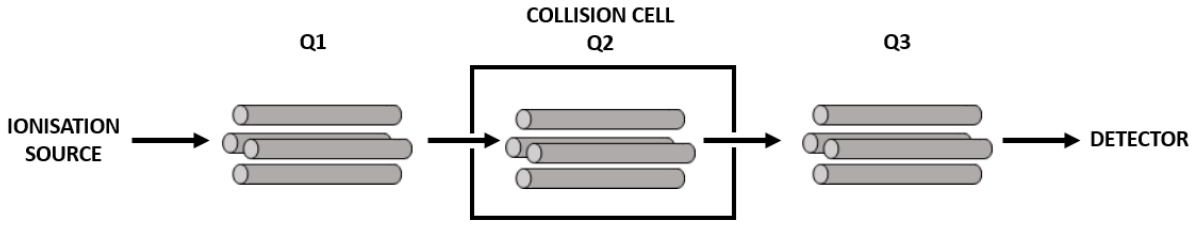

Figure 10: Triple-quadrupole mass analyser. Q1 is the primary quadrupole mass analyser, Q2 the non-mass resolving collision cell, and Q3 the secondary quadrupole mass analyser.

For natural dye detection, the MS/MS system was utilised in multiple reaction monitoring acquisition (MRM). This mode means that the system is optimised to detect several parent ions and their resulting fragmentations. To optimise the voltages and understand the fragmentation patterns, the diluted analytical standards were first injected directly into the

mass spectrometer. The targets obtained through the optimisation process were then programmed into the HPLC-MS/MS method.

For some of the synthetic dye standards, sufficient fragmentation was not observed for the ions – owing to their stability by resonance. For this reason, single ion monitoring acquisition (SIM) – whereby only the parent ion is detected – was used. Where possible, MRM is preferred to SIM as the use of SIM means that interference from unwanted ions is more prominent and that the signal to noise ratio of the spectrum is lower. [53] In this case however, SIM mode enabled quantitative data to be obtained about the recoveries of several variations on the clean-up method, and hence the best conditions to be evaluated.

Whilst all preliminary testing of the new protocols was carried out using the triple-quadrupole mass spectrometer, it should be noted that some final analyses reported in this thesis were performed with the Orbitrap spectrometer described below for untargeted analysis. This was due to malfunctions with the triple-quadrupole mass spectrometer towards the end of the research and time constraints meaning that analyses had to be completed before a technician was available.

2.2.4 Untargeted Mass Spectrometry

For the unknown dyes from the ACNA collection, targeted analysis is not a useful approach as it requires the user to already know the m/z ratio of the molecules they are seeking to detect. For this case study, qualitative data is more important than quantitative data as the aim of the investigation is to understand the masses of the dye molecules.

The Orbitrap is a new model of ion trap mass analyser offering extremely high sensitivity. The principle for analysis is that a voltage is applied to three electrodes forming an electrostatic field. The molecules are injected into the system, and are directed towards the central electrode by the electric field. This movement towards the central electrode is opposed by a centrifugal force. This opposition of forces means that the molecules move in complex spiralling motions, defined by the m/z of each molecule and the parameters of the electrostatic field (generated by the electrodes). [55]

The movement of these charged ions generates a response in the electrode (for example, as a positive ion moves closer to the electrode, negative charges build up on its surface). This phenomenon is referred to as the “image current” and by measuring this it is possible to detect the motion of the ions. [55] As this motion is dictated by the known parameters of the field and the m/z of the molecules, complicated Fourier Transformations by computer software mean that this data can be used to deduce the m/z of the molecules. [55]

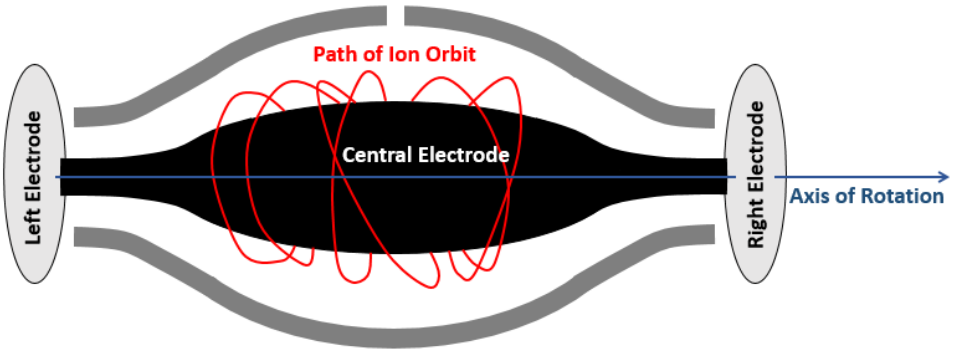

Figure 11: Orbitrap Mass Analyser

The result is a highly sensitive mass analysis which can obtain excellent resolution even in full scan mode (FSM). This equipment was hence chosen for the untargeted analysis necessary for the dyes from the ACNA collection.

2.2.5 Raman Spectroscopy

Raman spectroscopy is an extremely useful technique for cultural heritage due to its non-destructive nature. In this thesis, Raman spectroscopy was used to obtain some non-destructive preliminary information about the dyes from the museum case study before they were subjected to extraction for HPLC-MS. Raman spectroscopy was performed on each of the 15 case study samples as well as for the 3 analytical synthetic dye standards previously mentioned, and also an analytical standard of another acid azo dye – Tartrazine.

Raman spectroscopy detects molecular vibrations which give rise to Raman scattering. In principle, monochromatic light is applied to the sample – which provides energy for electrons to be excited from their ground vibrational levels up to an excited virtual energy state. In most cases the electrons return to their original vibrational levels after excitation, and emit a photon of the same frequency as the incident light – this is referred to as Rayleigh scattering. In a small quantity of cases, the electron falls to a vibrational level either above (stokes) or below (anti-stokes) the original vibrational level. This information can be used to obtain the energy differences between the vibrational levels, which are intrinsically linked to the types of bond within a molecule. This means that each molecule has a distinct Raman spectrum, and that the peaks observed are indicative of specific functional groups.

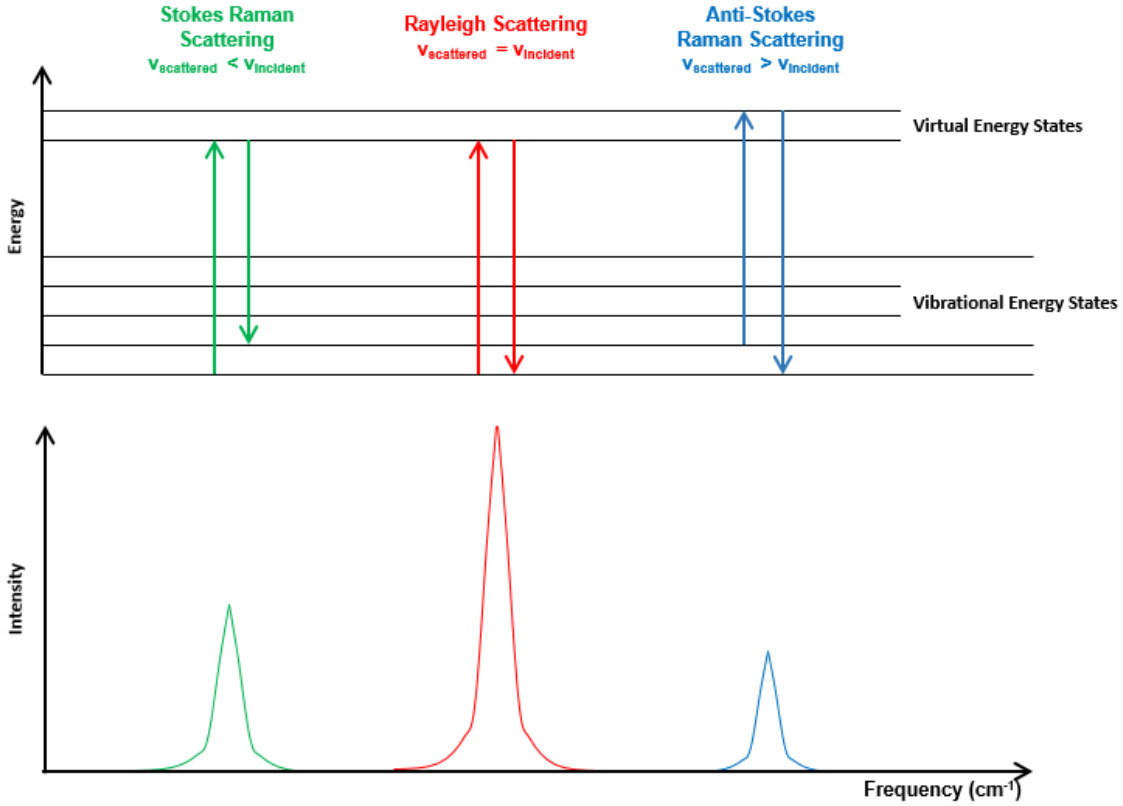

Figure 12: Illustration of the energy transitions involved in Raman spectroscopy (top) and the relationships between the transitions and the frequencies observed in Raman spectra (bottom)

2.3 Development of Novel DLLME Protocol

2.3.1 Optimisation of Clean-Up for Natural Dyes

Optimisation of the clean-up protocol was performed on a mixed reference sample containing 500ppb of alizarin, purpurin, carminic acid and luteolin O-7-β-D-glucoside. The analytes were dissolved to 500ppm and diluted to 100ppm in ethanol and the final dilution to 500ppb was then performed in methanol. Standards were stored in the freezer between uses. All experiments were repeated three times and each replicate analysed twice by mass spectrometry to obtain an average.

2.3.1.1 Optimising Disperser and Extracting Solvents for Natural Dyes

The best conditions for the clean-up of natural dyes were evaluated by trialling three extraction solvents: 1-pentanol, chloroform and dichloromethane, with four different dispersers: methanol, isopropanol, acetone and acetonitrile to evaluate which combination produced the highest recovery for the four analytes. For the purposes of optimisation of these particular analytes, the DLLME clean-up was performed directly from the standard solution, without the application of the ammonium-EDTA extraction, which was subsequently added to the best performing combination of disperser and extracting solvent, and trialled to verify coherence of the method.

2.3.1.1.1 1-Pentanol Disperser Trials

Trials with 1-pentanol as the extracting solvent were devised using the traditional LLE methodology as a backbone to define solvent quantities and ratios [4] and performed as follows. 100µl of the 500ppb reference mixture was placed in an Eppendorf tube and made up to 1ml with Millipore water. 100µl 1M HCl was then added to the solution followed by 200µl of disperser. A 500µl syringe was then loaded with 200µl 1-pentanol and 100µl of disperser and rapidly injected into the aqueous phase forming the cloudy solution. The Eppendorf was then closed and vortexed before being placed in an ultrasonic bath for 10min. After sonication, the mixture was centrifuged at 12500rpm for 10min to separate the layers. The lower aqueous layer was removed with a syringe leaving the organic layer in the Eppendorf.

Tests with litmus paper on some samples showed that the organic phase still had acidic character after the extraction, so a comparison was made between the DLLME recovery of the analytes with and without washing. For washing, 500µl Millipore water was added to the organic layer in the Eppendorf, shaken, centrifuged and extracted. This process was repeated with 250µl Millipore water for a second washing. After extraction, the organic layer was transferred into a vial and the extract dried under N_2 flow and 65°C heat. The extract was reconstituted with 100µl methanol for analysis by HPLC-MS.

2.3.1.1.2 Chloroform and Dichloromethane Disperser Trials

Trials with dichloromethane and chloroform were adapted from an approach based on anthraquinone derivative detection in urine analysis from Shi et al. [56] It should be noted that despite their success with carbon tetrachloride, only chloroform and dichloromethane were

tested to avoid the extreme toxicity of the solvent. In this approach, 100µl 500ppb mixed standard was added to a Falcon tube. 1.7ml Millipore water was added to the tube alongside 200µl 1M HCl. A syringe was loaded with 800µl disperser and 80µl extracting solvent, and rapidly injected into the aqueous phase, forming the cloudy solution. The mixture was then vortexed for 10 seconds and sonicated for 10 minutes before undergoing 5 minutes of centrifugation at 4200rpm. The bottom organic layer was then removed with a syringe and placed in a vial where it was dried under N_2 flow. The extract was reconstituted with 100µl methanol for analysis by HPLC-MS.

2.3.1.2 Comparison of Novel DLLME with Traditional LLE

To verify that the recovery obtained by DLLME was superior to that achieved by the traditional LLE clean-up procedure the recoveries from the best disperser and extracting solvent conditions were compared to the LLE process recorded in literature. [4] The traditional LLE was performed using the following protocol. 100µl of the 500ppb standard mixture was made up to 1ml with Millipore water and 100µl HCl was added. This solution was transferred to a small separating funnel, and then 250µl 1-pentanol was added to the funnel. The mixture was shaken and left to separate before draining the aqueous and organic layers into separate containers. The aqueous layer was then returned to the funnel and the process repeated with another 250µl 1-pentanol. The resulting organic layers were combined and washed with 500µl of Millipore water, and then three further 250µl aliquots of Millipore water until washings were neutral. The organic phase was then extracted and dried under N_2 at 65°C. The extract was reconstituted with 100µl methanol for analysis by HPLC-MS.

2.3.1.3 Trial of the Complete Analytical Protocol for Natural Dyes

After the DLLME procedure was optimised and verified through the above experiments, the chosen method was paired with the initial extraction protocol outlined by Serafini et al. [4] In this protocol, 100µl of 500ppb mixed standard was placed into a vial alongside an extraction solution of 4.4mg NaCl, 0.8ml 30% NH_3 and 0.8ml previously made up 1mM Na_2EDTA. The solution was then placed under N_2 flow to facilitate the evaporation of the ammonia, and this was performed for several hours until a neutral solution was obtained. Following this, the extraction solution was made up to 1ml with water, and the 1-pentanol clean-up protocol was applied. Two sets of trials were performed with methanol and isopropanol as the dispersers.

2.3.1.4 Recovery Analyses by HPLC-MS

Recovery analysis was carried out using the results from targeted HPLC-MS. For the chromatographic analysis a Series 200 Perkin Elmer micro-LC system equipped with an autosampler was used. The system was coupled to a PE-Sciex API 2000 triple quadrupole mass spectrometer equipped with a TurboIon-Spray ionisation source, operating in negative ionisation mode. The column was chosen after trialling a Kinetex XB-C18 2.6µm core-shell particle column and a Luna-C18 5µm column. To optimise the mobile phases for natural dyes, three trials were performed:

i. Phase A: Methanol
   Phase B: 5mM ammonium acetate in water
ii. Phase A: Acetonitrile:Methanol (1:1)
   Phase B: 2.5mM ammonium acetate and 2.5mM formic acid in water
iii. Phase A: 0.1% formic acid in acetonitrile
   Phase B: 0.1% formic acid in water

The mobile phases chosen were 0.1% formic acid in acetonitrile (ACN) and 0.1% formic acid in Millipore water in a gradient elution programme as shown in Table 1. The MRM targets are listed in Table 2.

Table 1: Gradient elution programme for natural dyes

Time (min)	**Phase A: 0.1% HCOOH in ACN**	**Phase B: 0.1% HCOOH in H_2O**
0.0	5%	95%
2.0	5%	95%
8.0	100%	0%
11.0	100%	0%
11.5	5%	95%
14.5	5%	95%

Table 2: Natural dye standard mass spectrometry targets

Dye	Exact Mass	Parent Ion (m/z)	Fragments (m/z)	DP (V)	FP (V)	EP (V)	CEP (V)
Alizarin	240.2106	239.1	210.1; 166.9	-35	-360	-10	-22.7
Purpurin	256.2100	255.1	226.9; 171.0	-23	-360	-11	-23.09
Carminic acid	492.3856	491.1	447.2; 357.1	-60	-360	-10	-28.81
Luteolin O-7-β-D-glucoside	448.3761	447.2	285.2; 133.00	-60	-360	-11	-27.75

Recoveries were calculated by performing peak area integrations with the Sciex Analyst software, and these areas were compared to results from the 500ppb standards in Microsoft Excel.

2.3.2 Optimisation of Clean-Up for Synthetic Dyes

Optimisation of the clean-up protocol was performed on a mixed reference sample containing 500ppb of Acid Yellow 25, Congo Red, and Red 2G. The analytes were dissolved to 500ppm and diluted to 100ppm in Millipore water and the final dilution to 500ppb was then performed in methanol. Standards were stored in the refrigerator between uses. All experiments were repeated three times and each replicate analysed twice by mass spectrometry to obtain an average.

2.3.2.1 Optimising Disperser for Synthetic Dyes

The DLLME method was adapted from a protocol outlined by Faraji et al [37] for the determination of azo dyes from ice cream samples. Chloroform was used as the extracting

solvent for the tests and methanol, isopropanol, acetonitrile and acetone were all trialled as dispersers.

The disperser trials were carried out as follows. 500µl of 100ppb mixed standard was placed in the bottom of a Falcon Tube. The sample was then made up to 5ml with Millipore water, and 150µl previously made up 2M tetra-n-butylammonium bromide (TBAB) in water was added as the ion-pair reagent. The Falcon tube was lightly swirled to ensure homogeneity. 750µl of the desired disperser and 100µl chloroform was then drawn up into a syringe and injected rapidly into the aqueous phase forming the cloudy solution. The mixture was then vortexed for 10 seconds and sonicated for 10 minutes before undergoing 5 minutes of centrifugation at 4200rpm. The bottom organic layer was removed with a syringe and placed in a vial where it was dried under N_2 flow. The extract was reconstituted with 100µl methanol for analysis by HPLC-MS.

2.3.2.2 Trial of the Complete Analytical Protocol for Synthetic Dyes

After the DLLME procedure was optimised, the chosen method was paired with the initial extraction protocol to ensure functionality of the whole protocol when joined together. The initial extraction method is exactly as described for natural dyes in §2.3.1.3 Trial of the Complete Analytical Protocol for Natural Dyes and the DLLME method was performed exactly as described in §2.3.2.1 Optimising Disperser for Synthetic Dyes with methanol used as the disperser.

2.3.2.3 Recovery Analyses by HPLC-MS

Recovery analysis was carried out using the results from targeted HPLC-MS. The instrumental set-up and chromatographic column used were the same as for natural dyes but the system was used in SIM instead of MRM, and the mobile phases and target m/z were changed. To optimise the chromatographic conditions for synthetic dyes, three trials were performed on the mobile phases:

i. Phase A: 0.1% formic acid in acetonitrile
   Phase B: 0.1% formic acid in water
   (as used for natural dyes)
ii. Phase A: 5mM ammonium acetate in acetonitrile
   Phase B: 5mM ammonium acetate in water
iii. Phase A: Methanol
   Phase B: 5mM ammonium acetate in water

The mobile phases chosen were methanol and 5mM ammonium acetate in Millipore water in a gradient elution as shown in Table 3.

Table 3: Gradient elution programme for synthetic dyes

Time (min)	Phase A: MeOH	Phase B: 5mM Ammonium Acetate in H_2O
0.0	0%	100%
3.0	100%	0%
4.5	100%	0%
5.0	0%	100%
7.0	0%	100%

The SIM targets are listed in the table below:

Table 4: Synthetic dye standard mass spectrometry targets

Dye	Exact Mass	Parent Ion (m/z)	DP (V)	FP(V)	EP (V)	CEP (V)
Acid Yellow 25	549.5537	526.2	-101	-140	-9	-29.66
Congo Red	696.6622	325.3	-38	-365	-9	-24.79
Red 2G	509.4200	231.5	-15	-326	-7	-22.52

As for natural dyes, recoveries were calculated by performing peak area integrations with the Sciex Analyst software, and these areas were compared to results from the original standards in Microsoft Excel.

2.4 Case Study: Application to Collection of ACNA Dyes from Sapienza University of Rome's Museum of Chemistry

2.4.1 Sampling

Sampling of the ACNA Museum dye collection was carried out on 22nd July 2020 after a preliminary viewing of the collection on 17th February 2020. The preliminary visit was used to take photographs and note commercial names of dyes in the collection which were then researched in the subsequent months to identify possible assignments for the dyes based on the nomenclature. The card backed fibres and some powdered samples were then selected as the case study for this research.

Figure 13: Photograph of a selection of the dyes on display from the ACNA synthetic dye collection based at Sapienza University of Rome.

Powders were sampled from glass jars using a clean spatula and stored in labelled Eppendorf tubes until analysis. Fibres were snipped using small laboratory scissors taking care to only remove the small quantity required for analysis and placed using tweezers into labelled Eppendorf tubes.

Figure 14: A photograph of the fibres being carefully sampled for analysis by the novel extraction procedure.

2.4.2 Preliminary Raman Analysis

All measurements were performed on a Horiba Jobin-Yvon HR-Evolution micro-Raman spectrometer and used a 633nm red laser to induce Raman scattering. A Raman shift range of 200-2000cm^{-1} was acquired for each point by combining two acquisitions centred at 650cm^{-1} and 1600cm^{-1} respectively. The specific acquisition settings were varied depending on the fluorescence of each particular sample and its sensitivity to degradation. Magnification was 50 or 100x depending on the sample, laser power varied from 0.1-100%, accumulation time was a maximum of 40s and a minimum of 3s; whilst number of scans per acquisition was a maximum of 240 and a minimum of 10. Spectra were averaged, combined, backgrounds subtracted and smoothed using both Origin Pro 9.0 and the Horiba software LabSpec6.

A motorised mapping stage equipped with a video camera was used to select and focus on three points on each sample which were acquired and subsequently averaged. For powdered samples, a small grain was placed on a microscopic slide and brought under focus under magnification for acquisition. For the fibre samples, a fine fibre was placed upon a small quantity of reversible adhesive putty on the microscope slide to ensure the fibre was flat and stationary for analysis.

2.4.3 Extraction and Application of DLLME

The fibre samples were extracted precisely through the method optimised by §2.3.2.2 Trial of the Complete Analytical Protocol for Synthetic Dyes. Specifically, the fibre sample was placed in a vial containing 4.4mg NaCl, 0.8ml 30% NH_3 and 0.8ml previously made up 1mM Na_2EDTA. The samples were left in the extraction mixture and covered in aluminium foil for 2 days and left to extract at room temperature. The solution was then pipetted out, using a Pasteur pipette to avoid the fibres. The sample was then placed under N_2 flow to facilitate the evaporation of the ammonia, and this was performed for several hours until a neutral solution was obtained.

The neutral solution was then placed in a Falcon tube and made up to 5ml with Millipore water to which 150µl previously made up 2M TBAB was added. The Falcon tube was lightly swirled to ensure homogeneity. 750µl of methanol and 100µl chloroform was then drawn up into a syringe and injected rapidly into the aqueous phase forming the cloudy solution. The mixture was then vortexed and sonicated for 10 minutes before undergoing 5 minutes of centrifugation at 4200rpm. The bottom organic layer was removed with a syringe and placed in a vial where it was dried under N_2 flow.

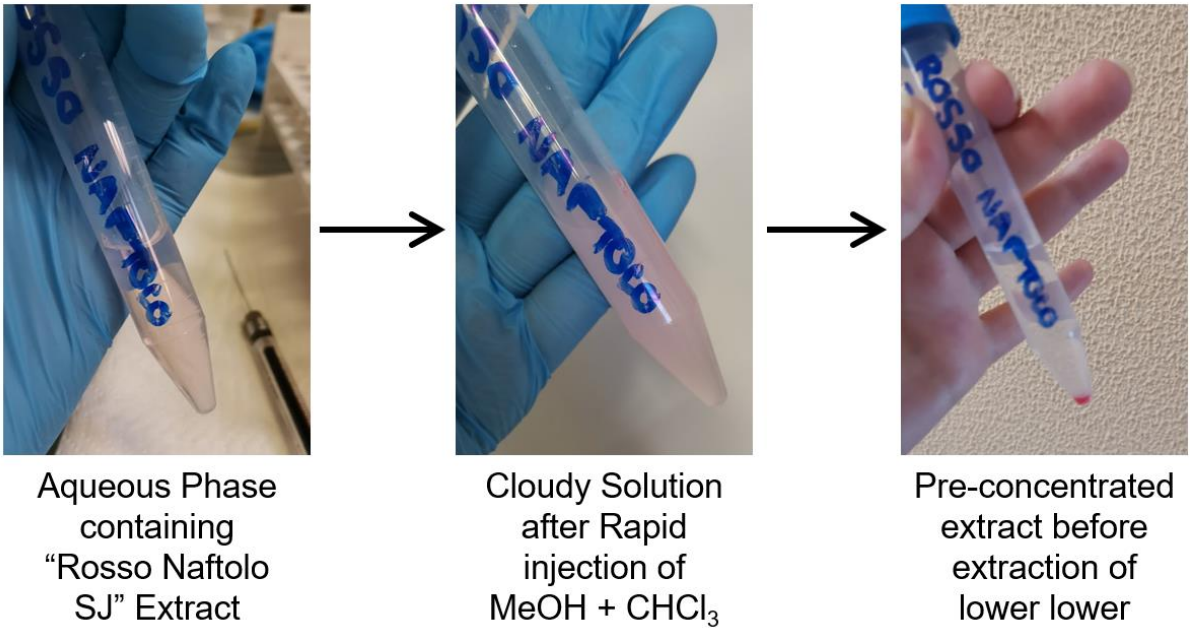

Figure 15: A photographic sequence showing the aqueous solution obtained after the extraction of "Rosso Naftolo SJ" from the fibre (left), the cloudy solution obtained after rapid injection of the disperser and extraction solvent (middle), and the pre-concentrated sample obtained after the phases were separated by centrifugation (right)

For the powder samples, it was not necessary to perform the initial extraction step and therefore the samples were dissolved in 5ml water in a Falcon tube and the DLLME protocol then performed directly on the sample.

The HPLC-HRMS was then carried out using untargeted analysis by the instrumental set-up reported below.

2.4.4 Untargeted HPLC-MS

Untargeted mass spectrometric data of the unknown museum samples was acquired using a Thermo Fisher Scientific Dionex™ UltiMate™ 3000 (RSLC) UHPLC system equipped with an RS auto sampler and coupled with a high-resolution Q-Exactive Orbitrap mass spectrometer equipped with a heated electrospray ionisation source (H-ESI).

The H-ESI source was operating in negative ionization mode with tuning parameters set at: sheath gas flow rate (nitrogen) = 45 units, auxilliary gas flow rate (nitrogen) = 20 units, spray voltage = -3.00 kV, capillary temperature = 350˚C, source temperature = 350˚C. MS experiments were carried out in Full scan-data dependent acquisition mode (Full-dds). A full scan was conducted with a scan range between 100-800 m/z with a resolution of 70,000 FWHM; Automatic Gain Control (AGC) was 1×10^6, maximum injection time was 100 ms. For MS/MS experiments, resolution was 17,500 FWHM, AGC was 5×10^6 and Maximum injection time was 80 ms, loop count and TopN was 5, isolation window was set to 2.0*m*/*z*, the fixed first mass was 50 *m*/*z*. Minimum AGC target was 8×10^3 and the intensity threshold was 1×10^6. The dissociation of molecular ions was induced in the high-energy collision cell (HCD) by means of nitrogen; simultaneous experiments were conducted at three different normalized collision energies, 10, 30 and 50%.

The Luna C18 column used for the optimisation of all of the methods and the mobile phase conditions optimised for synthetic dyes were used for analysis.

3. Results and Discussion

3.1 Optimisation of Chromatographic Conditions

The chromatographic conditions used for HPLC were optimised by trialling a range of mobile phases, columns and gradient elution programmes to obtain the best separation and elution of the analytes.

3.1.1 Chromatographic Conditions for Natural Dyes

Of the three trials performed on the mobile phases, only 0.1% formic acid in acetonitrile and 0.1% formic acid in water provided adequate separation of the analytes, and they were therefore used as the mobile phases. The gradient elution programme was optimised by trial and error based on the measured retention times of the analytes to ensure adequate separation.

For the trials of the chromatographic columns, whilst both columns achieved adequate separation of the analytes, the Luna-C18 column tolerates a flow rate of 0.8ml/min; whilst the Kinetex XB-C18 only tolerates a 0.2ml/min flow rate. As such, the run time of samples in the Luna-C18 column was significantly lower and it was hence chosen as the column for analyses.

An example of the chromatograph of the 500ppb mixed natural dye reference is presented in Figure 16.

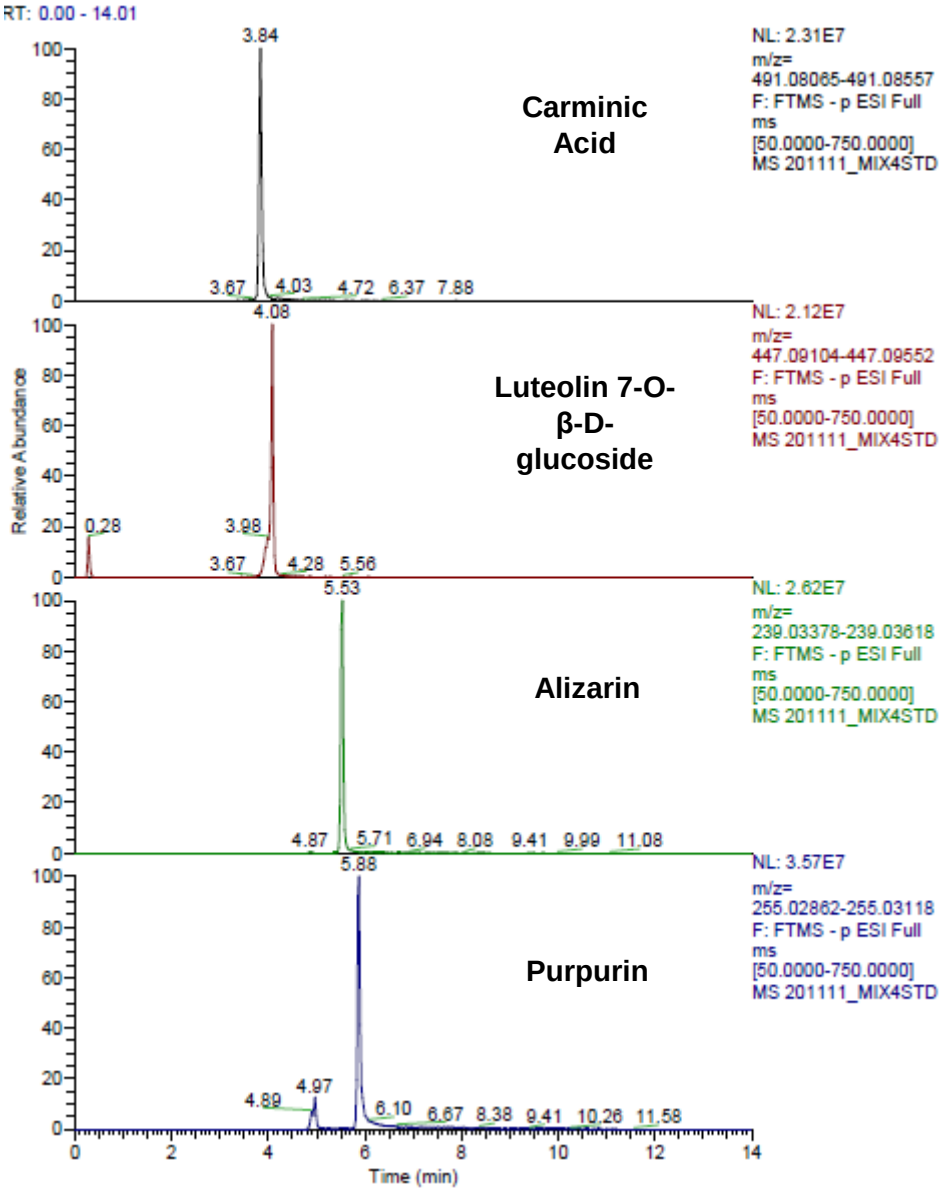

Figure 16: Extracted ion mass chromatogram (XIC) of natural dye analytes showing their retention times.

3.1.2 Chromatographic Conditions for Synthetic Dyes

For synthetic dyes, first trials were performed with the same chromatographic conditions that were optimised for natural dyes. It was found that the Luna-C18 5µm column was also well-suited to the synthetic dyes, so this was chosen as the chromatographic column.

The decision to trial mobile phases using an ammonium acetate additive was based on chromatographic conditions reported in literature for the analysis of azo dyes from food samples [37]. When acetonitrile was used as Mobile Phase A, the interference due to the solvent was extremely high – this can be a particular problem when using SIM detection mode

as was required for the synthetic dye samples. The use of methanol for Mobile Phase A and ammonium acetate in water for Mobile Phase B suffered less interference effects than observed for acetonitrile, and was highly effective for the separation of the analytes. The elution gradient used followed a similar pattern to that used for the natural dyes, however the separation was more significant for the synthetic dyes meaning that adequate separation could be obtained in a shorter run time.

3.2. Development of DLLME for Clean-Up of Natural Dyes

For the development of the clean-up of natural dyes, quantities were decided by referring to literature from DLLME studies of anthraquinone derivatives from urine [56] as well as considering the quantities in the traditional LLE protocol which has been used in recent research. [4] Acid was added to the aqueous phase before DLLME to make the phase less favourable to the natural dye analytes.

3.2.1 Results of Disperser and Extracting Solvent Optimisation for Natural Dyes

DLLME trials were performed with a variety of extracting and dispersing solvents and compared to find the best conditions to optimise the protocol. The results of these disperser and extracting solvent trials are displayed in Figure 17.

It should be noted that as described in the experimental section, dichloromethane and chloroform trials were performed using a slightly different methodology than was used for trials with 1-pentanol. A trial was performed combining the methodology for chloroform and dichloromethane (derived from a methodology by Shi et al. in 2013 [56]) and the use of 1-pentanol as the extracting solvent. In this case, 1-pentanol was not separated from the aqueous phase and this method was therefore not applicable.

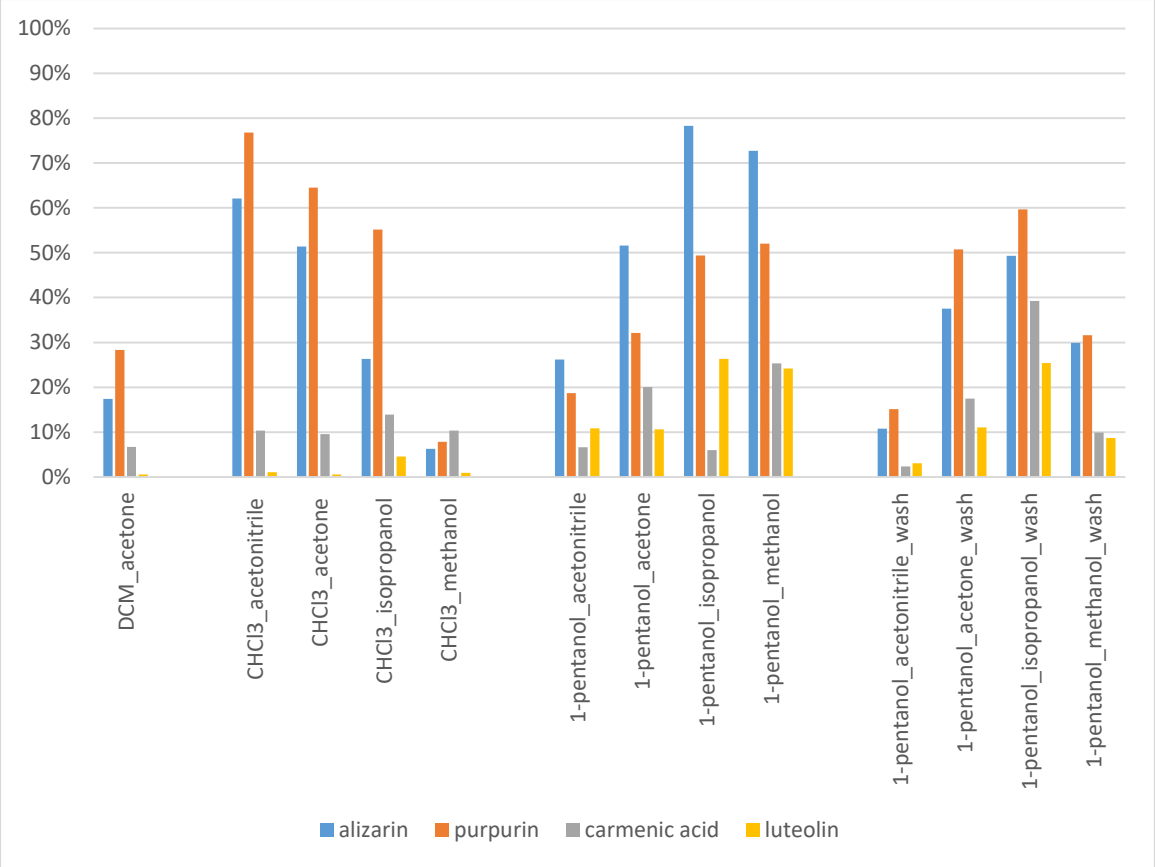

Figure 17: Results of the disperser and extracting solvent trials.
Results are grouped into extracting solvents, left to right: dichloromethane; chloroform; 1-pentanol.
1-pentanol trials are split into 2 sets: left: without final wash; right: with final wash

In trials with dichloromethane as the extracting solvent there was no separation of the organic phase in three of the four disperser trials; meaning that there was no layer to extract and hence no results listed. This confirms the observations of Shi et al. [56]. Where separation was observed – in the case of acetone – recoveries were low for all analytes. Dichloromethane was therefore disregarded as a candidate for further research.

With chloroform as the extracting solvent, separation of the organic layer was achieved for all dispersers. Where chloroform was combined with the use of acetonitrile or acetone as the disperser, excellent recoveries were obtained for alizarin and purpurin. In all cases however, very poor recovery was observed for carminic acid and luteolin, with less than 5% recovery of luteolin with all dispersers. The low recovery of these analytes is attributed to their high relative polarity compared to alizarin and purpurin. This high polarity gives carminic acid and luteolin an affinity for water, and the non-polar nature of chloroform means they are unlikely to migrate into the organic phase; despite the ample opportunity offered by the high surface area

provided by DLLME. The recovery of these analytes was considered insufficient for the protocol and hence chloroform was disregarded as a candidate for further research.

When 1-pentanol was used as the extracting solvent – the method was trialled both with and without washing. The washing was considered as in the traditional LLE extractions; washings were required to deacidify the organic phase. If acidic characteristics are present when the extract is dried, it is likely that the glucoside bonds in carminic acid and luteolin will not be well-preserved, as was the case for the previous acid based initial extraction methods [4, 23, 28] However, this effect works in opposition to a loss of the volume of organic solvent observed during washings; which decreases the quantity of the analytes present in the final samples. The effects of these two phenomena appear to vary depending on the disperser used – for example methanol undergoes a significant loss of recovery for all analytes after washing; whilst isopropanol displays a significant increase in the recovery of carminic acid when washing is used. These different effects could be related to differing acidity of the organic phase after rapid injection depending on the disperser solvent.

In general, trials using 1-pentanol as the extracting solvent achieved higher recovery of carminic acid and luteolin than was observed with chloroform. A likely hypothesis for this is that these polar analytes have more affinity for 1-pentanol than chloroform due to its higher relative polarity. Following the results, acetonitrile and acetone were immediately disregarded as dispersers as their recoveries were exceeded by methanol and isopropanol.

The two best sets of recoveries for all four analytes were obtained in the following conditions:

- Isopropanol as a disperser with 1-pentanol as the extracting solvent and the use of a final wash of the organic phase.
- Methanol as a disperser with 1-pentanol as the extracting solvent without the use of a final wash of the organic phase.

For isopropanol, the deacidification offered by the final washing meant that more of the carminic acid's glycosidic bonds were preserved than in the trial performed without washing. For methanol, the effects of any deacidification achieved by washing was less prominent than the loss of materials in the unrecovered extraction phase lost by washing, and therefore the trials without a final wash offered a higher recovery for all analytes.

From these preliminary trials, it appears that the use of isopropanol with the addition of a final wash was the most effective for the recovery of the analytes. However, for trials verifying the coherence of the method as a whole, both of the best performing combinations described

above were trialled with the ammonia-EDTA extraction protocol to understand the best possible conditions for the complete analytical protocol.

3.2.2 Comparison of Novel DLLME with Traditional LLE

To verify that the novel DLLME approach is an improvement on existing methods, the results from the optimised DLLME conditions were compared to the recovery obtained by the traditional LLE approach using a separating funnel. The results are shown in Figure 18.

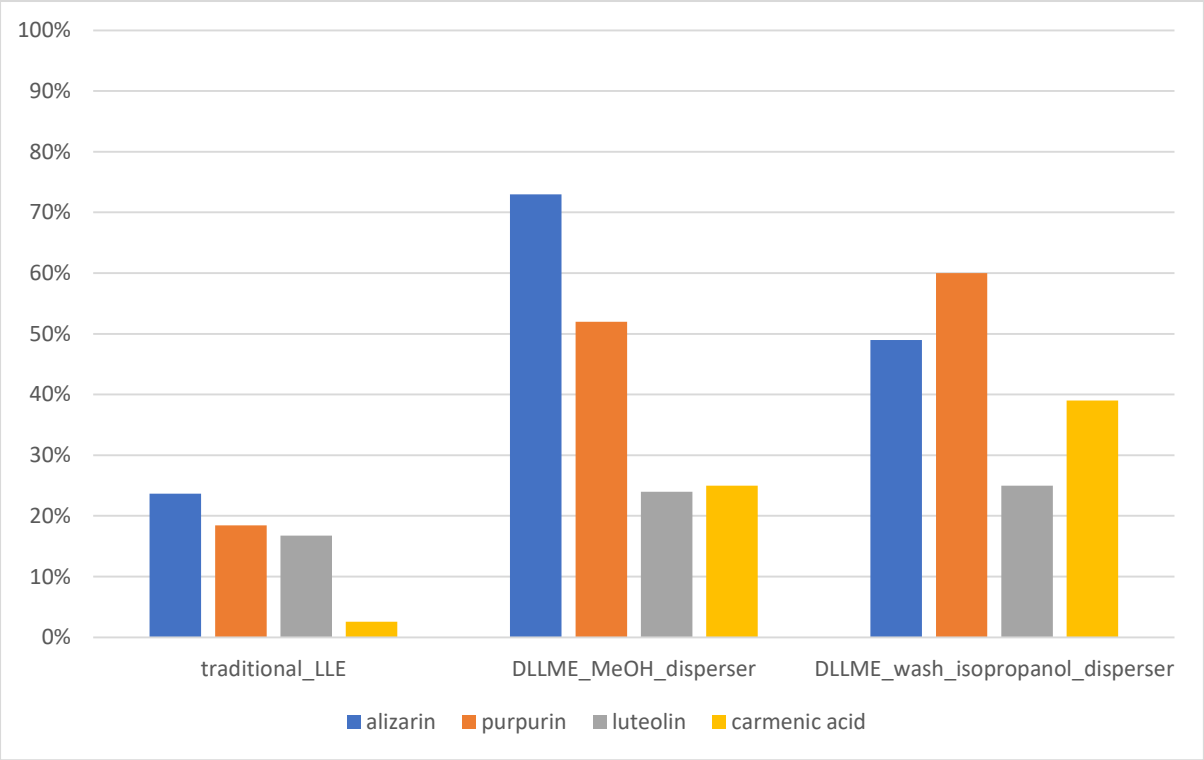

Figure 18: Comparison of recoveries obtained by traditional LLE clean-up and the optimised novel DLLME method. Left to right: traditional LLE with 1-pentanol as extracting solvent; novel DLLME with 1-pentanol as extracting solvent and methanol as disperser – performed without a final wash; novel DLLME with 1-pentanol as extracting solvent and isopropanol as disperser – performed with a final wash.

This comparison displays a clear increase in recovery by the novel DLLME method when compared to the traditional LLE method for all analytes. This increase in recovery is attributed to the high surface area afforded by the DLLME technique. These results illustrate clearly that the novel method produced by this research is a significant improvement on the existing methodology for the post-extraction clean-up of natural dyes. In practice this means that a smaller initial sample size can be used to obtain sufficient detection by HPLC-MS. This is a significant contribution to the cultural heritage goal of minimising the destructiveness of techniques.

Alongside the quantifiable results, the practical application of the LLE was considerably more time consuming and difficult to precisely control than that of the DLLME. Both of these factors mean that as well as obtaining better results, the new method is easier to apply and hence more well-suited to the wide range of disciplines involved in cultural heritage analysis.

3.2.3 Results of Trials of the Complete Analytical Protocol for Natural Dyes

Following optimisation of the analytical protocol and verification of improvement on current methods, the application of the two best clean-up protocols were combined with the ammonia-EDTA initial extraction protocol described in literature. [4] The recoveries of each analyte using methanol as a disperser (excluding a final wash of the organic phase); and isopropanol as a disperser (including a final wash of the organic phase) are presented in Figure 19.

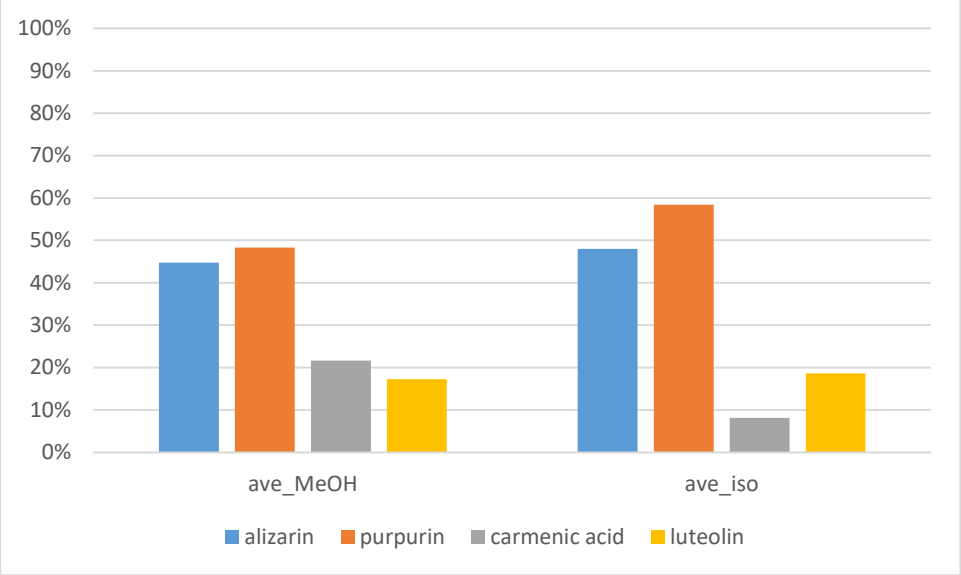

Figure 19: Recoveries of the trials of the complete analytical protocol combining the ammonia-EDTA extraction protocol with the two best clean-up protocols as defined by the disperser and extracting solvent tests

Although it is clear that in both cases there is some loss of recovery on the addition of the ammonia extraction protocol, the samples most strongly affected are alizarin and purpurin which still have a high recovery. When isopropanol was used as disperser, the recovery of carminic acid also experienced a significant reduction whilst only a small decrease in recovery was observed for the trial with methanol.

Using all the recovery analyses performed and presented by this thesis, the author therefore puts forward that the best conditions for the post-extraction clean-up of natural dyes are the use of 1-pentanol as an extracting solvent alongside methanol as a disperser and omitting a

final wash of the organic phase. Whilst a recovery of <20% for luteolin 7-O-β-D-glucoside may not immediately seem high, it is a clear improvement on the methodologies that are currently documented in literature.

3.3 Development of DLLME for Clean-Up of Synthetic Dyes

For the development of the clean-up of synthetic dyes, quantity ratios were decided by referring to a study on DLLME extractions of azo dyes from ice cream samples presented by Faraji et al. [37]

3.3.1 Ion-Pair Reagent

As mentioned in the introduction of this thesis, traditional liquid-liquid extractions are not useful for the clean-up of the azoic acid dyes focussed on by this research, which are anionic in nature. This ionic character means that the dyes have a very high affinity for water and are hence very unlikely to transition to a less polar extracting solvent during liquid-liquid extractions.

Ion-pair reagents can be used to facilitate this transition. They are always bulky reagents which have the opposing ionic charge to the target analyte. Tetra-n-butylammonium bromide (TBAB) was used as the ion-pair reagent to facilitate the extraction of the synthetic dye molecules analysed in this thesis. The quaternary character of TBAB means that it has significant steric hindrance and through this can form an “ion-associate” with an anionic molecular structure-such as the azoic acid dyes [57]. These ion-associates are bound by the steric effects of the ion-pair reagent and the molecules together create a pseudo-apolarity, meaning that the ion-associate can transition into the non-polar extraction solvent.

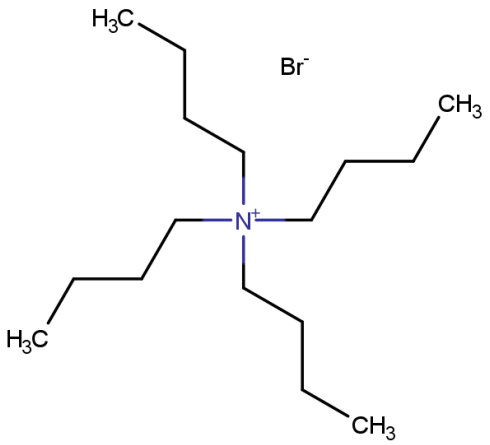

Figure 20: Structural formula of tetrabutylammonium bromide, a quaternary ammonium salt and the ion-pair reagent used for the extraction of synthetic dyes into the organic phase during DLLME

The use of this ion-pair reagent enables the application of the DLLME clean-up protocol to synthetic dyes, and whilst it can also be used with traditional LLE; the combination of the two concepts enhances extraction recovery, efficiency and precision. TBAB was specifically chosen as it is reported to be significantly more efficient than the chloride and iodide countered quaternary ammonium salts. [58] It was also successfully applied to azo dyes from food samples by Faraji et al. [37] in the protocol used as the basis for this research

3.3.2 Results of Disperser Optimisation for Synthetic Dyes

The results of the disperser tests for synthetic dyes are displayed in Figure 21. Under the DLLME protocol, all dispersers display good recoveries for all analytes. The recovery of Congo Red – over 100% for all dispersers – is explained by a combination of the error margins, and the matrix effect of the TBAB ion-pair reagent. Despite the errors in recovery attributed to matrix effects, the disperser trials were sufficient to evaluate that the best recovery was achieved with methanol as the disperser. Methanol was therefore used as the disperser for further tests.

To counter the matrix effects, further analyses used a spiked sample as a reference – which takes into account the matrix. The spiked sample was prepared identically to the experimental conditions used in the experiments but using 100µl methanol instead of 500ppb reference mix. After drying, the residue was reconstituted with the 500ppb reference mix before analysis.

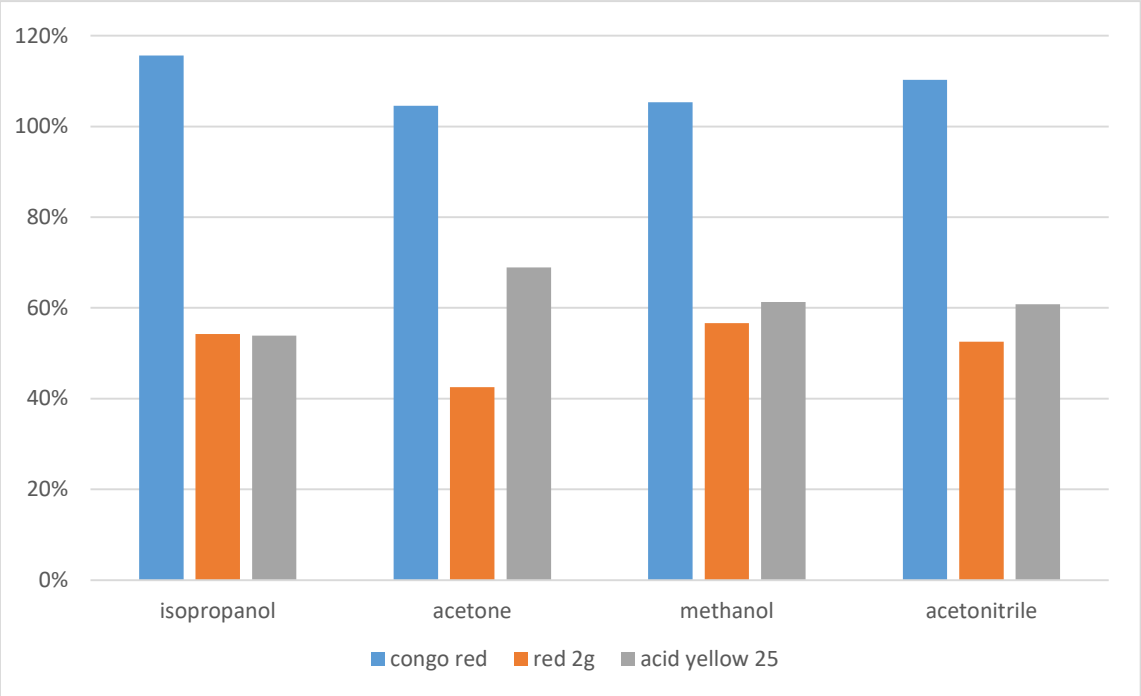

Figure 21: Comparison of the recoveries of three azo dye standards after the performance of DLLME with chloroform as the extracting solvent trialling 4 dispersers: isopropanol, acetone, methanol, and acetonitrile

3.3.3 Results of Trials of the Complete Analytical Protocol for Synthetic Dyes

After optimising the disperser conditions for the extraction of the synthetic azo dyes, the state-of-the-art ammonia-EDTA protocol was applied alongside the DLLME to assess its effects on the recovery of the samples. The results of this analysis are presented in Figure 22.

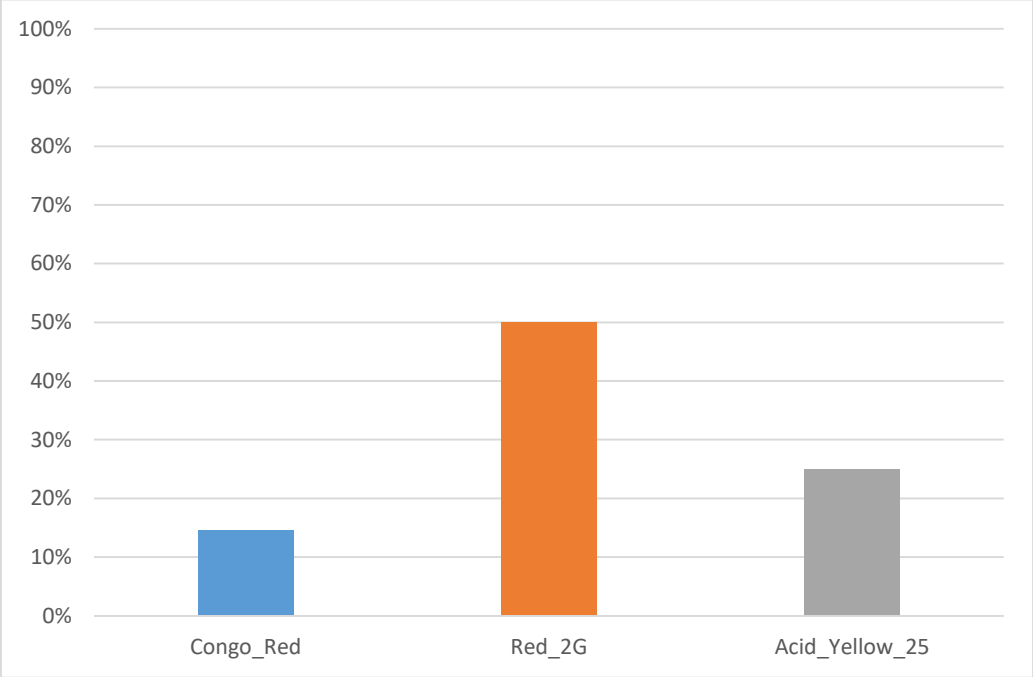

Figure 22: Recoveries of the three trialled synthetic dye analytes with the complete analytical protocol combining the ammonia and Na_2EDTA extraction method with the novel DLLME clean-up protocol developed by this research.

These results display a significant reduction in recovery than was observed when the DLLME was performed alone – particularly with reference to Congo Red. This reduction is likely to be somewhat attributed to the reference in this trial accounting for the matrix effect, but also could be related to the application of the extraction protocol. However, despite this reduction recoveries are sufficient to verify the coherence and effectiveness of the complete analytical protocol – the first to use a clean-up protocol for the analysis of synthetic dyes from cultural heritage samples. This means that the ammonia-EDTA extraction method can now be applied to synthetic dyes as well as natural dyes, and brings together a coherent extraction methodology that requires only one sample to be removed from artefacts suspected to contain both natural and synthetic dyes. This is a significant contribution to the field of cultural heritage research.

3.4 Results of Case Study on ACNA Dyes from Sapienza University of Rome's Museum of Chemistry

3.4.1 Predictions Based on Nomenclature and Preliminary Raman Analyses

Prior to the sampling of the museum artefacts, research was performed using historical documents and chemical databases on the commercial names of the museum samples. Using these resources, it was possible to tentatively assign likely connections between the museum samples under investigation and possible chemical compositions. All possible molecular attributions based on nomenclature were recorded in order to compare them with the interpretations of results from analytical techniques. For some of the dyes in the collection, this study of the nomenclature provided very little information. For example, extensive research into the use of the peculiarly spelled "Italana" labelled dyes in the collection (which also considered the common spelling – "Italiana") found no reference to them in any bibliographic resources. These difficulties could be partly related to the fact that the collection was obtained directly from the ACNA factory, and it is not clear whether all the dyes in the collection were commercially available or were simply in-house trials of possible new dye types. Nonetheless, for some of the dyes in the collection useful information was obtained through this bibliographical research, and the results of these preliminary studies are noted in Table 5. After this preliminary investigation, the samples were taken from the Sapienza University of Rome Museum of Chemistry and investigated using Raman analysis as described in the experimental protocol.

As previously mentioned, the Raman spectra of organic textile dyes – particularly on those fixed to fibres – can in some cases be overwhelmed by fluorescence due to the relative weakness of the Raman effect when compared to other electromagnetic transitions. In these cases, SERS can be used to amplify the Raman Effect and obtain a well-defined spectrum. SERS does however require the irreversible application of a metal colloid to the sample, and is hence a destructive technique. As such, Raman spectroscopy is generally applied before moving to SERS if the spectra obtained are insufficient. For the ACNA case study presented in this thesis, despite fluorescence being present for every sample, well-defined Raman spectra were obtained for 13 of the 15 museum samples. Therefore, SERS was not carried out on the group as part of this thesis. For the two compounds for which the fluorescence effect meant that no spectra could be obtained, further work by the group hopes to apply SERS to a small sample of the extracted dye prepared for the analysis by HPLC-MS.

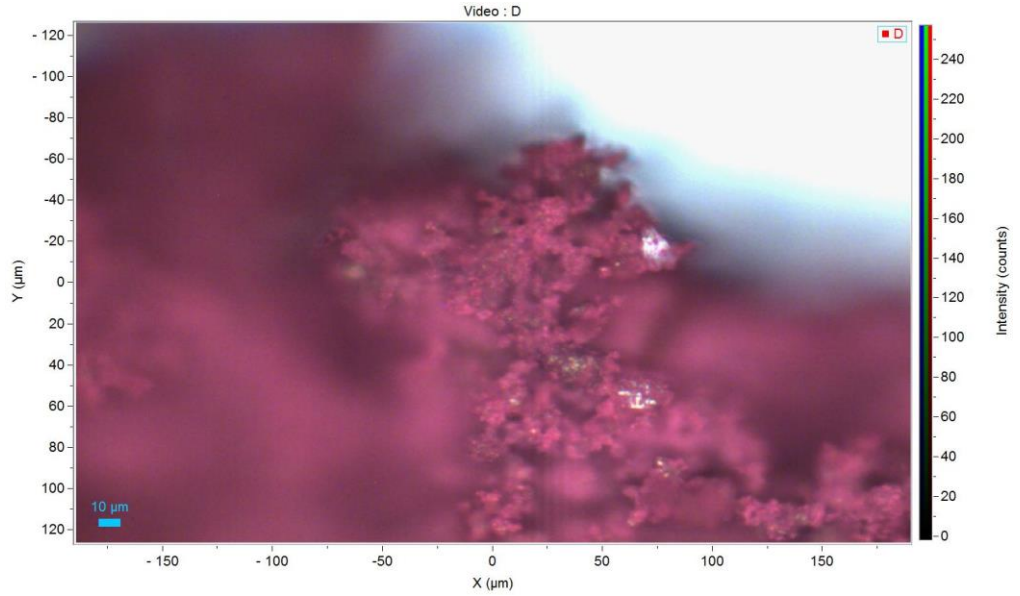

Figure 23: Point 1: Rosso Amidonaftolo 2G powder. An example of an image of one of the points brought under focus by the motorised mapping stage for the powder dyes studied from the collection.

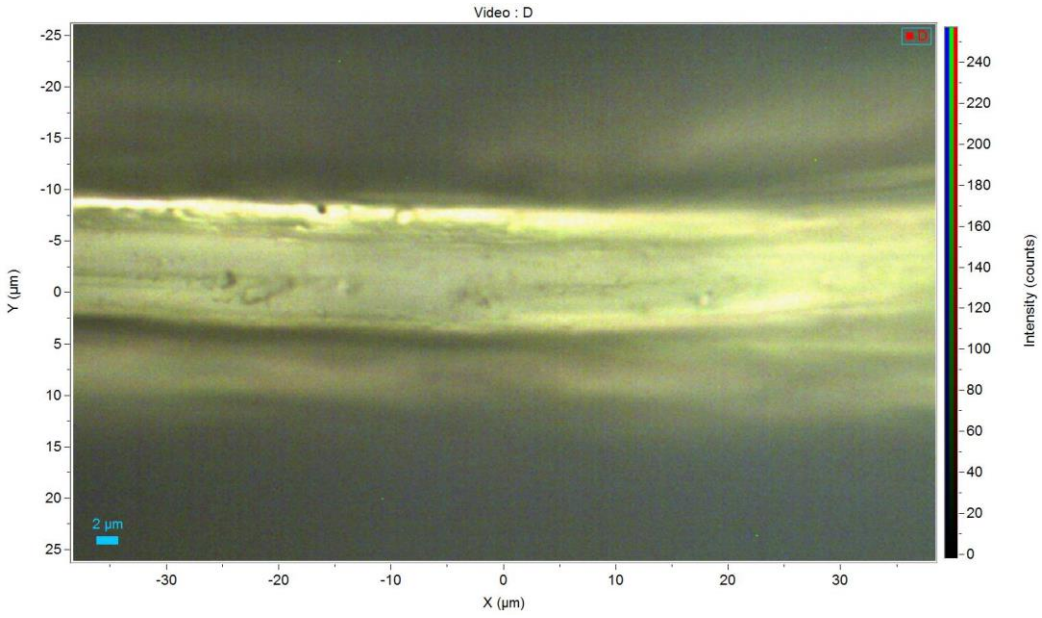

Figure 24: Point 1: Tartrazina J fibre. An example of an image of one of the points brought under focus by the motorised mapping stage for the dyes fixed to fibres studied from the collection.

The Raman spectra of all the dyes are recorded in the Annex. For the fibres, the Raman spectra of the yellow and orange dyes were significantly impacted by the Raman signals of the wool. For the red dyes, there was some interference from the wool, however the effects were much less pronounced. A spectrum of undyed wool was obtained under the same conditions as were used for analysis of the dyes and compared to the spectra by overlaying in LabSpec6. The peaks which were not attributed to contributions from the wool were recorded and are presented in Table 5. These peaks were compared to spectra recorded in a variety of scientific studies [59, 60, 61, 62] alongside the database of organic dyes from the Infrared and Raman Users Group (IRUG). Possible assignments based on the similarity of these spectra to those recorded in literature is presented in the table. Due to aforementioned problems with the lack of databases for synthetic dyes – owing to their wide-ranging diversity – it was not possible to find well-matching Raman spectra for all of the dyes in the collection. For example, a strong link was found in literature between the nomenclature “Rosso Novamina 2G” and the sulfonated azo dye Acid Orange 19, however Raman spectra were not available for this species for comparison. In other cases, such as “Tartrazina J” where nomenclature clearly invokes a commonly used synthetic dye – in this case the disulfonated azo dye Tartrazine – it was particularly surprising when the spectra obtained was poorly matched to a reference spectrum of Tartrazine obtained using an analytical standard. This variability and unpredictability of nomenclature is a key marker of the complexity of synthetic dye studies mentioned in the introduction to their research. In the case of “Tartrazina J”, the Raman spectrum of the disulfonated azo dye Acid Yellow 17 reported by Baker in 2011 [59] was found to be more closely matching to the peaks observed by the spectral acquisition of the sample in the laboratory.

Table 5: Preliminary Predictions based on nomenclature and Raman spectroscopic analysis.

Sample Name	Sample Type	Molecular Predictions Based on Nomenclature	Raman Spectral Peak Positions	Molecular Predictions Based on Raman Spectra
Arancio Luce G	Fibre	N/A	258 cm^{-1} (w), 280cm^{-1} (m), 396cm^{-1} (w-m), 470cm^{-1} (w), **512cm^{-1} (m),** 547cm^{-1} (s), **641cm^{-1} (m)**, 712cm^{-1} (m-w), 789cm^{-1} (v.w), 900cm^{-1} (v.w), 937cm^{-1} (w-m), 976cm^{-1} (v.w-w), **1001cm^{1} (m),** 1040cm^{-1} (v.w), **1084cm^{-1} (v.s), 1171cm^{-1} (m), 1233cm^{-1} (s), 1302cm^{-1} (m), 1314cm^{-1} (m,sh), 1326cm^{-1} (m,sh)**, 1372cm^{-1} (m-w), **1495cm^{-1} (m), 1596cm^{-1} (v.s)**, **1655cm^{-1} (m)**	Significant similarities with Sample "Giallo Italana 2g" in the <800cm^{-1} range
Giallo Eliamina RL	Powder	Direct Yellow 29 [63] Direct Yellow 44 [64] Direct Yellow 49 [65] Direct Yellow 50 [66]	431cm^{-1} (w), 477cm^{-1} (w), 541cm^{-1} (w), 588cm^{-1} (w), 720cm^{-1} (v.w), 736cm^{-1} (w), 749cm^{-1} (v.w), 774cm^{-1} (w), 788cm^{-1} (w), 825cm^{-1} (w), 854cm^{-1} (v.w), 902cm^{-1} (v.w), 932cm^{-1} (v.w), 956cm^{-1} (v.w), 1015cm^{-1} (v.w), 1077cm^{-1} (v.w), **1151cm^{-1} (m),** 1175cm^{-1} (w), 1218cm^{-1} (w-m), **1249cm^{-1} (m-s), 1316cm^{-1} (m-s,sh)**, **1338cm^{-1} (v.s),** 1401cm^{-1} (v.w), 1436cm^{-1} (w-m), 1463cm^{-1} (v.w), 1490cm^{-1} (v.w), 1516cm^{-1} (v.w), 1 573cm^{-1} (w,sh), **1615cm^{-1} (s)**	Pigment Yellow 74 [67]
Giallo Italana 2G	Fibre	N/A	257 cm^{-1} (w), 282cm^{-1} (m); 309cm^{-1}(v.w), 319cm^{-1} (v.w), 331cm^{-1} (v.w), 396cm^{-1} (v.w), 470cm^{-1} (w), **512cm^{-1} (m,sh), 548cm^{-1} (s),** 582cm^{-1} (w-m), **637cm^{-1} (m)**, 713cm^{-1} (m-w), 740cm^{-1} (w), 801cm^{-1} (v.w), 916cm^{-1} (w), 935cm^{-1} (w), 977cm^{-1} (v.w), 1034cm^{-1} (w,sh), 1050cm^{-1} (m-w), **1086cm^{-1} (v.s)**, 1123cm^{-1} (w), 1155m^{-1} (w), 1204cm^{-1} (v,w), 1226cm^{-1} (m-w), 1256cm^{-1} (v.w), 1414cm^{-1}(m-w), **1448cm^{-1} (m)**, **1519cm^{-1} (m)**, 1555cm^{-1} (v.w), **1604cm^{-1} (v.s)**	Significant similarities with Sample "Arancio Luce G" in the <800cm^{-1} range
Giallo Luce Solido 2G	Fibre	Acid Yellow 11 [68]	282cm^{-1} (v.w); 395cm^{-1} (w), 406cm^{-1} (w), 469cm^{-1} (v.w), 493cm^{-1} (v.w) 516cm^{-1} (v.w), 547cm^{-1} (w-m), 568cm^{-1} (v.w), 579cm^{-1} (v.w), 621cm^{-1} (w-m), **642cm^{-1} (m)**, 693cm^{-1} (w,br), 711cm^{-1} (w,br), 748cm^{-1} (v.w,br), 757cm^{-1} (v.w,br), 874cm^{-1} (w,br), 881cm^{-1}(w,br), 919cm^{-1} (v.w), 955cm^{-1} (w), **1003cm^{-1} (m)**, **1032cm^{-1} (m)**, 1040cm^{-1} (m-w,sh), 1061cm^{-1} (v.w), **1081cm^{-1} (m)**, 1099cm^{-1} (w,br), 1110cm^{-1} (w,br), **1125cm^{-1} (m)**, 1158m^{-1} (v.w), **1462cm^{-1} (s,sh)**, **1518cm^{-1} (m)**, 1559cm^{-1} (m-w), **1602cm^{-1} (v.s)**,	N/A

Sample Name	Sample Type	Molecular Predictions Based on Nomenclature	Raman Spectral Peak Positions	Molecular Predictions Based on Raman Spectra
Giallo Luce Solido 2G	Powder	Acid Yellow 11 [68]	228cm^{-1} (v.w-w), 243cm^{-1} (v.w-w), 293cm^{-1} (w), 384cm^{-1} (v.w), **467cm^{-1} (m-s),** 505cm^{-1} (v.w), 571cm^{-1} (w-m), 616cm^{-1} (v.w-w), 631cm^{-1} (v.w), **795cm^{-1} (m),** 837cm^{-1} (v.w), 917cm^{-1} (w-m), **999cm^{-1} (m), 1054cm^{-1} (m),** 1093cm^{-1} (v.w-w), 1130cm^{-1} (v.w-w), **1168cm^{-1} (m), 1221cm^{-1} (m),** 1277cm^{-1} (w-m,sh), 1289cm^{-1} (w-m), 1306cm^{-1} (w,sh), 1342cm^{-1} (v.w-w), 1371cm^{-1} (v.w), 1436cm^{-1} (w-m), 1488cm^{-1} (w-m,sh), **1517cm^{-1} (s),** 1559cm^{-1} (w-m,sh), **1600cm^{-1} (v.s),** 1655cm^{-1} (w)	Acid Yellow 11 [59]
Giallo Novamina R	Fibre	Acid Yellow 61 [69, 70] Acid Yellow 39 [71] Acid Yellow 25 [72]	**512cm^{-1} (m-s)**, **548cm^{-1} (m-s,sh)**, 620cm^{-1} (w), **643cm^{-1} (m)**, 665cm^{-1} (w), *712cm^{-1} (w),* ***764cm^{-1} (m)***, 806cm^{-1} (w), **828cm^{-1} (m)**, ***852cm^{-1} (m-s)***, *$^{-1}$ (m-w)*, 958cm^{-1} (w), 975cm^{-1} (w), ***1002cm^{-1} (s)***, 1032cm^{-1} (w), 1049cm^{-1} (m-w), 1086cm^{-1} (w), 1125cm^{-1} (v.w), 1206cm^{-1}(m-w), **1465cm^{-1} (s,sh)**, **1515cm^{-1} (m)**, 1552cm^{-1} (m-w), **1609cm^{-1} (v.s)**	Acid Yellow 25 (performed on an analytical standard in the laboratory comparison shown in Figure 26)
Tartrazina J	Fibre	Tartrazine	260cm^{-1} (v.w); 282cm^{-1} (w); 401cm^{-1} (v.w,br), 484cm^{-1} (v.w), **550cm^{-1} (m)**, 610cm^{-1} (v.w), 621cm^{-1} (v.w), 635cm^{-1} (w,sh), 643cm^{-1} (w-m), 695cm^{-1} (w), *712cm^{-1} (w), 719cm^{-1} (v.w,sh), 761cm^{-1} (v.w-w)*, 807cm^{-1} (v.w.), 876cm^{-1} (w), 921cm^{-1} (v.w), 957cm^{-1} (v.w), 976cm^{-1} (v.w), ***1003cm^{-1} (s)***, 1032cm^{-1} (w-m), 1040cm^{-1} (w,sh), **1086cm^{-1} (m-s)**, **1123cm^{-1} (m-s) 1463cm^{-1} (s,sh)**, **1503cm^{-1} (m-s)**, **1600cm^{-1} (v.s)**	Acid Yellow 17 [59]
Rosso Amido-naftolo 2G	Fibre	Red 2G [73]	339cm^{-1} (v.w), **482cm^{-1} (m), 502cm^{-1} (m),** 549cm^{-1} (w), 570cm^{-1} (w), 898cm^{-1} (w-m), 998cm^{-1} (w-m,br), 1014cm^{-1} (v.w), 1086cm^{-1} (v.w), 1111cm^{-1} (w-m), 1171cm^{-1} (m), **1225cm^{-1} (m,br), 1281cm^{-1} (v.s),** 1361cm^{-1}(w-m), **1405cm^{-1} (s), 1455cm^{-1} (m,sh), 1493cm^{-1} (s), 1574cm^{-1} (s,sh), 1595cm^{-1} (v.s),** 1620cm^{-1} (w-m,sh)	Red 2G (performed on an analytical standard in the laboratory – comparison shown in Figure 25)
Rosso Amido-naftolo 2G	Powder		350cm^{-1} (w), 438cm^{-1} (v.w), 533cm^{-1} (v.w), 582cm^{-1} (w), **904cm^{-1} (m-s),** 988cm^{-1} (v.w-w), 1012cm^{-1} (w), 1086cm^{-1}(v.w), 1115cm^{-1} (v.w), 1165cm^{-1} (v.w), **1208cm^{-1} (m-s), 1277cm^{-1} (v.s), 1362cm^{-1}(m), 1408cm^{-1} (m), 1501cm^{-1} (m-s), 1562cm^{-1} (m), 1596cm^{-1} (m-s), 1620cm^{-1} (m,sh)**	Red 2G (performed on an analytical standard in the laboratory – comparison shown in Figure 25)

Sample Name	Sample Type	Molecular Predictions Based on Nomenclature	Raman Spectral Peak Positions	Molecular Predictions Based on Raman Spectra
Rosso Italana B	Fibre	N/A	549cm^{-1} (m), 683cm^{-1} (w), 713cm^{-1} (w), 829cm^{-1} (w), 1017cm^{-1} (w), 1044cm^{-1} (w-m), 1087cm^{-1} (w-m), 1114cm^{-1} (m-s), 1142cm^{-1} (w,sh), 1372cm^{-1} (w-m), 1413cm^{-1} (w-m), **1458cm^{-1} (m-s), 1482cm^{-1} (m), 1583cm^{-1} (s,sh)**, **1604cm^{-1}(v.s)**	Acid Red 26 Significant similarities with Sample "Rosso Italana R"
Rosso Italana R	Fibre	N/A	**1086cm^{-1} (w,sh), 1113cm^{-1} (m-s),** 1142cm^{-1} (w,sh), 1180cm^{-1} (w), **1238cm^{-1} (m-s), 1265cm^{-1} (m-s), 1310cm^{-1} (m), 1372cm^{-1} (m), 1415cm^{-1} (m-s,sh), 1437cm^{-1} (m-s, sh), 1457cm^{-1} (s), 1485cm^{-1} (s), 1507cm^{-1} (m-s,sh), 1582cm^{-1} (s,sh), 1606cm^{-1} (v.s)**	Acid Red 26 Significant similarities with Sample "Rosso Italana B"
Rosso Luce Solido BL	Fibre	Likely to be a naphthol dye e.g. Para Red, Pigment Red 3 [74]	254cm^{-1} (m-w), 242cm^{-1} (v.w), 398cm^{-1} (v.w), 504cm^{-1} (w), 546cm^{-1} (w), 637cm^{-1} (w), 711cm^{-1} (v.w), 732cm^{-1} (v.w), 899cm^{-1} (v.w), 967cm^{-1} (v.w), 1146cm^{-1} (m), **1228cm^{-1} (m-s), 1279cm^{-1} (m-s), 1353cm^{-1} (v.s),** 1475cm^{-1} (m-w), 1562cm^{-1} (w,sh), **1594cm^{-1} (m)**	Pigment Red 4 [75] Pigment Red 253 [76]
Rosso Naftolo	Fibre	Likely to be a naphthol dye.	Spectrum not obtained due to overwhelming fluorescence.	N/A
Rosso Novamina	Fibre	Acid Red 62 [69, 70] Acid Orange 19 [70]	281cm^{-1} (v-w), 390cm^{-1} (v-w), 459cm^{-1} (w), 486cm^{-1} (v.w), 502cm^{-1} (w), 549cm^{-1} (v.w), 643cm^{-1} (v.w-w), 680cm^{-1} (v.w), 713cm^{-1} (v.w), 733cm^{-1} (v.w), 767cm^{-1} (v.w), 1003cm^{-1} (w-m), 1033cm^{-1} (w), 1045cm^{-1} (w), 1086cm^{-1} (v.w), 1115cm^{-1} (v.w), 1168cm^{-1} (v.w), **1238cm^{-1} (m), 1278cm^{-1} (m,sh), 1296cm^{-1} (m),** 1319cm^{-1} (w.sh), 1327cm^{-1} (w), 1410cm^{-1} (w-m), **1449cm^{-1} (s),** 1496cm^{-1} (w) **1552cm^{-1} (m-s,sh), 1590cm^{-1} (m-s,sh), 1605cm^{-1} (v.s)**	N/A
SEII Azoic Acido Pag	Fibre	N/A	Spectrum not obtained due to overwhelming fluorescence.	N/A

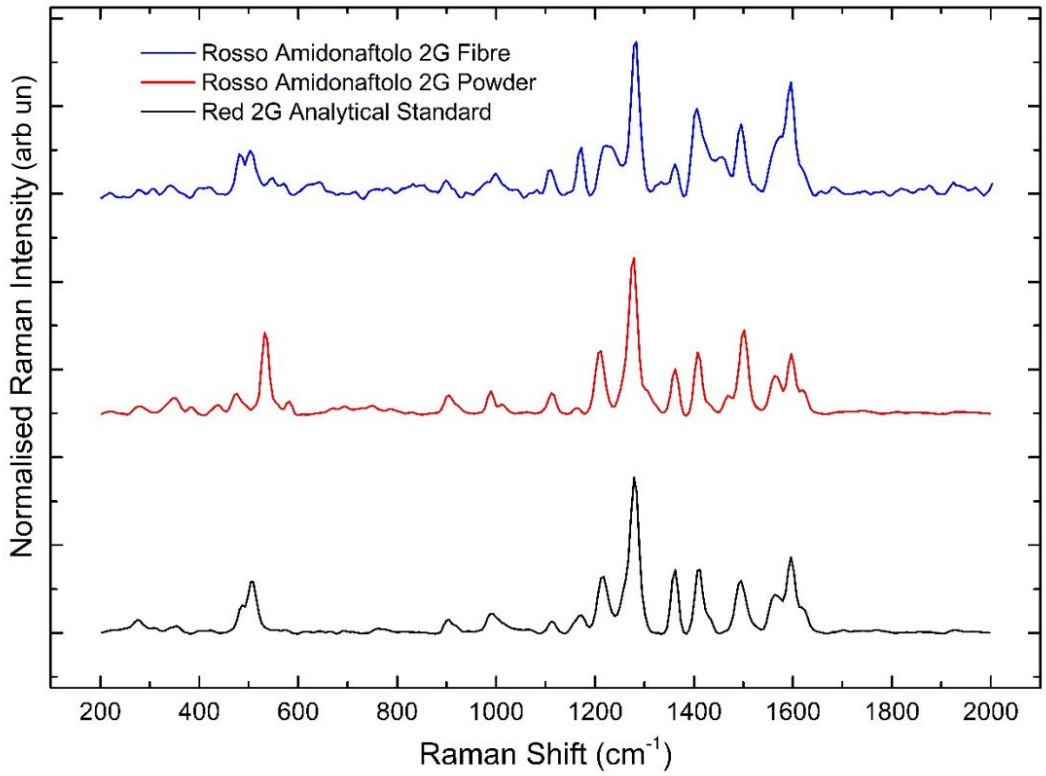

Figure 25: Comparison for the Raman spectra acquired for the fibre and powder of “Rosso Amidonaftolo 2G” with the Raman spectra acquired for Red 2G

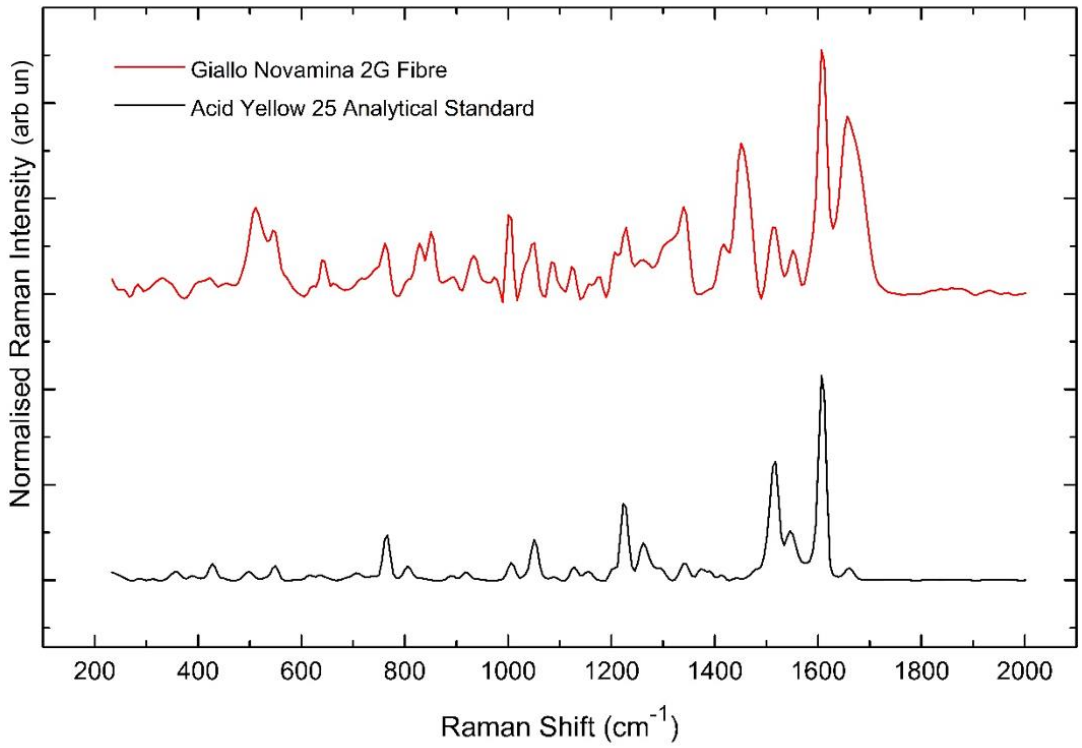

Figure 26: Comparison for the Raman spectra acquired for the museum sample “Giallo Novamina 2G” with the Raman spectra acquired for Acid Yellow 25. Significant differences observed at peaks 1600, 1452, 1341, 1000, 854 and 822cm^{-1} are attributed to the Raman signals of the wool matrix.

3.4.2 Assignments Based on Preliminary Predictions and Untargeted Mass Spectrometric Results

3.4.2.1 Analytical Challenges

To corroborate the data obtained by the Raman analysis and obtain further information about the dye compounds present on the museum samples; untargeted mass analysis was performed by an Orbitrap high resolution mass spectrometer. The mass spectrometry results contained several peaks which were present in all analytes and hence likely to be related to the matrix and these were therefore discounted when analysing the data.

Where assignments were in agreement with the predictions formulated from the Raman spectroscopy, it was concluded that the molecules were highly likely to correspond to the projected molecular structures. In cases where Raman spectroscopy was not sufficient to obtain formal predictions about the molecular structures (particularly when there was no corresponding spectra available in literature) but the mass analyses were able to provide possible predictions, the Raman spectroscopy was consulted to check for the presence of characteristic Raman peaks likely to be present if the assignment was correct. In some cases, characterisation of the specific species was not obtained and will require additional time and further research – which will hopefully be aided by the results from the SERS analysis of the extracted species that will take place after the submission of this thesis.

The reasons for the difficulties associated with this characterisation are the lack of databases available for the identification of synthetic dye molecules, and the fact that the dyes come from one specific company and therefore may not have been widely used or indeed ever commercially available. Further challenges with the mass spectrometry of the azoic acid dyes most likely to be present in this collection are the fact that these species contain varying degrees of charged character. This means that, when an m/z ratio is recorded, one can interpret the ratio in several ways. For example, if an m/z ratio of 231.72 is recorded, the reader could interpret this as: a singly charged molecule of mass 231.72u; a doubly charged molecule of mass 463.44u, a triply charged molecule of mass 695.16u and so on. Alongside this, where databases exist, they generally include the counter-ion mass when reporting the overall molecular mass. Whilst in most cases, ionic azo dyes utilise sodium as their counter-ion, it is possible that other cationic species can be used for this purpose. This means that alongside interpreting all the possible charges of the molecule, the interpreter must also project both which are the likely cations, and how many are likely to be present in order to search for the mass on chemical databases.

The cumulative data obtained by: the literary research on dye nomenclature, interpretation of the Raman spectra, and the untargeted mass spectrometry results for each of the dyes in the collection is presented and discussed.

3.4.2.2 Assignments

Arancio Luce G:

Preliminary research for “Arancio Luce G” was not indicative of any possible correlations between the dye and known species. Bibliographic research into the name turned up no results – which could be related to the simple translation of the name to “Light Orange”, which is a commonly used phrase and therefore not highly indicative. Whilst peaks were visible on the Raman spectrum, the presence of signals from wool were extremely prominent, making the overall shape of the dye peaks difficult to interpret. No similar spectra were found in the Raman databases correlating to the peaks observed.

Mass spectral data of the compound was also fairly weak, with three unique m/z peaks observed, all of which had relatively low intensity chromatographic peaks. The peaks observed were for m/z 360.3131, 361.2608 and 526.0877. The highest intensity of these peaks was observed at m/z 361.2608 and this species was therefore used to make some possible projections. Projections were made considering that “Arancio Luce G” is likely to be an azoic acid dye, and that the counter-ion is likely to be Na^+ (as is usually the case for this class):

- If the species is a singly charged azoic dye, the m/z is likely to represent $[M\text{-}Na]^-$, where M would represent the molecular mass and be equal to 384.2506u.
- If the species is a doubly charged azoic dye, the m/z is likely to represent $[M\text{-}2Na]^{2-}$, where M would represent the molecular mass and be equal to 361.2608.

When these dyes were searched on chemical databases, a tentative possible assignment was made: Acid Orange 31 which has a molecular mass of 384u. [77]

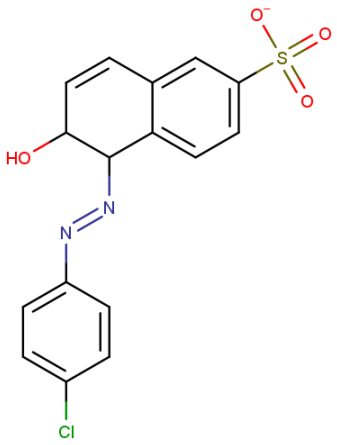

Figure 27: Structural formula of Acid Orange 31, tentatively assigned to the fibre “Arancio Luce G”

To corroborate this data, the Raman spectrum was returned to for further interrogation. Though a precise spectrum for Acid Orange 31 was not found in literature. According to a 2019 investigation by Lomax & Lomax [78], the presence of the Ar-Cl bond can be indicated by a sharp peak at 1090cm^{-1}, which is present in the Raman spectrum obtained for Arancio Luce G. The peak at 1496cm^{-1} in the spectrum also corresponds strongly to the azo-benzene stretch reported for Red 2G by Cesaratto et al [60]., a molecule which has a similar molecular template to Acid Orange 31.

Using this information it is tentatively suggested that the dye corresponding to “Arancio Luce G” may be the structure Acid Orange 31.

Giallo Eliaminia RL:

The preliminary investigations into the identification of “Giallo Eliamina RL” were based strongly upon the nomenclature. Several chemical databases listed Yellow Eliamina as a synonym for a variety of dyes: Direct Yellow 29, Direct Yellow 44, Direct Yellow 49 and Direct Yellow 50. With the exception of Direct Yellow 29, these molecules share some features. Specifically, they are diazo structures with the presence of a central carbamide group.

Figure 28: Carbamide group present in three of the structures investigated by the preliminary nomenclature research.

This class of dyes however has not been widely studied by Raman spectroscopy and hence it was not possible to carry out spectral comparisons on these dyes. It was identified that there were some spectral similarities with Pigment Yellow 74.

Mass spectral data of the “Giallo Eliamina RL” powder revealed several species present in the sample, however a very intense peak corresponding to m/z 388.7797 at a retention time of 2.25min was by far the most prominent. As was performed for “Arancio Luce G”, two projections were made on the possible masses of the molecule:

- If the species is a singly charged azoic dye, the m/z is likely to represent $[M\text{-}Na]^-$, where M would represent the molecular mass and be equal to 411.7695u.
- If the species is a doubly charged azoic dye, the m/z is likely to represent $[M\text{-}2Na]^{2-}$, where M would represent the molecular mass and be equal to 823.5390u.

Molecular weight searches into the two projected masses were performed but no yellow dyes were found to correspond.

Giallo Italana 2G:

Initial preliminary studies were not indicative for the identification of “Giallo Italana 2G”. As discussed, the “Italana” range does not appear in any of the bibliographic resources used for nomenclature research. In the first instance, Raman data was also not very indicative – with the only similarities noted those with “Arancio Luce G”.

The mass spectral data for “Giallo Italana 2G” presented three m/z species, and by far the most intense was m/z 236.9883 which had a retention time of 3.35min. As was performed before, two projections were made about the possible molecular masses:

- If the species is a singly charged azoic dye, the m/z is likely to represent $[M\text{-}Na]^-$, where M would represent the molecular mass and be equal to 259.9781u.

- If the species is a doubly charged azoic dye, the m/z is likely to represent $[M-2Na]^{2-}$, where M would represent the molecular mass and be equal to 519.9562u.

Molecular weight searches into the two projected masses were performed but no yellow dyes were found to correspond. However, it is possible to infer from the Raman spectrum that the spectral template for “Giallo Italana 2G” is likely to share structural details with Acid Orange 31. In particular, the peaks appear to correspond well with relation to the presence of a chloride substituted aromatic group.

Giallo Luce Solido 2G:

Preliminary research on both the nomenclature and Raman spectra agreed that it was highly likely that that “Giallo Luce Solido 2G” powder was likely to be the dye Acid Yellow 11. Whilst there were some spectral similarities between the fibre sample and the powder sample, several of the peaks in the $<1000cm^{-1}$ range do not strongly correspond. The preliminary analyses considered that this may be due to the dyeing process, but also introduced the possibility that the fibre and powder may have different molecular structures despite sharing the same name.

<u>Powder:</u>

In mass spectrometry of the powder, the prediction that the dye was Acid Yellow 11 was corroborated. The results from the mass spectrometry indicated the presence of a very intense peak corresponding to m/z 357.0572 between retention times 2.59-2.99min. The m/z corresponds to the following species: $[M-Na]^-$ where M is the mass of Acid Yellow 11 and is equal to 380.0555u. [79] The spectrum of the “Giallo Luce Solido 2G” powder therefore considered highly likely to be Acid Yellow 11.

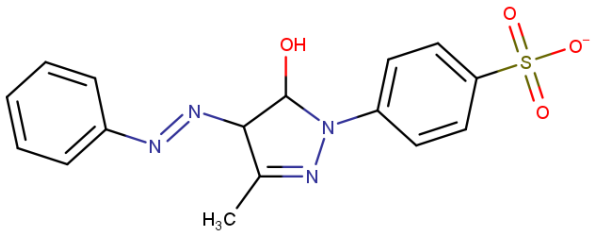

Figure 29: Structural formula of Acid Yellow 11, corresponding to the powder “Giallo Luce Solido 2G” [79]

Fibre:

For the fibre species, there is no significant peak present corresponding to the m/z 357.0572. This agrees with the preliminary Raman data in which the spectra did not appear to completely match despite some similarities. This indicates that the powder and fibre samples named "Giallo Luce Solido 2G" are different molecular species despite having the same commercial names.

The fibre sample did however present a very intense chromatographic peak corresponding to an m/z 417.3234 at retention time 3.76min. From this m/z it is possible to draw several interpretations, but if it is considered likely that the fibre is an azoic acid (which the similarities in the >1000cm^{-1} range of the Raman spectrum to the powdered sample would point towards); and that the counter-ion is likely to be Na^+ some projections for possible masses can be put forward:

- If the species is a singly charged azoic dye, the m/z is likely to represent $[M\text{-}Na]^-$, where M would represent the molecular mass and be equal to 440.3132u.
- If the species is a doubly charged azoic dye, the m/z is likely to represent $[M\text{-}2Na]^{2-}$, where M would represent the molecular mass and be equal to 880.6264u.

Molecular weight searches into the two projected masses were performed but no yellow dyes were found to correspond.

Giallo Novamina 2G:

By the preliminary research on nomenclature, three molecules were proposed as possible identifications of the dye "Giallo Novamina 2G". Whilst no Raman spectra were available for Acid Yellow 61 and 39 for comparison, the Raman analysis on an analytical standard of Acid Yellow 25 performed in the laboratory was found to correspond strongly to the non-wool related peaks of the "Giallo Novamina 2G" Raman spectrum.

On mass spectrometric analysis, an intense chromatographic peak was observed corresponding to m/z 526.0876 at retention time 2.86min, which is exactly as observed for the Acid Yellow 25 analytical standard. The m/z corresponds to the following species $[M\text{-}Na]^-$ where M is the mass of Acid Yellow 25 with a sodium counter-ion and is equal to 549.0774u.

The chemical structure of "Giallo Novamina 2G" is therefore understood as highly likely to be that of Acid Yellow 25.

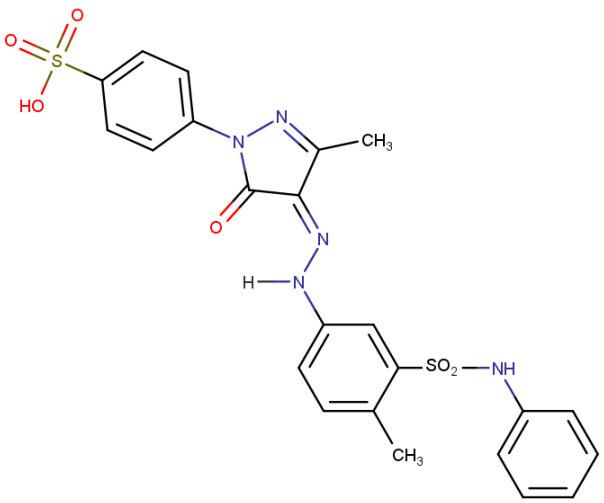

Figure 30: Structural formula of Acid Yellow 25, corresponding to the fibre “Giallo Novamina 2G”

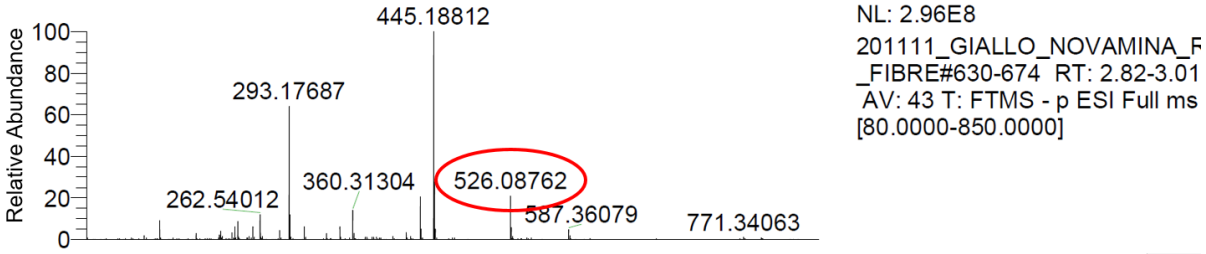

Figure 31: Peak Corresponding to Acid Yellow 25 $[M-Na]^-$

Tartrazina J:

The preliminary predictions for “Tartrazina J” were Tartrazine (based on nomenclature however significant spectral differences were observed between the Raman spectra for these compounds) and Acid Yellow 17 (based on a very strongly similar Raman spectrum).

For Acid Yellow 17, the expected m/z peak is likely to exist at m/z 251.6546 corresponding to the species $[M-2Na]^{2-}$ where M is the mass of Acid Yellow 17 and is equal to 551.2888u. This peak was not present in the spectrum of Tartrazina J, and it was therefore inferred that the dye molecule is unlikely to correspond to Acid Yellow 17.

For Tartrazine there are significant problems in detection with mass analysis owing to a triple charge on a very small molecule and the existence of the molecule in several states. In trials undertaken as part of this thesis even an analytical standard of Tartrazine was unable to be detected in targeted analysis, so it is unlikely that it would be possible to detect the species in untargeted analysis – which is less sensitive for specific compounds. Alongside this, the

Raman spectrum of “Tartrazina J” showed some significant spectral peaks which did not correspond to the peaks observed on the analytical standard of Tartrazine.

However, one of the recorded m/z peaks in literature for Tartrazine is m/z 233.1550 owing to the following species $[M-3Na+H]^{+}$, and this peak was present in the mass spectrum of “Tartrazina J”, alongside another equally intense peak at m/z 228.9509. Due to the problems with analysing Tartrazine, it was not possible to conclude whether there may have been Tartrazine present in the sample, but if it is present, it is possible that the dyed fibre may contain a mixture of dyes which would account for the extra peaks on the Raman spectrum.

Rosso Amidonaftolo 2G

The predictions proposed by the preliminary research on both the nomenclature of the dye “Rosso Amidonaftolo 2G” and the comparison of the Raman spectra obtained meant that Red 2G was predicted as a likely candidate for the identification of the molecular structure of both the powder and fibre samples.

This identification was corroborated by the presence of an intense chromatographic peak corresponding to the m/z 231.5072 at retention times 2.11 and 2.14min for the fibre and the powder respectively, which are close to those observed for the Red 2G standard. The m/z corresponds to the following species: $[M-2Na]^{2-}$, where M is the mass of Red 2G with two sodium counter-ions and is equal to 508.9939u.

Both the powder and fibre samples of Rosso Amidonaftolo 2G are hence identified as highly likely to be Red 2G, which has the following chemical structure:

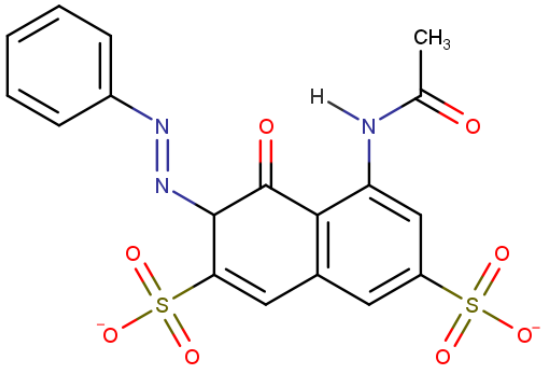

Figure 32: Structural formula of Red 2G, corresponding to the powder and fibre “Rosso Amidonaftolo 2G”

Rosso Italana B

As previously discussed, preliminary research on the nomenclature of the “Italana” dyes yielded no results. Spectral comparisons with databases indicated possible correlations with structures similar to Acid Red 26. On corroboration with the mass spectral data however, this possible attribution was found to be unlikely, as the following predictions were made based on the only diagnostic peak which appeared on the spectra which had an m/z of 236.9884 and a retention time of 3.36min.

- If the species is a singly charged azoic dye, the m/z is likely to represent $[M\text{-}Na]^-$, where M would represent the molecular mass and be equal to 259.9782u.
- If the species is a doubly charged azoic dye, the m/z is likely to represent $[M\text{-}2Na]^{2-}$, where M would represent the molecular mass and be equal to 519.9564u.

Molecular weight searches into the two projected masses were performed but no red dyes were found to correspond.

Rosso Luce Solido BL & Rosso Italana R

For both “Rosso Luce Solido BL” and “Rosso Italana R”, the m/z peaks corresponding to the major peaks in the chromatogram were all present in a wide range of the spectra acquired from the whole set of museum dyes, and were hence not considered to be indicative of the dye compounds present in the samples. It is possible that on further analysis of the chromatograms in the laboratory that other chromatographic peaks may be identified, with more diagnostic m/z values. For “Rosso Italana R” in particular, a very broad peak was acquired from 1.45-2.41min, which unfortunately includes the retention time of an extremely intense peak at m/z 360.3130 which appears in every spectrum. It is therefore likely that if the diagnostic peak elutes within this range that its signal may be overwhelmed by the intensity of the m/z 360.3130 peak in the mass spectrum and therefore not be visible. For both “Rosso Luce Solido BL” and “Rosso Italana R”, the tentative data obtained from the preliminary predictions was not adequate to make any informed decision about the identification of dyes without the mass spectrum.

Further analysis of the chromatogram to check for other possible diagnostic peaks is a recommended next step.

Rosso Naftolo SJ

Naphthol reds are a very common and significantly varied range of azoic acid dyes, so the name “Rosso Naftolo SJ” is not particularly indicative to obtain preliminary predictions from. Unfortunately, the extremely high fluorescence of the sample when subjected to the Raman

laser meant that it was not possible to obtain a Raman spectrum for “Rosso Naftolo SJ” and hence no preliminary predictions were possible based on Raman comparisons.

This meant that the interpretation of the mass spectral data was approached only with the understanding that the dye was likely to be a species containing a naphthalene group. For interpretation of the chromatogram, the only intense peak considered likely to be indicative of the molecular structure of the compound was the peak recorded at m/z 200.9741 and retention time of 3.08min. This was therefore used to make a tentative projection for the possible mass of the molecule as follows:

- If the species is a singly charged azoic dye, the m/z is likely to represent $[M\text{-}Na]^-$, where M would represent the molecular mass and be equal to 223.9308u.
- If the species is a doubly charged azoic dye, the m/z is likely to represent $[M\text{-}2Na]^{2-}$, where M would represent the molecular mass and be equal to 447.8616u.

Molecular weight searches into the two projected masses were performed but no red dyes were found to correspond. The author presents that due to the likelihood that the dye molecule contains: a naphthalene group (as suggested by the name) as well as at least one sulfonate group and one azo group (as is the case for the other dyes identified from the sample set), it is presented that a molecule of mass 223.9308u is unlikely to correspond as these components have a cumulative mass >223.9308u.

Rosso Novamina 2G

Preliminary research on the nomenclature of “Rosso Novamina 2G” found two related azoic acid dye species – however a historical document [70] strongly indicated that “Rosso Novamina 2G” is likely to be Acid Orange 19. No Raman spectra were available in literature for the standard for spectral comparisons to be performed, and the Raman spectrum of “Rosso Novamina 2G” was also very strongly affected by the signals of the wool compared to the other red dyes, meaning that only very few peaks were visible.

As such, the prediction was made solely on the nomenclature for this sample and it was predicted that if the sample corresponded to Acid Orange 19, which has a mass of 519.0535u [80], then the major m/z peak in the mass spectral data should correspond to the following species: $[M\text{-}Na]^- = 496.0655$. Indeed, an intense peak corresponding to this m/z was observed at a retention time of 2.88min.

It is hence proposed that the identification of the dye “Rosso Novamina 2G” is likely to be Acid Orange 19.

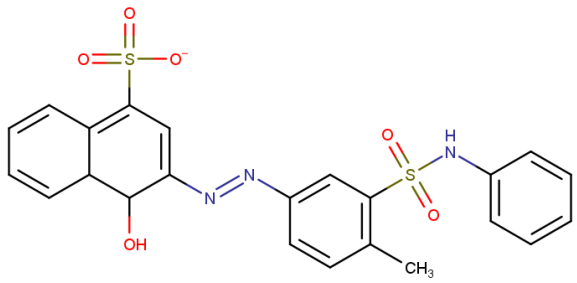

Figure 33. Structural formula of Acid Orange 19, corresponding to the powder and fibre “Rosso Novamina 2G” [80]

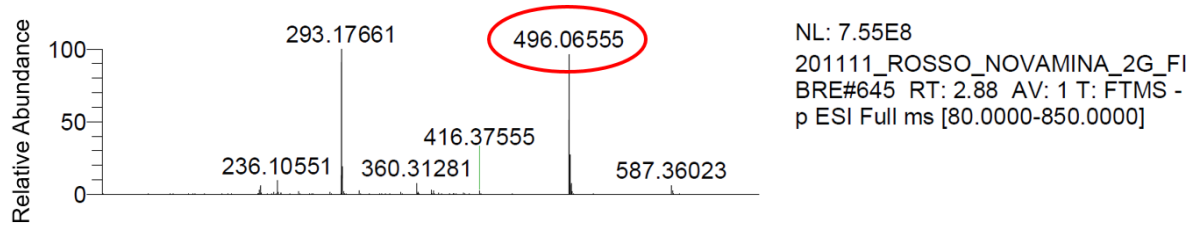

Figure 34. Peak Corresponding to Acid Orange 19

SEII Azoico Acido Pag:

For “SEII Azoico Acido Pag”, unlike the other samples, the label on the glass jar was simply handwritten. Due to this (alongside the fact that the naming format was very different to the others in the set) it was deemed likely that the label may have corresponded simply to an internal sample management system and not the commercial dye nomenclature. Research on the name found no bibliographic references, and Raman spectroscopy of the powder was not useful due to being overwhelmed with fluorescence.

This meant that the mass spectrometry data was faced without any prior knowledge of the possible molecular structure and hence was difficult to interpret. There were several very high intensity m/z peaks observed in the data. A fairly intense chromatographic peak was observed corresponding to the m/z 231.5072 associated to Red 2G at a retention time of 2.14 (as observed with the standard); as well as intense peaks at m/z 217.01257, 253.1350, 327.0452, 355.0767 and 577.4851. The peak at m/z 327.0452 – recorded at a retention time of 2.68min was by far the highest peak observed, however all peaks were of a significant magnitude, indicating that it is possible that the sample is a mixture. The most intense peak was used to make a projection for the possible mass of the molecule as follows:

- If the species is a singly charged azoic dye, the m/z is likely to represent $[M\text{-}Na]^-$, where M would represent the molecular mass and be equal to 350.0350u.
- If the species is a doubly charged azoic dye, the m/z is likely to represent $[M\text{-}2Na]^{2-}$, where M would represent the molecular mass and be equal to 700.0700u.

After consulting molecular databases, the dye Acid Orange 7 (also known as Orange II) was found to have a mass of 350.0337u [81]. Unfortunately, without the availability of Raman data or information based on nomenclature, it is not possible to present this information as anything but a suggestion. It is hoped that it is possible to obtain more informative spectra by future studies of the purified sample using SERS. However, if this powder is a mixture – as the mass spectral data appears to suggest, then SERS performed on the sample may be very complex to interpret.

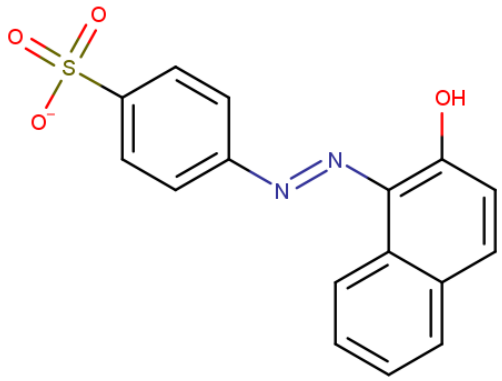

Figure 35: Structural formula of Acid Orange 7, a possible majority component of the SEII Azoico Acido Powder [81]

3.4.3 Summary of Findings

It is evident from the data presented by this case study that the application of the novel analytical protocol for the extraction and pre-concentration of synthetic dyes was highly effective for this research, and is therefore a significant contribution to the study of synthetically dyed artefacts.

Several interesting findings were presented by the case study regarding the naming conventions and content of the ACNA's synthetic dye collection. Particularly interesting was the case of "Giallo Luce Solido 2G", where it appears that despite being labelled with the same name and being from the same company, the dyed fibre and powdered sample are likely to

have different chemical structures. In contrast, in “Rosso Amidonaftolo 2G” the chemical structure was consistent between the powder sample and the dyed wool. This is a strong exemplification of the complexity of the study of synthetic dye collections – when even within a single company naming conventions can vary so widely.

The case study also perfectly illustrates the benefits and necessity of the multi-analytical approach to the analysis of unknown artefacts. In some cases, Raman spectroscopy was highly indicative of the likely assignment of the structure, but when compared with the mass spectral results the preliminary assignment was shown to be incorrect. This was illustrated with the “Tartrazina J” sample, which appeared strongly similar to the spectrum for Acid Yellow 17 but the expected m/z was then not observed in the mass spectrum. Contrastingly, for some samples the indicative nature of the Raman meant that mass spectra could be rapidly interpreted to corroborate the data, for example with “Rosso Amidonaftolo 2G”. In other cases, such as for “Arancio Luce G”, the preliminary data was not indicative of a particular compound, but could be used to corroborate the data obtained from the mass spectrum. Overall, this multi-analytical approach meant that several samples from the collection could be analysed with only a very small textile sample. It is also hoped that application of SERS to the extracted samples from the collection in the future will enhance the research even further, and hopefully provide more indicative data for the unidentified samples – particularly “SEII Azoico Acido Pag” and “Rosso Naftolo” which were unable to be analysed by traditional Raman due to fluorescence effects.

4. Conclusions

The primary aims of this thesis were to develop a novel extraction strategy, based on the state-of-the-art ammonia-EDTA initial extraction, and adding a novel clean-up protocol for the analysis of natural and synthetic dyes from historical and artistic matrices. As a secondary aim, the protocol developed for synthetic dyes was applied to a real case study of historical synthetic dyes from industry.

For natural dyes, this new methodology sought to enhance the recovery and efficiency of a state-of-the-art ammonia-EDTA based extraction method. This method has allowed observation of never-before detected glycoside moieties and has hence significantly enhanced the understanding we can obtain about the source species of dyes. As textile dyes are so informative about the societies from which they originate, it is essential to extract the maximum quantity of information possible from the dye in each step of the analysis. The novel DLLME protocol displays a significant improvement in the recovery of the dyes when

compared to the traditional LLE protocol previously used, as illustrated by quantitative comparison between the two methods. This increased recovery means that we have a higher quantity of dye analytes to analyse under HPLC-MS, and that the quality of the results are therefore significantly improved. This is particularly important for natural dyes, where a single sample can constitute a range of chromophoric compounds, often in low concentrations, and therefore it is important to achieve as high a recovery as possible. This research was based upon four specific natural dye standards: two aglycone standards and two glycosidic standards, chosen to be broadly representative of the natural dyes. For these standards, it was clearly defined that the performance of DLLME with methanol as a dispersing agent and 1-pentanol as the extracting solvent was the best condition for the highest recovery of the standards. To build further upon the research in this thesis, it is recommended that further DLLME trials are performed on more of the glycosidic compounds, as they are so widely represented in the natural dyes and hence informative to natural dye studies. It is key to undertake significant research on this class because they are so easily broken down by the presence of acids, and therefore the protocol must be specifically tailored to accommodate their requirements.

For the synthetic textile dyes, this thesis marks the very first time a clean-up protocol has been developed for use in cultural heritage studies. The research adapted a methodology from the analytical science of food and successfully developed a protocol well-suited to cultural heritage. It was clear from the results of the trials carried out that the best conditions for the extraction was the use of methanol as the disperser solvent with chloroform as the extracting solvent. One of the main benefits of the development of the clean-up protocol for synthetic dyes is that they can now be extracted alongside the natural dyes in a single extraction step. This means that artefacts thought to contain both types of dyes now only require the acquisition of a single sample instead of two, and hence minimises the destructiveness of analysis. The new research also opens up an opportunity to investigate the best extraction conditions specifically for synthetic dyes, and a review and comparison of all current methods of extraction for synthetic dyes could be an excellent next research step.

The coherence and functionality of the new clean-up protocol for synthetic dyes was clearly illustrated by the case study of the ACNA dye collection from Sapienza University of Rome's Museum of Chemistry, where all dyes were successfully extracted and analysed using a multi-technical high sensitivity approach and combining this with analysis of bibliographic resources. Information was obtained about all of the compounds using this analysis, proving that the novel protocol proposed by this research effectively extracted and recovered the synthetic dyes. The results obtained from the application to the case study illustrated the strength of the multi-technical approach for this type of analysis, and the purification and pre-concentration

effects afforded by the novel DLLME clean-up procedure meant that the m/z of the dye species was clearly visible in high resolution mass spectrometry, with minimal interference from unwanted contaminants. The case study marks an excellent first step in the analysis of the vast array of synthetic dyes present in the collection housed in Sapienza University of Rome's Museum of Chemistry and provides interesting information regarding the consistency of naming protocols used historically in the manufacturing industry. The comparison of the dataset to results from SERS analyses on the extracted dye samples prepared for the HPLC-MS protocol will be an interesting next step which may aid the identification of some of the unknown compounds. As a goal for future research, similar case studies on the other classes of dye in the collection could be performed.

Overall, this thesis has achieved its goals of developing new extraction methods for the analysis of both natural and synthetic dyes by defining novel clean-up protocols for the purification and pre-concentration of textile dye samples obtained from artefacts of cultural heritage. This work is a significant contribution to the field of cultural heritage and is a first step which the author hopes will prompt further experimentation on the value of clean-up protocols for the identification of natural and synthetic textile dyes from historical and artistic matrices.

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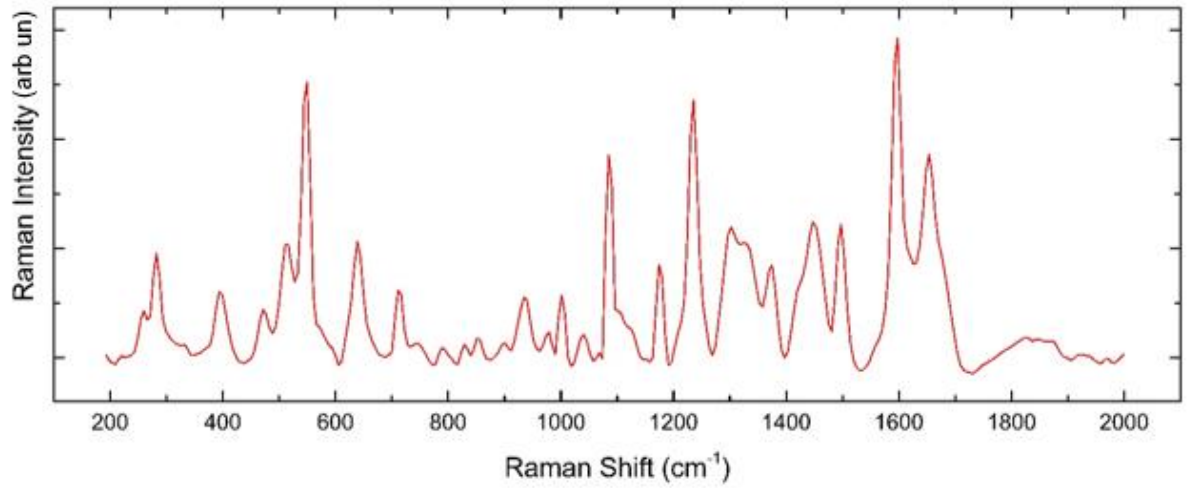
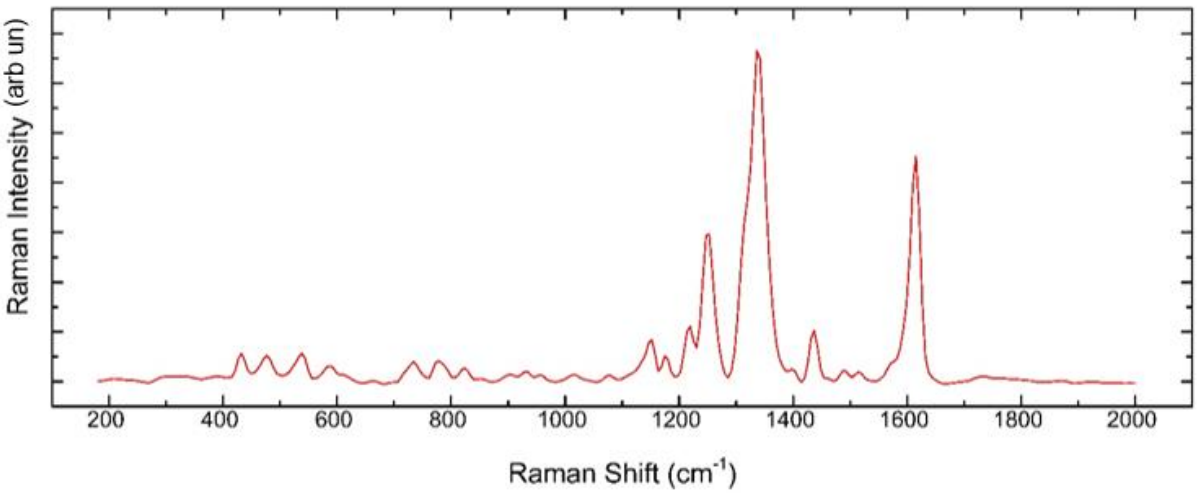
Annex: Raman Spectra for ACNA Dyes from Case Study

It is noted that the units for Raman intensity presented in all the below spectra are arbitrary.

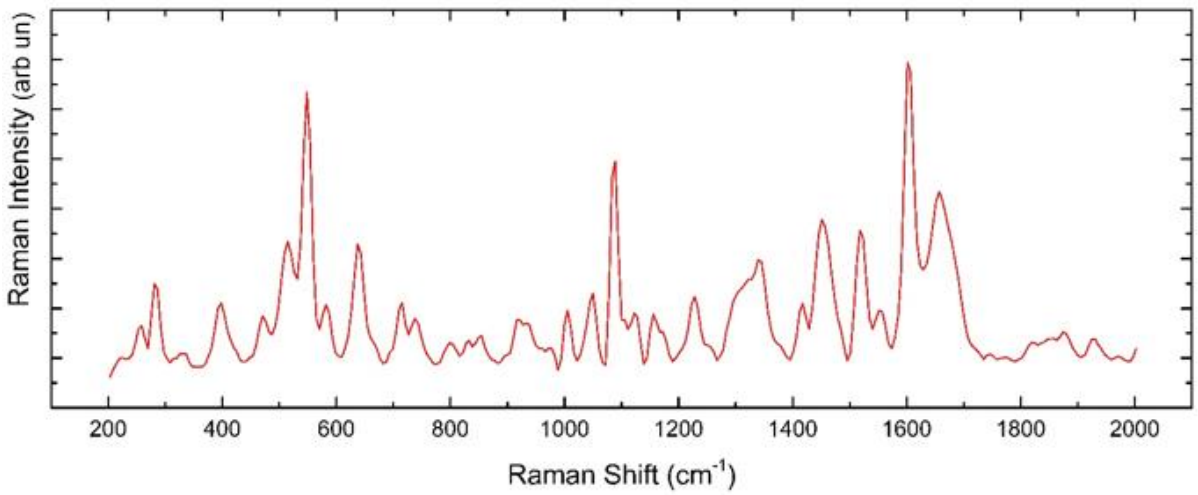
Arancio Luce G Fibre:



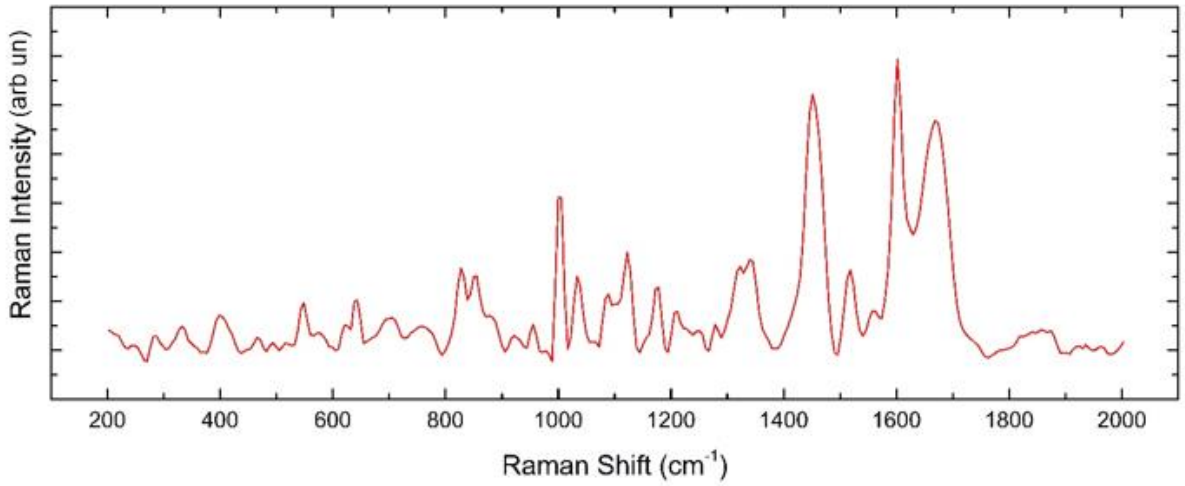
Giallo Eliamina RL Powder:



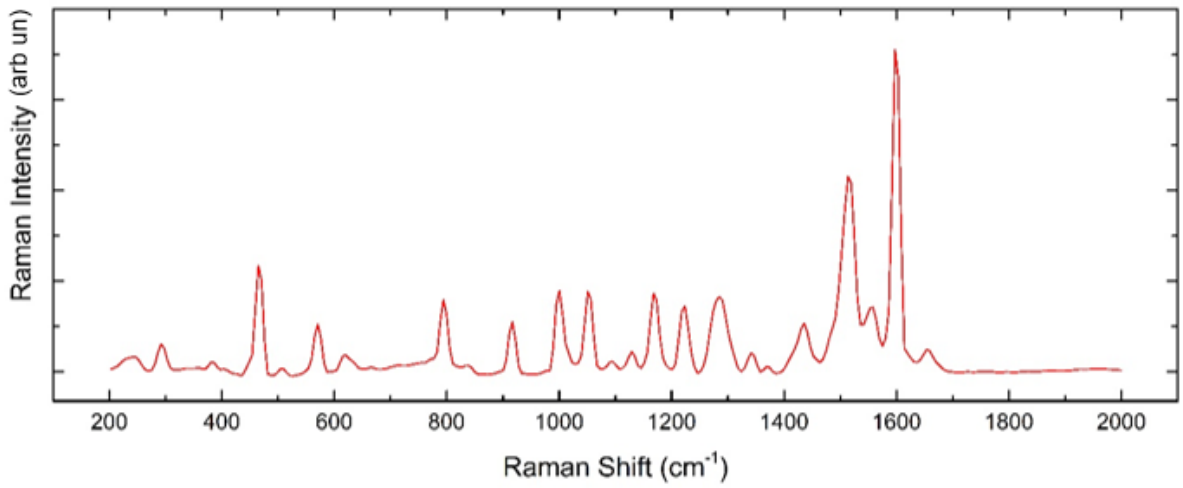
Giallo Italana B Fibre:



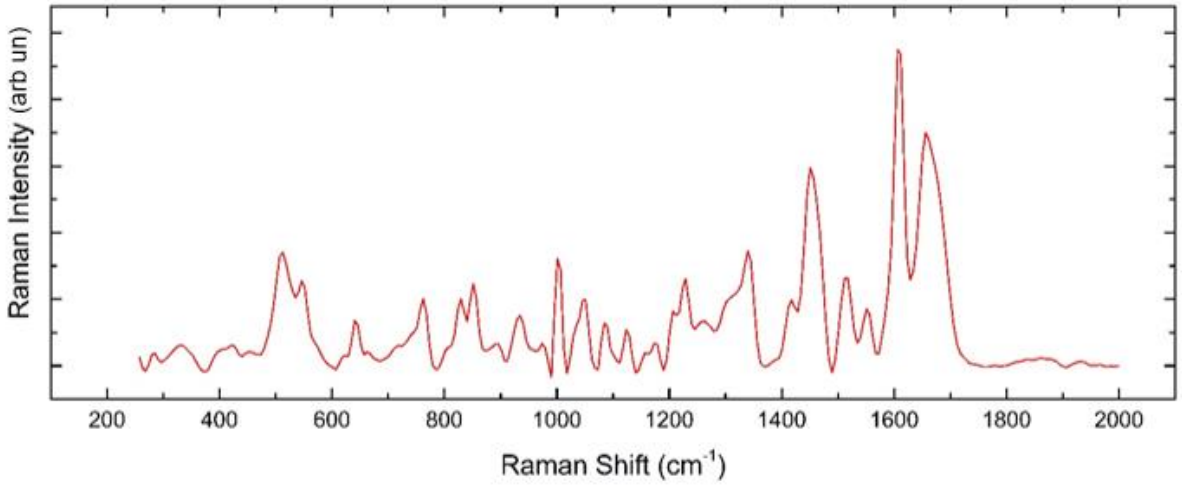
Giallo Luce Solido 2G Fibre:



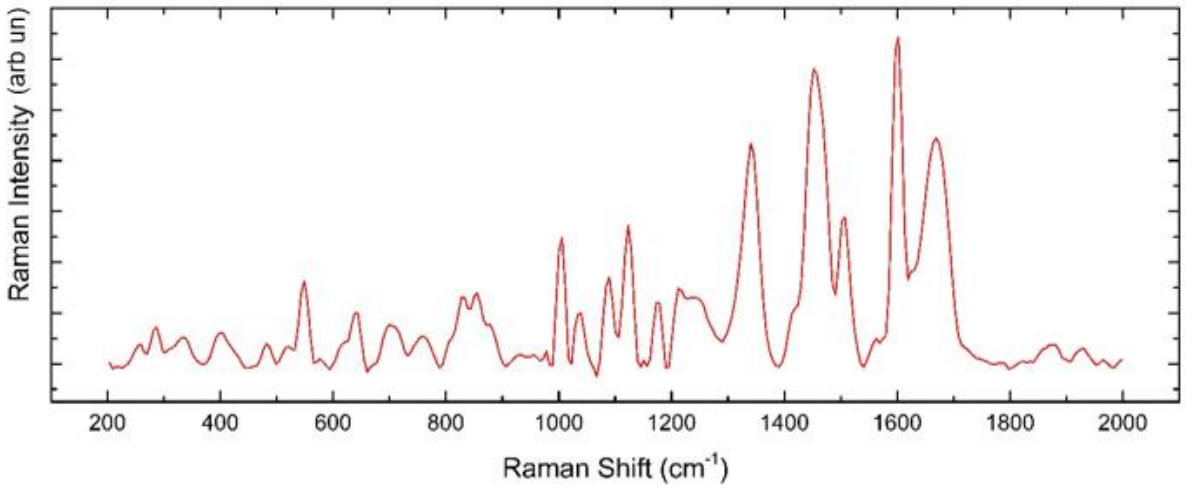
Giallo Luce Solido Powder:



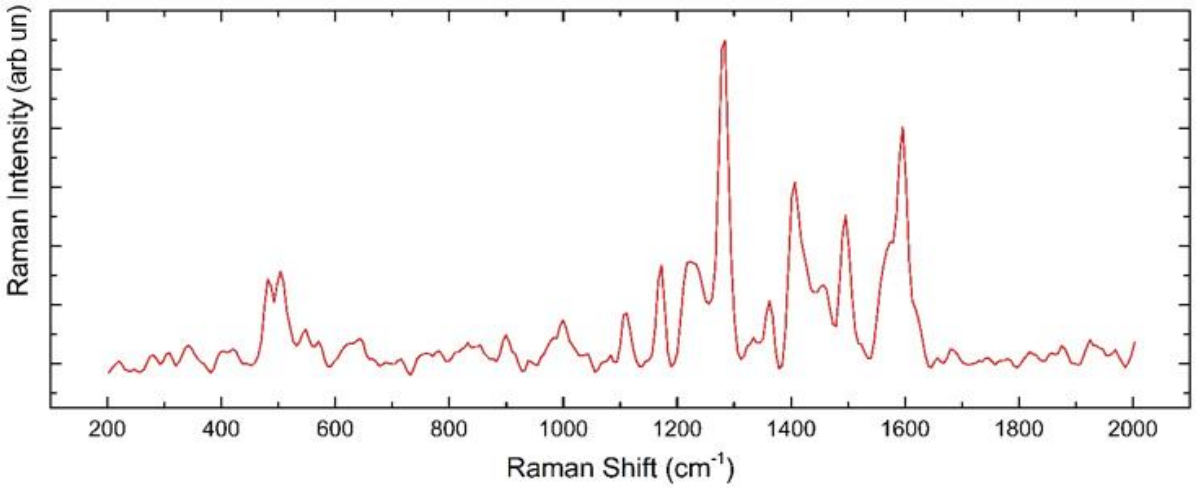
Giallo Novamina R Fibre:



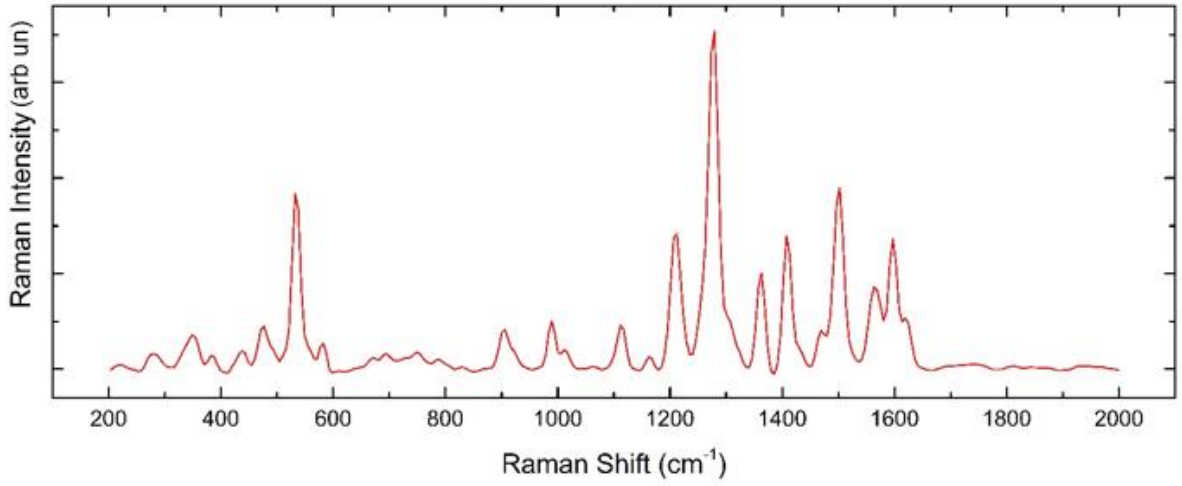
Tartrazina J Fibre:



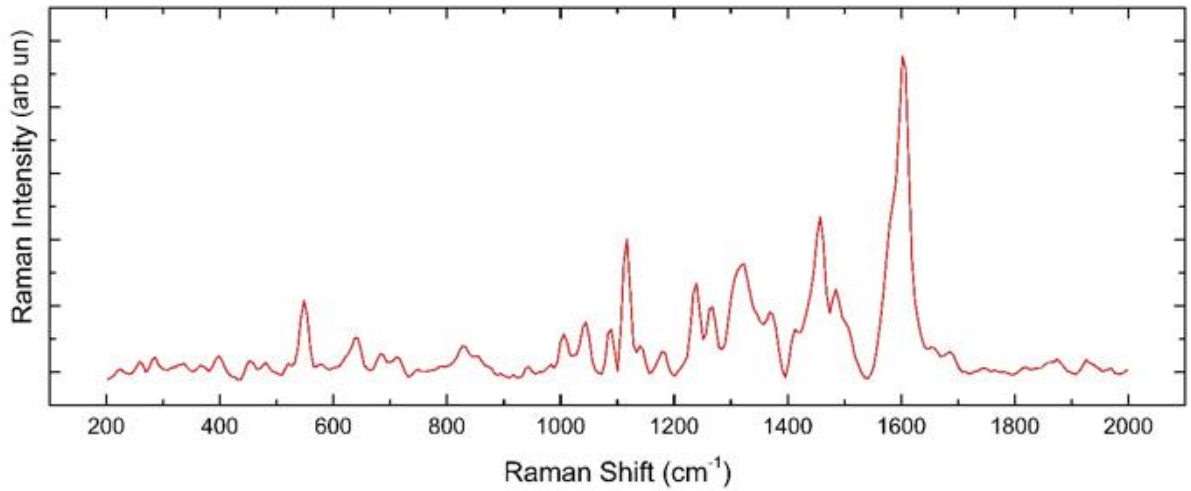
Rosso Amidonaftolo 2G Fibre:



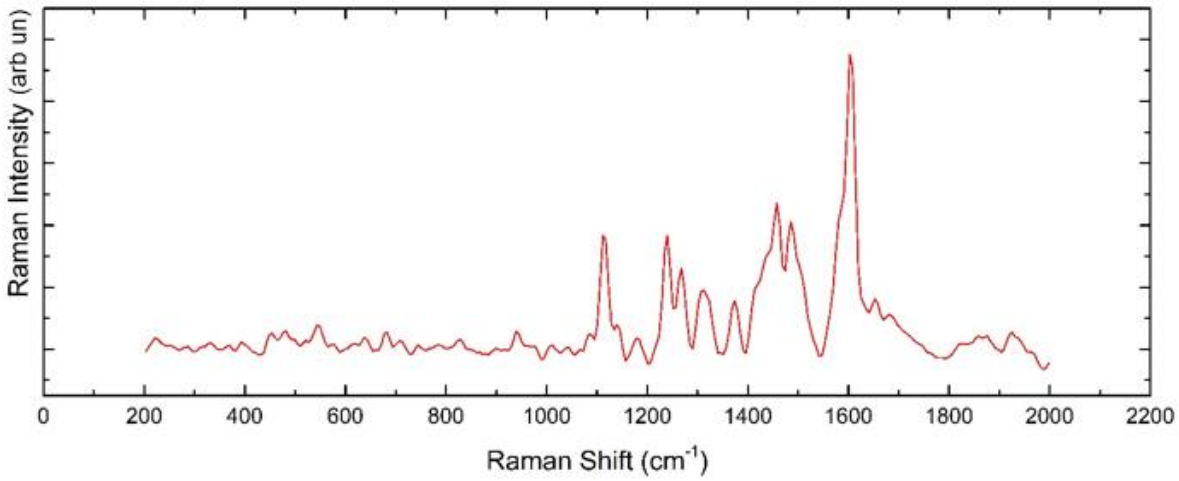
Rosso Amidonaftolo 2G Powder:



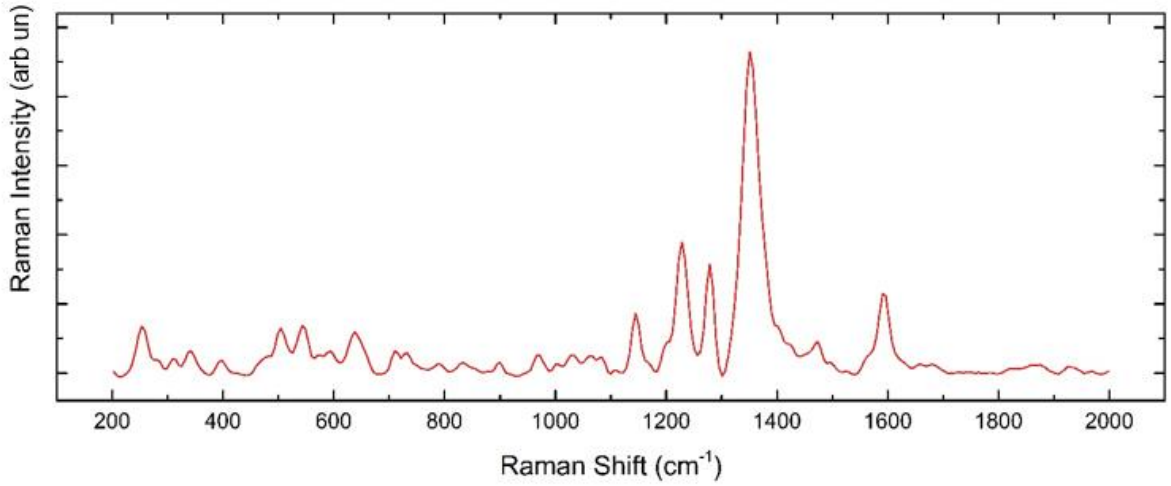
Rosso Italana B Fibre:



Rosso Italana R Fibre:



Rosso Luce Solido BL Fibre:



Rosso Novamina 2G Fibre: