Staining and fluorescent staining techniques for the characterization of binding media within paint cross – sections. Examination of post – Byzantine icons from the National Gallery of Athens – Alexandros Soutzos Museum's collection as a case study.

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ABSTRACT

This paper is a study of the diagnostic capacity of Visible Light Microscopy (VLM - Staining) and Fluorescence Light Microscopy (FLM) for proteinaceous binding media of works of art. The information obtained with this technique on organic compounds is extremely useful in the preliminary studies necessary for diagnosis, to decide on the conservation process and to validate the painter's technique. From another point of view, they are also useful to decide on the methods to be used in analytical chromatographic techniques especially in the case of unique small sample amounts like the one's of works of art.

In the present study reference samples were studied by optical microscopy in order to establish a more in depth understanding of staining mechanisms and create a preliminary database. As a next step authentic samples from two post – Byzantine icons belonging to the National Gallery of Athens- Alexandros Soutzos museum were studied, in order to identify the nature of their binding media.

INTRODUCTION

Staining methods constitute a promising approach to the study of organic materials in icons, like dyes, proteinaceous binding media, drying oils, waxes and resins.

Their main advantage is that they allow the visual localization of analytical information on the layered structure of a paint section. They are a relatively low – cost method that can be proved extremely useful to a museum's trained staff.

Visible light microscopy staining (VLM – staining) has been long –ago introduced in the field of diagnostic methods. Systematic study and appliances of visible light staining techniques on reference samples and samples from works of art were broadly developed in the 1970's. M. Cay, M. Johnson, E. Packard and E. Martin were among the first to introduce innovative staining methods through the use and appliance of various dyes.

Fluorescent staining techniques of cross-sections (FLM) were initially introduced by A. Rinui (1989) and R.Wolbers (1991), as an alternative, more sensitive method for the detection of proteins in paint media. Usually, fluorescent compounds, that have the ability to react selectively with proteins, are used in order to amplify the fluorescence of the proteinaceous binding media. Such compounds are Fluorescamine, Fluorescein Isothiocyanate (FITC), Lissamine Rhodamine (LISSA), and others.

Furthermore B. Ramirez – Barat and de la Vina (2001) reported on characterization of proteins in model samples of paint media by immunofluorescence.

This contribution will discuss the application of VLM and FLM on reference materials and authentic samples from two post – Byzantine icons belonging to the National Gallery of Athens- Alexandros Soutzos museum, in order to identify the nature of their binding media.

EXPERIMENTAL PROCEDURE

Methods and instrumentation

Two methods were used for the characterization of both the reference material and the real icons' samples: Visible Light microscopy (VLM - staining) and Fluorescence Light Microscopy (FLM). In both case the observation was done under a Leica DM/LM microscope equipped with a digital infrared camera DC 300 F and a high pressure mercury lamp (500W).

Observation of cross -sections by VLM

The staining of cross-sections methods takes advantage of the proteins property to bind selectively to some organic colorants, like acid Fuchsine, Noir amide and others. The dyes are directly applied on paint cross sections and their observation is done with reflectance light microscopy.

Observation of cross -sections by FLM

Fluorescence microscopy is a technique by which the fluorescence of materials is observed under an optical microscope equipped with a system of excitation and suppression filters. The fluorescence is caused by excitation of the sections with ultraviolet or visible light. Fluorescent compounds (like Fluorescamine, Fluorescein Isothiocyanate and others) that have the ability to react selectively with proteins are used in order to amplify the fluorescence of the proteinaceous binding media.

A Filter Cube is always used. The filters' cube characteristics that were used for this study are described in *Table 1*

Filter cube (LEICA)	**Filter Cube characteristics**		
	Excitation Range	**Excitation filter**	**Suppression filter**
A	UV	BP 340 -380	LP 425
I3	Blue	BP 450 -490	LP 515
N 2.1	Green	BP 515 - 560	LP 590

Table 1: Filter blocks characteristics

Fluorochrome solutions were applied directly on the cross sections, were given a 10 minute reaction time and were rinsed off each time with the proper solution.

Reference Materials

Staining was applied first on reference egg tempera layers with various pigments. The preparation of reference samples was based on the historical information we have concerning the materials and painting techniques of post Byzantine icons.

The ground layer (of the reference samples) consists of a mixture of Gesso, and rabbit glue (3/2,5 w/w) while the paint layers consists of egg yolk and one of the following pigments: Lead white, malachite, cinnabar and cobalt blue/ ultramarine at a 60% proportion relevantly to the binding media.

Authentic Samples

Cross-sections from two icons of the National Gallery of Athens –Alexandros Soutzos Museum were studied. The icons that were examined are:

A) "Evangelist Lukas is painting the Virgin", end of 16th century.

B) "The Virgin of the Powerful Mantle", 18th century.

Three samples were taken from the icon"Evangelist Lukas is painting the Virgin" and three from "The Virgin of the Powerful Mantle". The positions of the microsampling and the labelling of samples are shown in *Figure 1 and 2.*

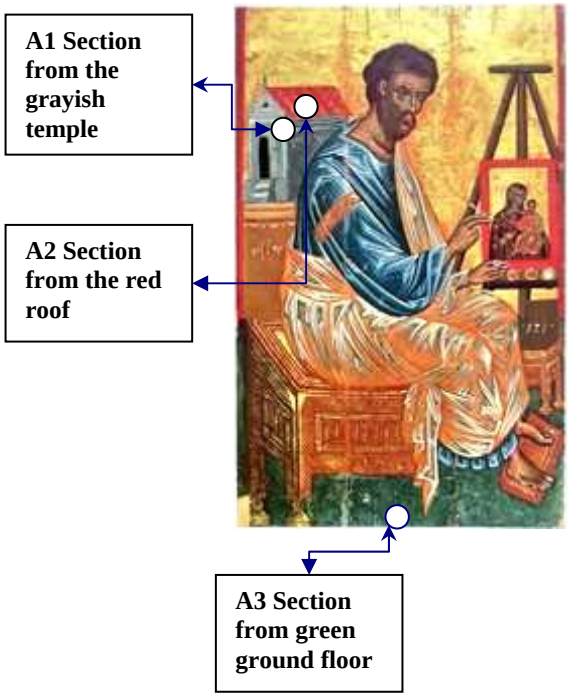

Figure 1: Micro sampling position and labelling of samples taken from the icon "Evangelist Lukas is painting the Virgin"

The detection of proteins in the various layers of the icons was based on the knowledge we acquired from the observation of reference materials.

Colorants and Fluorochromes

The visible light staining of cross - sections from both the reference materials and icons were done by acid Fuchsine and Noir Amide (Napthol Blue black or Amido Black) solutions of various pH(NA_2, $NA_{3,6}$ and NA_7). Both Fuchsine and Noir Amide were purchased by Fluka.

The fluorescent light staining of cross-sections was done by the use of two fluorochromes: Fluorescamine and Fluorescein Isothiocyanate (FITC), which were both purchased by Sigma.

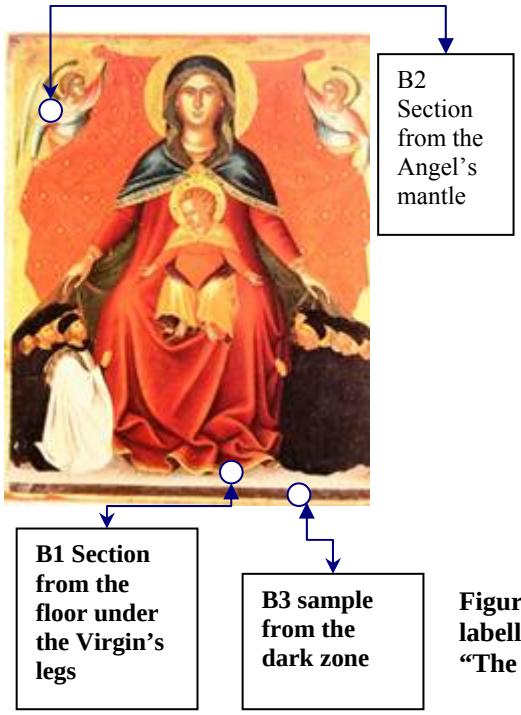

Figure 2: Micro sampling position and labelling of samples taken from the icon "The Virgin of the Powerful Mantle"

RESULTS

Results of the reference materials

A) Visible Light Microscopy (VLM– Staining)

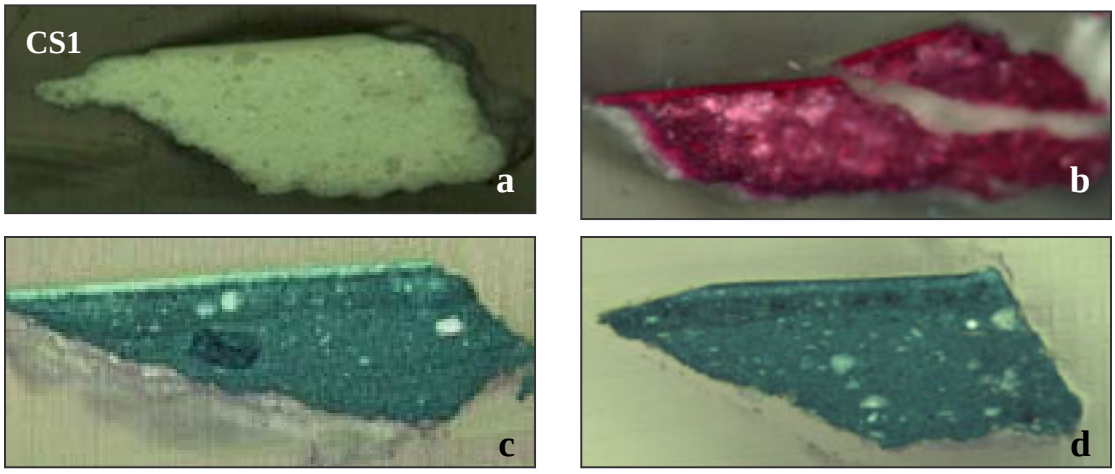

Figure 3: Photomicrographs of reference paint layers (VLM- Staining)

a) **Cross section CS1,** (10x) from bottom to top: 1) ground, 2) paint layer of egg yolk with lead white,
b) **CS1** under treatment with acid fuchsine, (10x)
c) **CS1** under treatment with Noir amide pH 7, (10x)
d) **CS1** under treatment with Noir amide pH 2, (10x)

During treatment of the reference materials with various colorants and observation with visible light microscopy various remarks were made.

Sample CS1 (Figures 3a – 3d): Treatment with acid Fuchsine colored intensely both the ground and the lead white paint layer of the sample (Figure 3b). On the other hand Noir Amide functioned differently depending on its pH. Treatment with Noir amide pH 7 colored intensely the ground layer of the sample and gave only a faint coloration to the paint layer (Figure 3c). Treatment with Noir amide pH 2 colored intensely both the ground and paint layer (Figure 3d). These remarks are in accordance with E. Martin's results that show that Noir amide[i] with pH 2 colors egg yolk better while Noir amide with pH 7 colors better animal glue.

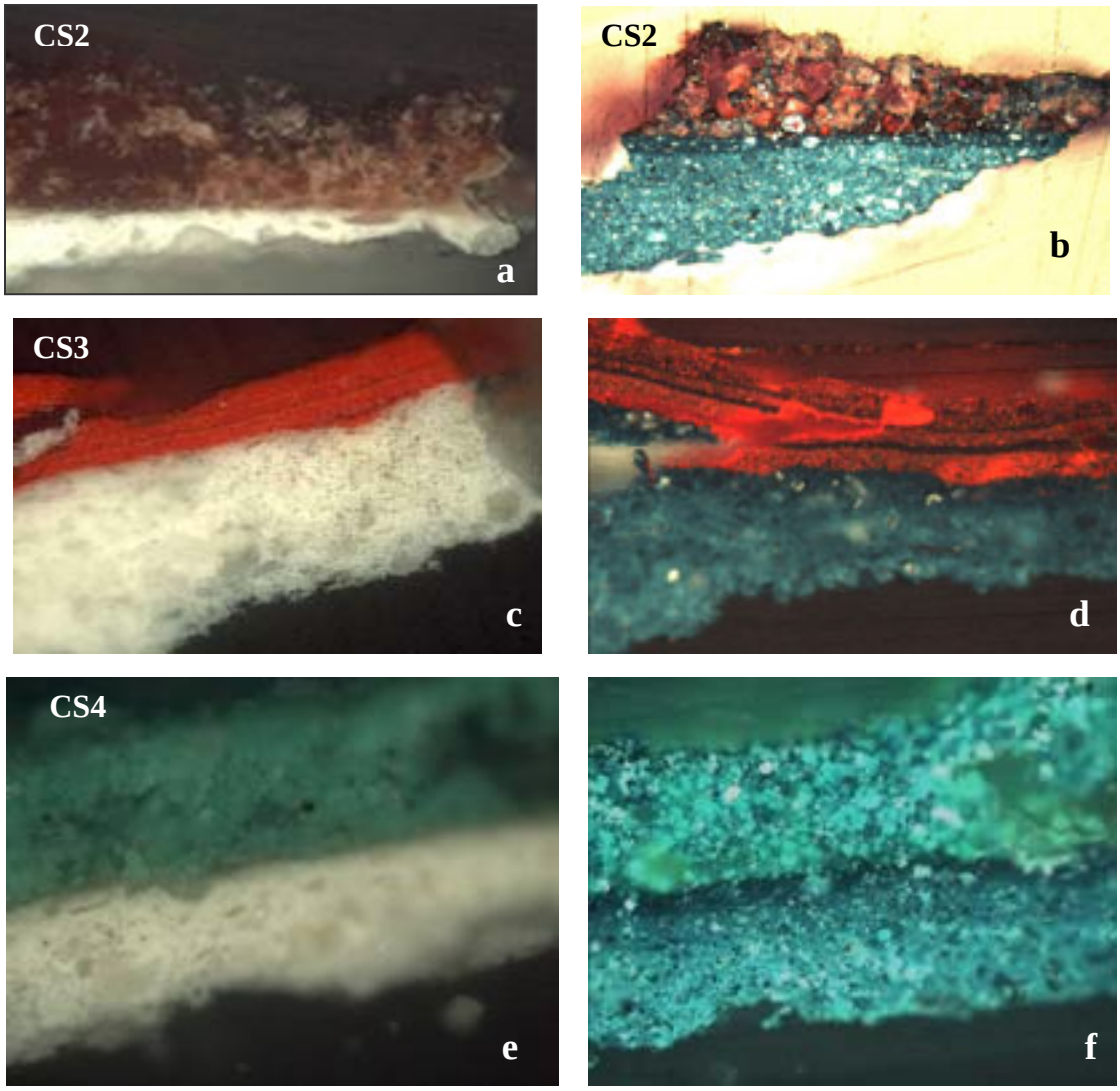

Figure 4: Photomicrographs of reference paint layers (VLM- Staining)

a) **Cross section CS2**, (50x) from bottom to top: 1) ground layer, 2) paint layer of egg yolk with red lake (madder)
b) **CS2** under treatment with Noir amide pH 3,6, (20x)
c) Cross section **CS3**, (20x) from bottom to top: 1) ground layer 2) ground layer rich in animal glue, 3) paint layer of egg yolk and cinnabar, 4) paint layer of egg yolk and cinnabar, 5) paint layer of egg yolk and cinnabar
d) **CS3** under treatment with Noir amide pH 3,6 (20x)
e) **Cross section CS4,** (20x) **from** bottom to top: 1)ground layer, 2) ground layer rich in animal glue, 3) paint layer of egg yolk and malachite
f) **CS4** under treatment with Noir amide pH 3,6, (20x)

Sample CS2 (Figures 4a & 4b): In the case of the sample with the madder paint layer, treatment with Noir amide pH 2 coloured intensely both layers. The intense

coloration of the binder made obvious the grains of aluminium oxide on which the dye of madder is absorbed (Figure 4b).

Sample CS3 (Figures 4c & 4d): In this case treatment with Noir Amide pH 3,6 coloured intensely the ground layer and less intensely the paint layer of cinnabar. The coloration with NA $_{3,6}$ allowed the localisation of the proteins of egg yolk in the paint layers. This way it became obvious that the proteinaceous part of egg yolk tends to gravitate to the bottom of its paint layer. Nevertheless, it can also be seen distributed in the whole paint layer area.

Sample CS4 (Figures 4e & 4f): In the case of this sample with the malachite paint layer treatment with Noir amide pH 3,6 gave an intense coloration of the ground layer and an almost unobservable result at the paint layer due to the chromatic similarity of the colorant and the paint layer's pigment.

B) Fluorescence Light Microscopy (FLM)

1) Autofluorescence

The observation of the reference samples' autofluorescence leads to the following remarks:

Sample CS1 (Figure 5a): The paint layer of egg yolk with lead white fluoresces intensely when irradiated with ultraviolet giving a yellow-bluish fluorescence. Its ground layer fluoresces less intensely in comparison with the paint layer giving a bluish fluorescence. Furthermore, when this sample is compared with CS5 (fig.5b) which has animal glue (as a binder) in the paint layer it becomes obvious that animal glue gives always a bluish fluorescence while egg yolk a yellow- bluish one.

Sample CS2 (Figure 5c & 5d): The sample of madder lake when irradiated with UV and observed with Filter cube A, presents an intense fluorescence at both the paint and the ground layer (bluish fluorescence). The total orange-yellowish fluorescence of the paint layer is due to the binder and the organic pigment. A partial diffusion of the paint layer in the ground layer is also observed.

The same sample when observed with Filter cube N 2.1 presents an intense red colored fluorescence at the paint layer, which is due to the madder lake and not to the binder. The ground layer does not fluoresce when observed with this filter.

Sample CS3 (Figure 5e): The reference sample of cinnabar when observed with Filter cube A, presents an intense fluorescence at the ground layer, and a localized fluorescence in the paint layers. The fluorescence of the paint layers allows the localization of the proteinaceous part of egg yolk. In addition, the chromatic

difference that is observed in the fluorescence of the paint and ground layer made it possible to observe a diffusion of the paint layer in the ground layer.

Sample CS4 (Figure 5f): The reference sample of malachite presents a light yellowish fluorescence, which makes possible the localization of the binder in the paint layer. Furthermore, the fluorescence of the ground layer is bluish.

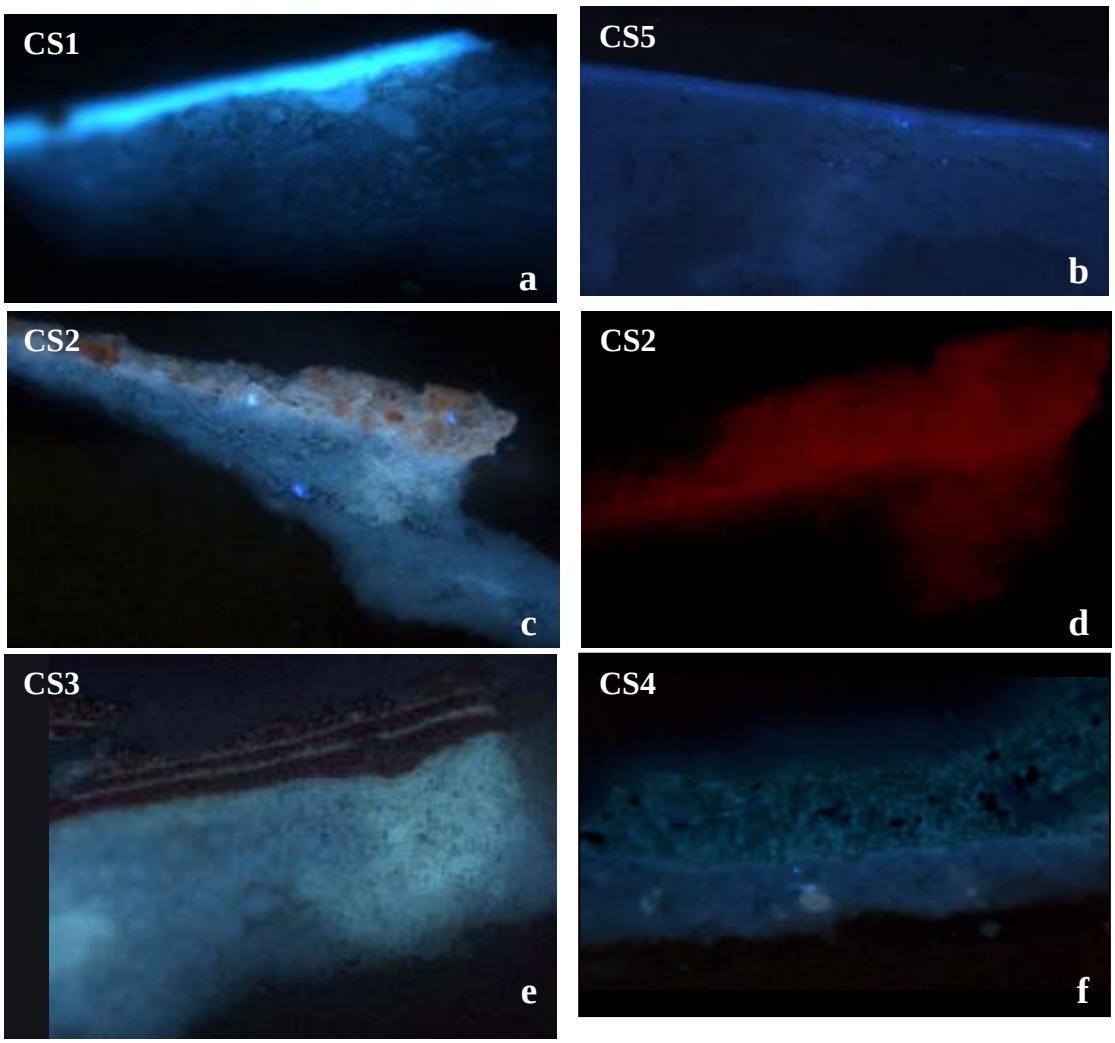

Figure 5: Photomicrographs of reference paint layers (FLM)

a) **CS1** under UV, (20x) – Filter cube A
b) Cross Section **CS5,** (10x) from bottom to top: 1) plaster layer, 2) white paint layer (chalk with animal glue) – Filter cube A
c) **CS2** under UV, (20x) – Filter cube A
d) **CS2** – observation with filter cube N 2.1, (50x)
e) **CS3** under UV, (20x)– Filter cube A
f) **CS4** under UV, (20x) – Filter cube A

2) FLM - staining

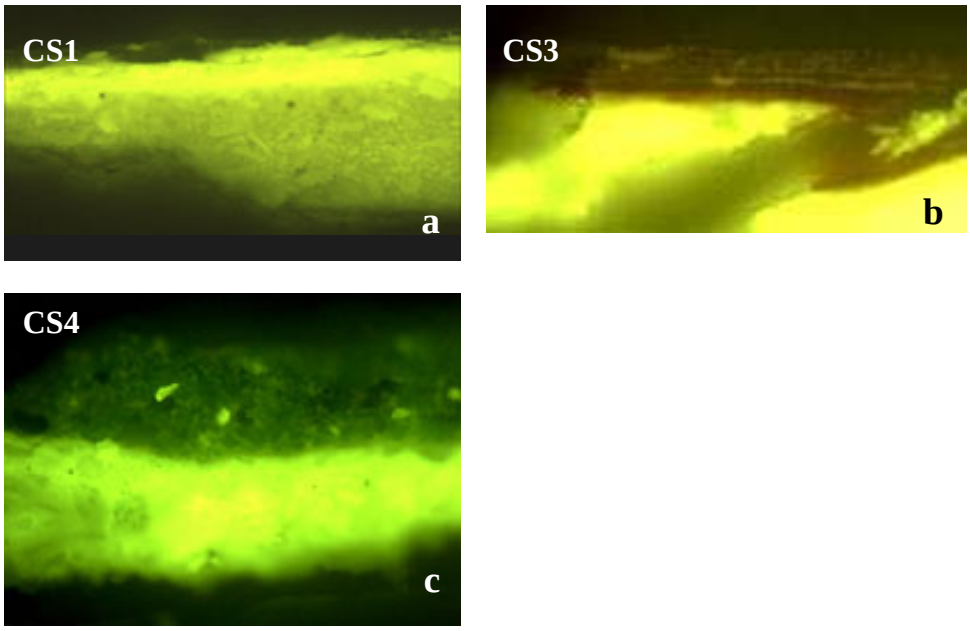

Figure 6: Photomicrographs of reference paint layers under treatment with FITC – Filter cube I3

a) CS1, (20x)

b) CS3, (20x)

c) CS4, (50x)

Sample CS1 (Figure 6a): Both the fluorescence of the paint layer and the ground layer is reinforced when compared with the results of autofluorescence. The color of fluorescence observed is intense yellow at the paint layer of lead white and intense yellowish –green at the ground.

Sample CS3 (Figure 6b): The treatment of the cinnabar reference sample with FITC leads to an intense yellowish fluorescence of the ground layer and an intense yellowish fluorescence of the paint layer.

Sample CS4 (Figure 6c): The treatment of malachite reference sample with FITC leads to an overall reinforcement of the fluorescence of the binding media.

3) Combination of VLM staining and FLM

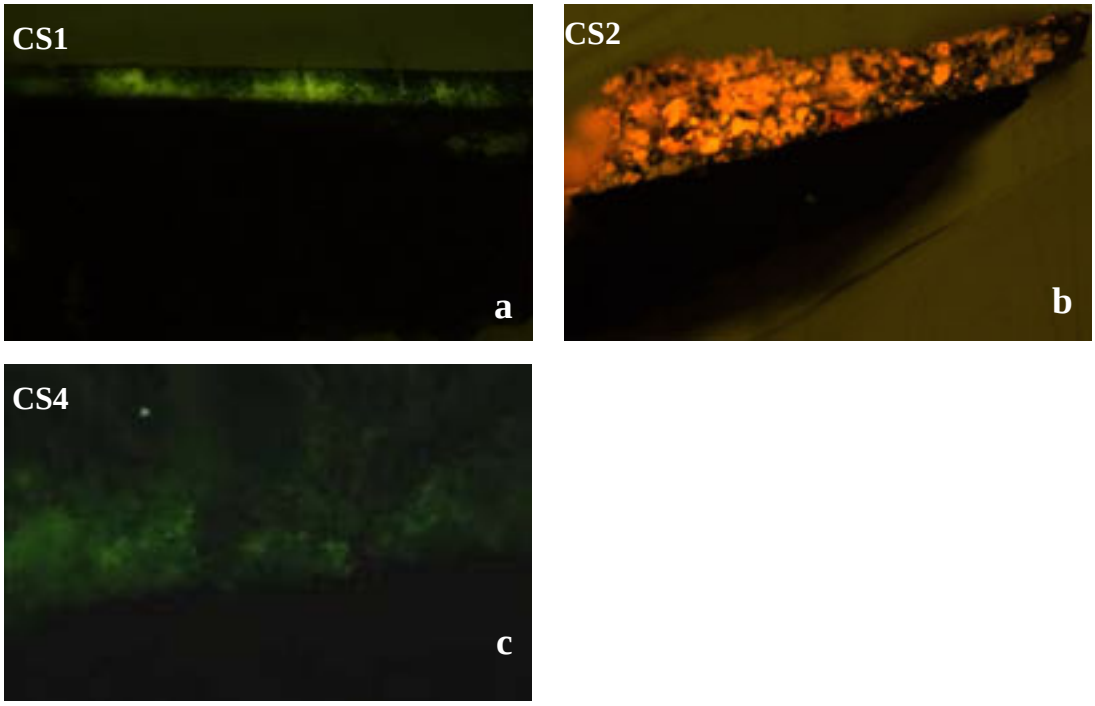

Figure 7: Photomicrographs of reference paint layers under treatment Noir Amide pH 2 and observation with filter I3

a) CS1, (50x) b) CS2, (20x), c) CS4, (50x)

A new method was applied having as an aim the differentiation of pure proteins from emulsion type binders (egg yolk or protein-oil emulsions). The general observation is that when the proteins of a sample are bonded with a colorant and are afterwards observed under a filter cube that does not allow the fluorescence of that colorant, then it becomes possible to detect the fluorescence of then non – proteinic elements of the binders.

Sample CS1 (Figure 7a): This reference sample when observed with this method, gives a yellow –greenish fluorescence at the paint layer due to the presence of egg yolk lipids, while the ground layer is depicted as a dark black area since it doesn't contain any lipids at all.

Sample CS2 (Figure 7b): The observation of the madder lake's reference sample makes it possible to localize the dispersion of proteins in the paint layer area (they have the picture of dark spots) and to see the organics pigments fluorescence.

Sample CS4 (Figure 7c): The observation of the malachite reference sample with this method presents similar results to sample CS1. A difference is only observed at the intensity of the paint layer's fluorescence, which is fainter.

Results of the real samples

1) **"Evangelist Lukas is painting the Virgin"**

Sample A1

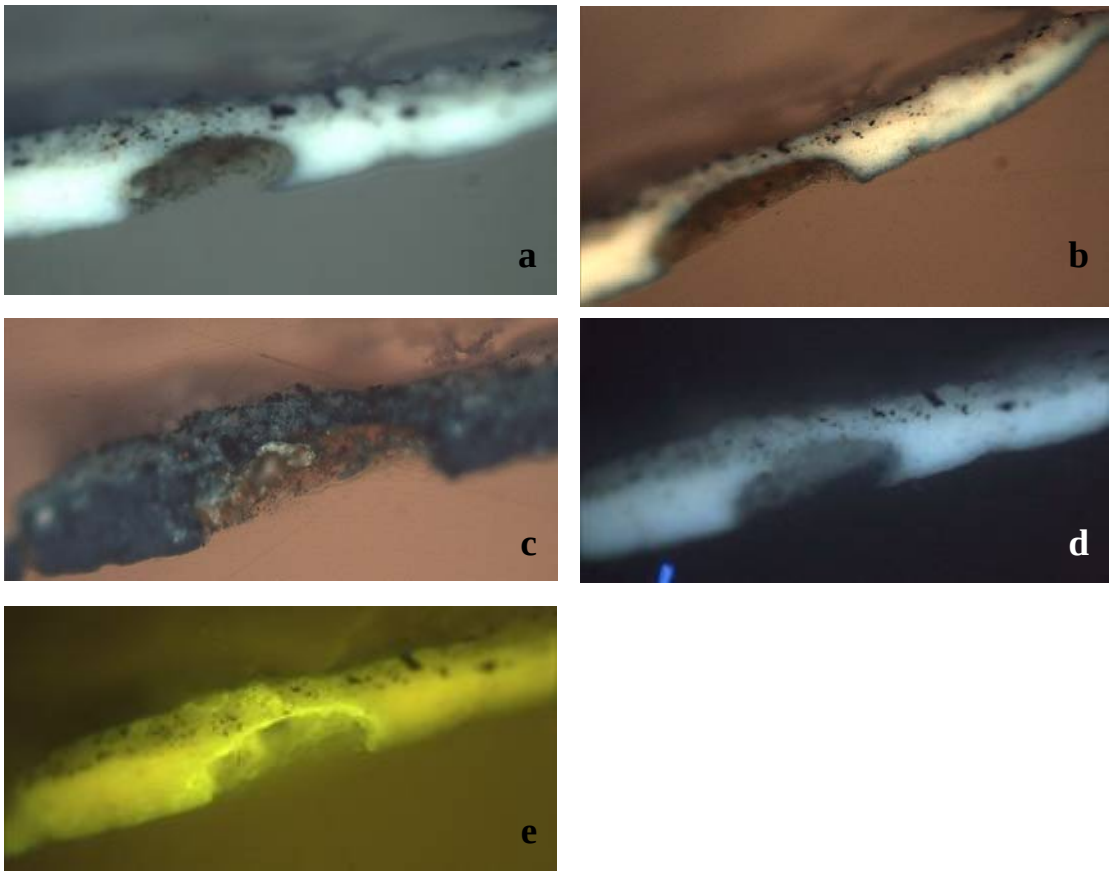

Figure 8: Photomicrographs of sampleA1 from the icon "Evangelist Lukas is painting the Virgin"

a) Cross section A1, (20x) from bottom to top: 1) White paint layer, 2) white paint layer with various pigments (black, brown, ochre)
b) A1 under treatment with Noir amide pH 7, (20x)
c) A1 under treatment with Noir amide pH 2, (20x)
d) A1 under UV, (20x) – Filter cube A
e) A1 under treatment with FITC, (20x) – Filter cube I3

Sample A1 (Figure 8a- 8e): sample A1 was colored with Noir amide of various pH (NA_2 and NA_7). The coloration with Noir Amide pH 7 (Fig.8b) had as a result a very faint coloration of the paint layers while on the other hand the treatment with Noir

amide pH 2 (Fig. 8c) gave an intense coloration at both layers. These observations are in accordance with the results of the reference sample of lead white and lead to the conclusion that probably egg yolk is the medium of the paint layer since it presents a more intense coloring when treated with Noir Amide pH 2.

The appliance of FLM techniques on the same sample revealed an equal intensity and an even chromatic fluorescence at both layers which is indicative of similarity in proteinaceous content.

Sample A2

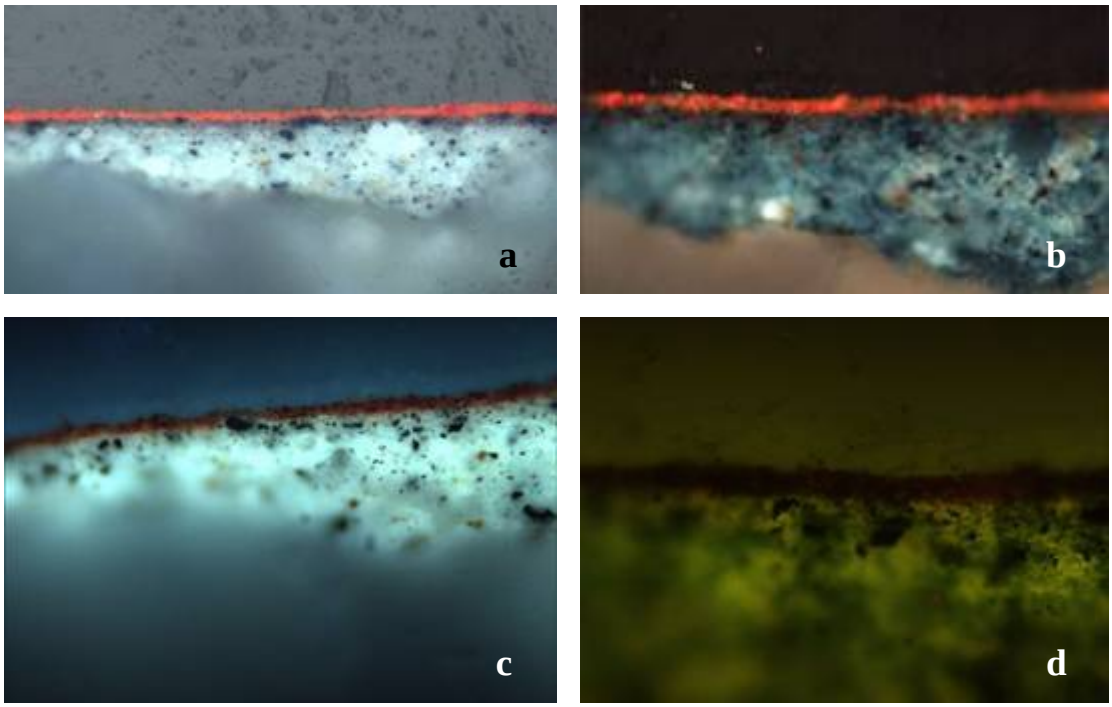

Figure 9: Photomicrographs of sample A2 from the icon "Evangelist Lukas is painting the Virgin"

a) Cross section A2, (20x) from bottom to top: 1) White paint layer with various pigments, 2) Very thin brownish layer, 3) Red paint layer
b) A2 under treatment with Noir Amide pH 2, (50x)
c) A2 under UV– Filter cube A, (50x)
d) A2 under treatment with Noir Amide pH 2 and observation with Filter cube I3, (50x)

Sample A2 (Figures 9a-9d): Sample A2 was treated with Noir Amide pH 2 and gave an intense coloration. At a later observation of the same colored sample with Filter cube I3, it became obvious that all the paint layers where colored with Noir amide. In addition in the white paint layer (with the various pigments), a yellow –greenish fluorescence is observed, due to lipid content.

The observation of the sample under UV enhances the differentiation of the various paint layers and makes it possible to distinguish a small line that gives a yellow fluorescence. A strong white – yellowish fluorescence is also observed throughout the whole sample indicative of rich protein content. Furthermore, the fluorescence seems to be less intense but more yellowish in the red paint layer.

Sample A3

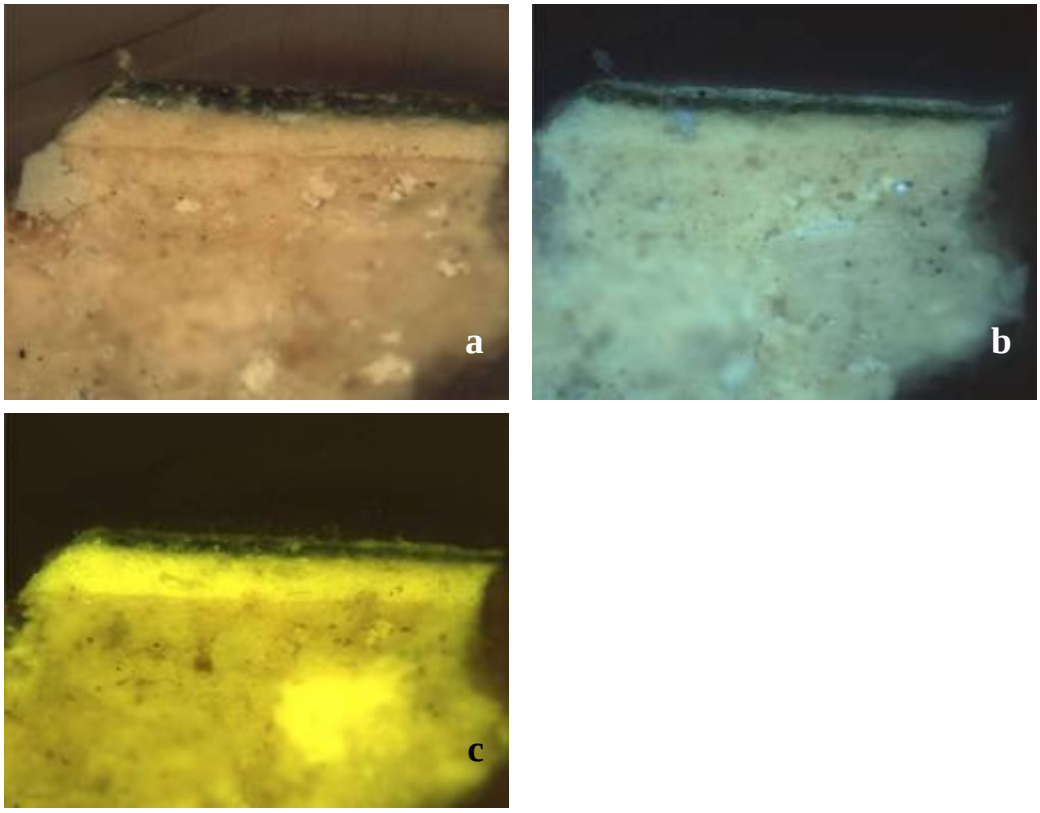

Figure 10: Photomicrographs of sample A3 from the icon "Evangelist Lukas is painting the Virgin

a) Cross section A3, (10x), from bottom to top: 1) Ground paint layer, 2) Ground layer with thinner granules, 3) Paint layer
b) A3 under UV, (10x) – Filter cube A
c) A3,(10x) – Filter cube I3

Sample A3 (Figures 10a – 10c): Sample A3 was observed only under UV with Filter Cube A and with Filter Cube I3. The observation of autofluorescence revealed the existence of two green paint layers that fluoresce differently, probably due to different pigment content. Observation with Filter cube I3 made also obvious the existence of two different types of grounds.

2) "The Virgin of the Powerful Mantle"

Sample B1

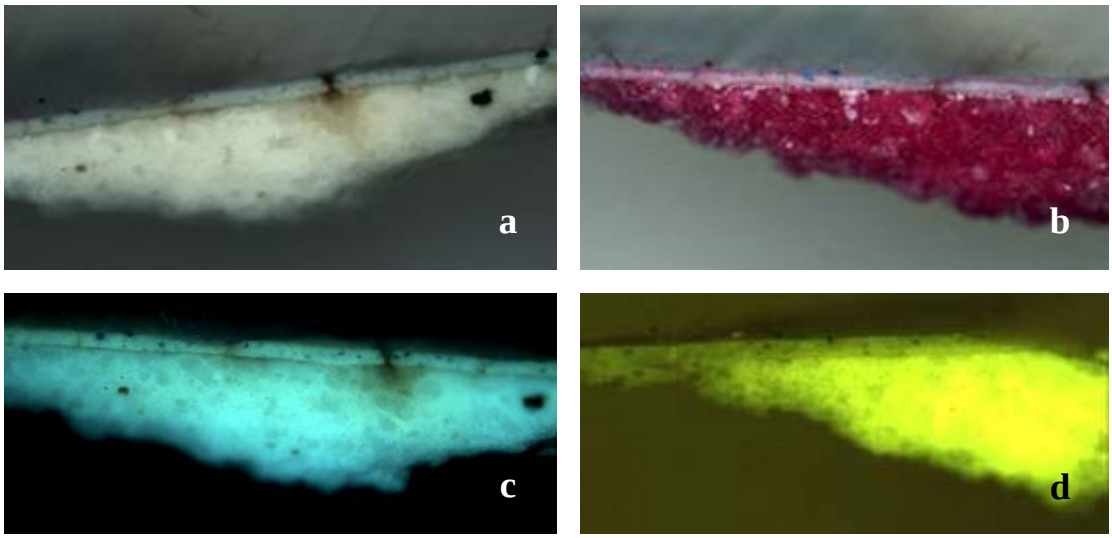

Figure 11 photomicrographs of sample B1 from the icon "The Virgin of the Powerful Mantle"

a) Cross section sample B1, (10x) from bottom to top: 1) ground layer, 2) white layer containing some blue grains of high reflectivity
b) B1 under treatment with acid Fuchsine, (10x)
c) B1 under UV – Filter cube A, (10x)
d) B1 under treatment with FITC, (10x) – Filter cube I3

Sample B1 (Figures 11a –11d): Treatment of sample B1 with acid fuchsine gave an intense coloration of the ground indicative of high protein content and a light coloration of the paint layer, indicative of low protein content.

The sample under UV (filter cube A) fluoresces strongly giving a yellow – bluish fluorescence at the paint layer, indicative of egg yolk presence in the layer. The ground layer gives a more bluish fluorescence.

Treatment of the sample with FITC enhances the fluorescence of the layer of the ground, revealing a high protein content. The paint layer fluoresces less intensely revealing a lower protein content. Both results the FLM results of the ground and the paint layer are in accordance with the results of the treatment of the sample with acid fuchsine.

Sample B2

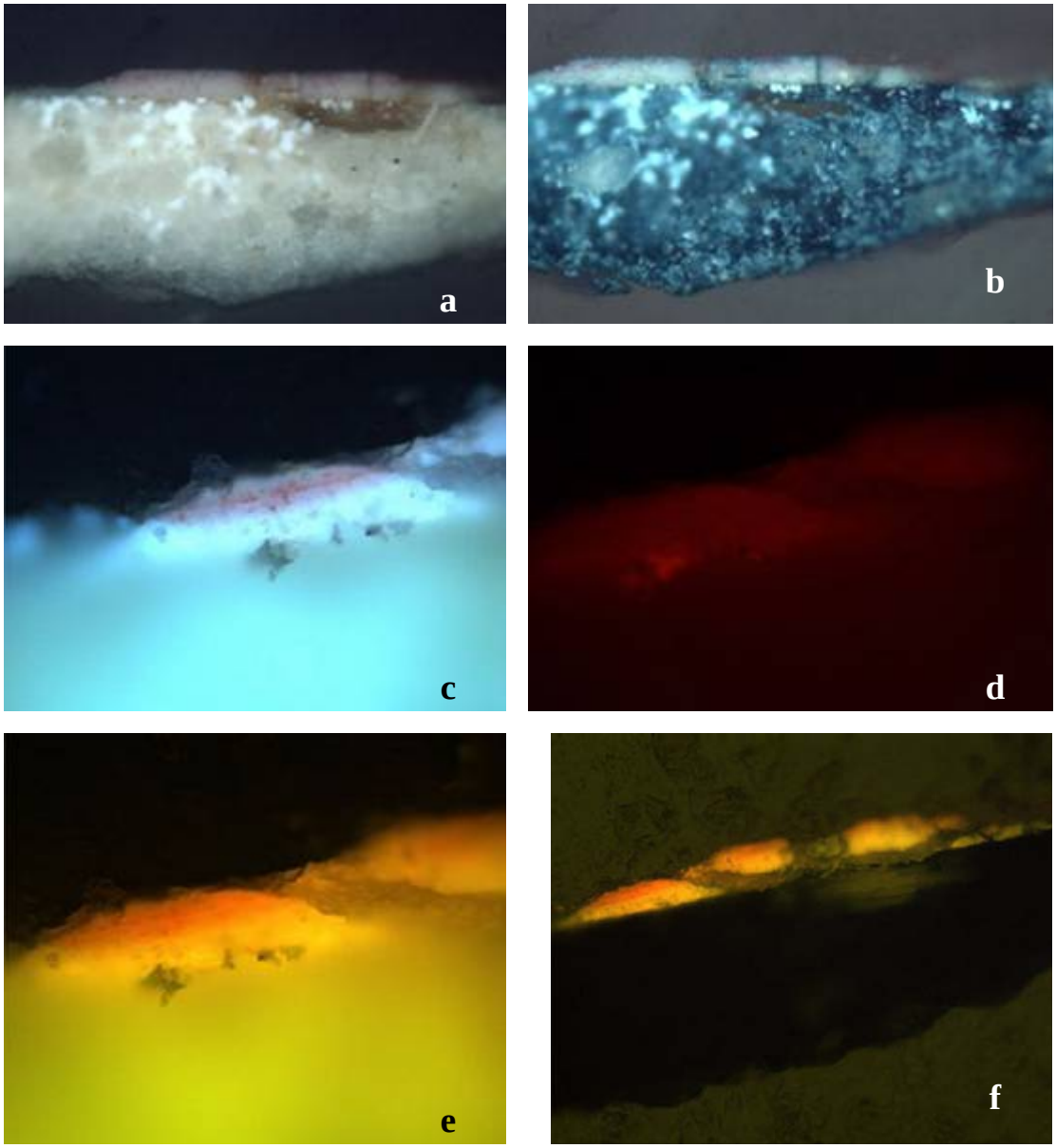

Figure 12: Photomicrographs of sample B2 from the icon "The Virgin of the Powerful Mantle

a) Cross section of B2 (20x), from bottom to top: 1) ground layer, 2) white paint layer, 3) Pink paint layer
b) B2 under treatment with Noir amide pH 2**,** (20x)
c) B2 under UV (50x) – Filter cube A
d) B2 observation with Filter cube N 2.1,(50x)
e) B2 under treatment with FITC, (50x) – Filter cube I3
f) B2 under treatment with Noir amide pH 2, (20x) – Filter cube I3

Sample B2 (Figures 12a – 12f): Sample B2 was treated with Noir Amide pH 2 and gave an intense coloration at the ground layer and a very faint coloration at the paint layer (fig. 12b). Observation of the colored (with Noir amide) sample with Filter-cube I3 (fig. 12 f) has as a result a strong yellow fluorescence of the paint layer, which is only topically and scarcely interrupted with bluish colored areas. The ground layer doesn't fluoresce and is depicted as a dark black area. According to the results of the reference material this reveals the existence of a pure protein at the ground layer (probably animal glue) and of non-proteinaceous (or of very low proteinaceous content) binding media at the paint layer.

The observation of the sample under UV revealed a bluish fluorescence throughout the sample. Furthermore, treatment with FITC had a result an equal in intensity yellowish fluorescence throughout the sample.

Observation of the sample with Filter cube N 2.1 revealed that the pink paint layer contain a lake since only lakes autofluoresce when observed with this filter cube.

Sample B3

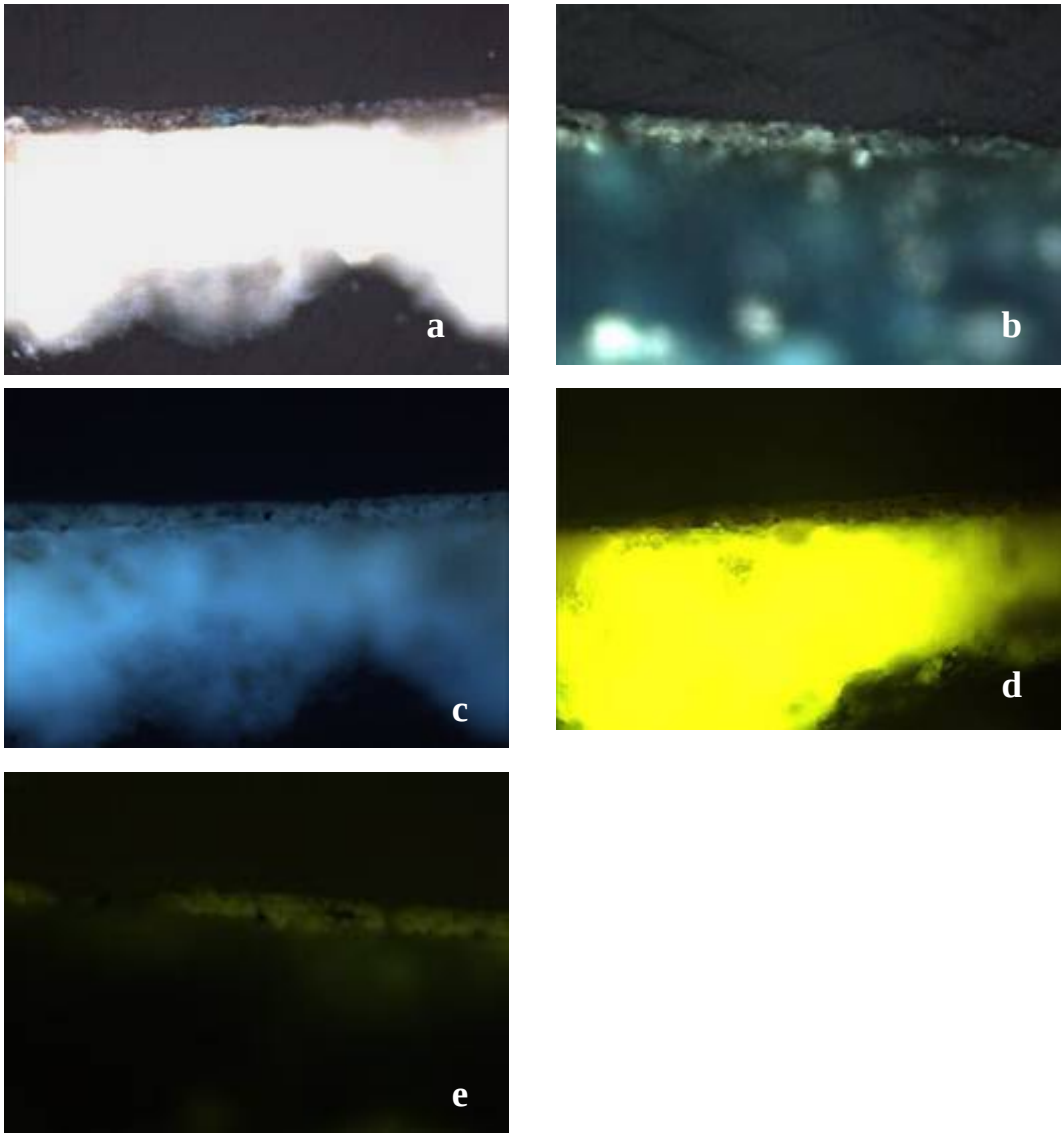

Figure 13: Photomicrographs of sample B3 from the icon "The Virgin of the Powerful Mantle"

a) Cross section of sample B3 (50x), from bottom to top: 1) ground layer, 2) brown layer with various grains.
b) B3 under treatment with Noir Amide pH 7(50x)
c) B3 under UV(20x) – Filter cube A
d) B3 under treatment with FITC (20x)– Filter cube I3
e) B3 under treatment with Noir amide pH 7 (50x) – Filter cube I3

Sample B3 (Figures 13a – 13e): The visible light microscopy revealed the presence of a thin brown layer consisting of various pigments and a thick ground layer. Treatment of the sample with Noir Amide pH 7 gave a strong coloration of both

layers (fig. 13b). The remarkable point here is that the brown paint layer became light-colored after treatment with Noir amide. Observation of the same sample (after treatment with Noir Amide) with filter cube I3 (fig. 13e) gave topically a greenish fluorescence at the paint layer due to the presence probably of the lipids of egg yolk. On the other hand, the ground layer doesn't fluoresce and is depicted as a dark black area. According to the results of the reference material this reveals the existence of a pure protein at the ground layer (probably animal glue) and the existence of natural or synthetic emulsion at the paint layer.

Observation of the sample under UV gave a strong yellow- bluish fluorescence at the paint layer and a more bluish one at the ground layer. According to the results of the reference samples these observations are indicative of the presence of egg yolk and animal glue respectively. One more notable point here is that the some of the inorganic pigments in the brown layer seem to give a white fluorescence.

CONCLUSIONS

Our research work, allowed us to:

1. To determine the possibilities and the limits that's involved in the use of traditional methods for detection of old proteinaceous (colorations by Fuchsine S and Noir Amide).
2. Having determined the limits of traditional colorations we chose to apply methods of fluorescence (autofluorescence and fluorescence of dyes binding proteins) that do not present the disadvantages of traditional methods.
3. To develop a new methodology based on the combination of the two methods and which in many cases leads to the identification of the binding media.

At a first step all the afore-mentioned methods were tested on reference materials and at then a second phase on real samples.

The systematic study of reference materials with the VLM technique lead us to the following conclusions:

a) The method of staining proteins with acid fuchsine presents great sensitivity

b) The method of staining proteins with Noir Amide Solutions of pH 7 and pH 2 allows differentiating between egg yolk and animal glue. Nevertheless, the usefulness of staining with Noir Amide depends on the color of the pigment. For example it is useful in the case of red pigments but not in the case of blue or dark green pigments.

c) These microchemical tests give satisfying results, but do not allow the identification of emulsion type binding media (for example egg or egg yolk – linseed oil)

The method of autofluorescence presents advantages in reference with the traditional methods of coloration, which has to do with the tracking sensitivity of proteins.

The method of identifying proteins with fluorescent dyes binding, presents the advantage to be more sensible than the traditional colorations, allowing the tracking of small amounts of proteins. The color of the inorganic pigments may affect the intensity of fluorescence.

By combining these two methods we may often distinguish the emulsions from pure proteins. Tests with coloration with Noir amide and afterwards observation at Filter cube I3 leads to the differentiation of pure proteins (like animal glue, casein) and emulsions (like egg yolk). The first ones' are presented as dark areas while the second as green areas due to the natural fluorescence of the lipids contained.

By these methods we also detected the diffusion of binding media from one layer to the other. This diffusion is caused either by the restoration cleaning process or by the absorption of the binding media in the intercourse during the execution of the painting.

The advantage of these methods are:

a) A very small amount of sample is required

b) The cost of maintaining and the consumables are much cheaper in comparison with the ones needed in other methods

But though they may seem easy to realize, these techniques demand the aid from a chemist to explain the results. Also for explaining the results, a deep knowledge of the construction techniques of works of art is needed in order to construct reference materials that are as similar as possible with the works of art (to be studied).

The study with micro-chemical test is only a small part of the whole technical study of works of art.

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[i] There is also the case of Noir Amide pH 3,6 which causes an equal in intensity coloration of both the paint and the ground layer.