Study of the aging of binders in proteinaceous media by high performance liquid chromatography

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Abstract. The technique for identification of proteinaceous binding media (egg yolk, animal glue and casein) based on the analytical technique Pico-Tag® and high performance liquid chromatography (HPLC) was implemented at the *Laboratório de Conservação e Restauro José de Figueiredo (LCR)*. According to this method, samples are hydrolyzed with hydrochloric acid; amino acids are derivatized with phenylisothiocyanate and separated, detected and analyzed by HPLC. Using this method, accelerated aging tests of paints were applied to study the influence of light, temperature, moisture and natural pigments in the aging process of proteins in tempers was determined. The results indicate that proteinaceous binding media are very stable, particularly the glue and casein media, not degradating considerably with time by the action of the aging factors. The egg yolk media shows chromatic changes along the aging, but probably these are not due to proteins but are related to other media components, such as lipids. Although pigments do not have, in general, a large effect on temper aging, they can slightly accelerate the process of degradation of proteins and perturb their identification. It is known that copper pigments interfere with the identification of tempers. So a methodology was studied, using EDTA, to inhibit the effect of some pigments in the characterization of tempers. It was concluded that the method used (addition of 0,2 M Na_2EDTA in a buffer ammonia/ammonium chloride) was not adequate, so more studies need to be done.

Keywords. Proteinaceous media, Egg yolk, Animal glue, Casein, Picotag+HPLC, Accelerated aging.

Introduction

The conservation and protection of our cultural heritage is essential to ensure that future generations have the opportunity to appreciate artistic masterpieces. Science, in turn, can help to understand the nature of materials used by the artists, assisting in the choice of procedures for storage. As far as paintings are concerned, the knowledge of chemical constituents may help conservators and art historians: i) giving information on the provenance of the objects, (ii) assisting the study of the technique of the artist, (iii) improving the conservation/restoration.[1]

A painting consists usually of several layers: support (wood, canvas, paper, etc.), ground layer (gypsum or chalk), one or more paint layers and in some cases a final varnish layer. Proteinaceous binding media are often used in a technique known as “temper”. The identification of protein binders such as egg, glue or gelatin, and casein reveal the original intent of the artist. Therefore, it is necessary to identify correctly the type of proteinaceous binding media used in a work of art. Nevertheless, this may be difficult due to the presence of other materials, such as pigments, and by aging under the influence of factors such as radiation, temperature and moisture.

To identify proteinaceous binding media colorimetric and spectroscopic techniques are often used, which allow the detection of proteins, but are not specific to each type of protein. To achieve this specificity is possible by using several techniques. In this study, a method for identification of proteinaceous binding media using a Picotag system + HPLC methodology coupled with UV detector[2, 3] for amino acid analysis was implemented. This is a popular method due to its reproducibility, flexibility, versatility and straightforward implementation in works of art.[2] The Picotag system consists in the analysis of amino acids in its simplest form. The proteins or peptides are hydrolyzed into their amino acid constituents by exposure to hydrochloric acid for 24 hours at 110ºC. The method is thus independent of form or function because the protein is completely degraded. The hydrolysis is followed by chromatographic separation of free amino acids. The chromatography requires the amino acid derivatization so they can be retained and detected under ultraviolet (UV) or visible (Vis) light. In this procedure, amino acids react with phenylisothiocyanate (PITC) to form PTC-amino acids that can be separated, identified and quantified by HPLC, at 254 nm.[4, 5, 6] Finally, the amino acid amount is determined and compared with protein standards to identify the proteinaceous binding media.

In addition to the implementation of the above technique, the influence of pigments in the degradation of proteins by

various types of aging was analyzed as well as the possibility of using EDTA as an agent to inhibit the influence of pigments in the identification of tempers.

In a painting, there are chromatic changes along aging. To complement this study, colorimetric analysis were also carried out to analyze the changes of color by action of various types of aging, to check if these changes are related with protein degradation. The color of a surface is the result of selective reflection of light by particles of pigments on the surface, and the chromaticity a measure of color quality. Thus, the most comprehensive method of measuring the color is to measure the reflection of the surface as a function of wavelength. This was done using a spectrophotometer which uses the coordinates system CIELAB (L*, a*, b).[7]

Experimental Section

This work comprised four parts. In the first part, temper films were prepared with different proteinaceous binding media to be analyzed. In the second part, the Picotag technique was implemented. In the third part, the degradation of proteins was studied, testing different types of artificial aging. Finally, in the fourth part the use of EDTA as an agent to inhibit the influence of pigments was evaluated.

Temper preparation

The preparation of tempers, paints and grounds was based on traditional recipes. The egg yolk temper is simply the yolk diluted in water (1:1).[8] The gelatin temper is a solution of 15,00 g of gelatin in 150 ml of hot water.[3] The glue temper was made by adding 7,5 ml of water to 0,511 g of animal glue (beads glue). To produce the casein temper, 25 ml of water g of gelatin in 150 ml of hot water.[3] The glue temper was heated, 1 ml of this hot water was added to 1,020 g of ammonium carbonate, to emulsify. Then, 4,004 g of casein were added to the remaining hot water. Finally, the solutions of ammonium carbonate and casein were mixed and the binder dissolved in 10 ml of hot water.[9]

Films Production

From all pigments available in the LCR, we chose 12 natural pigments (white lead, bindheimite, hematite, orpiment, goethite, realgar, vermilion, azurite, ultramarine, malachite, green earth and crisocola), as well as chalk and gypsum that are used in the ground layers of paintings.

The ground and paint films were made as followed: 1) Paint: to 1 ml of binder natural pigment powder was added to obtain a proper consistency of paint; 2) Ground: to 1 ml of gelatin or glue binder gypsum or chalk were added to obtain a proper consistency.

The paints were deposited in acrylic plaques with a brush to form homogeneous films, in a total of 37 types of films: 4 binders (egg yolk (pure), egg yolk temper, gelatin tempera and casein temper); 2 ground layers (glue temper with gypsum and chalk); 12 paints of egg yolk temper : (egg yolk temper with the 12 pigments mentioned above); 12 paints of gelatin temper (gelatin temper also with the 12 pigments mentioned above); 5 paints of casein temper (casein temper[1] with azurite, ultramarine, malachite, green earth and crisocola).

Four films were made from each paint or ground for the various types of aging. They were left to dry in the dark for 22 days before using them in various types of aging studies.

Implementation of the Picotag+HPLC technique for proteinaceous binding media identification

Instruments and Reagents

Waters Model 600E chromatograph, coupled with Millennium^{32} software, and Waters Model Alliance 2795, coupled with MassLynx 4.0 software were used. The chromatograph 600E is composed of the following Waters modules: Controller 600E, Pumps 600E, In-Line Degasser and 717 plus Autosampler. Both chromatographs share the oven and column detector PDA 996, both from Waters.

Pico-Tag A and B Eluent (A: CH_3CN/60mM CH_3COONa=6/94(v/v) (pH 6,0), B: CH_3CN/60mM CH_3COONa=60/40(v/v) (pH=6,0) (Waters), Hydrochloric acid 20-24% (w//w) (BDH), Phenol pa. (Merck), Triethylamine, 99.5% (Aldrich), Phenylisothiocyanate (Pierce), Sample diluent Picotag (Waters) were used. Pierce high purity amino acid calibration standard H (Pierce Biotechnology, Inc) was used. Other reagents and water were HPLC grade.

Analysis Method

Hydrolysis: Because of their small size, the samples were placed directly into the hydrolysis tubes. The internal standard (one nanomole of norleucine) and 400 µl of 6N hydrochloric acid were added to the hydrolysis tube and the samples were placed at 110 ºC for 24 hours.

Drying process: After hydrolysis the samples were pre-treated and dried. So, 10 µl of dried solution (water:ethanol:triethylamine, 2:2:1) were added to each sample, mixing thoroughly using a vortex mixer, after which the samples were dried in a vacuum chamber.

Derivatization The samples were then derivatized adding 20 µl of derivatization reagent (ethanol:water:trietylamine:

[1] In casein paints we used only the blue and green pigments because they are the most used with this type of binder, in mural painting.

PITC, 7:1:1:1), mixing using a vortex mixer for at least five seconds. The samples were then left for 20 minutes at room temperature before being dried under vacuum.

Analysis: The dried samples were stored in a freezer (–4ºC) until required for use. After adding the Picotag sample diluent buffer (5mM disodium hydrogen phosphate pH=7,40, with 5% acetonitrile), the samples were injected into the HPLC system and analyzed with the Picotag chemistry. A C–18 column was installed on the HPLC with the Picotag reversed phase buffer system: Eluent A, and Eluent B. The column temperature was 38ºC and the diode array detector was set to monitor at 254 nm wavelength. 20 µl of sample were injected. The Picotag gradient timetable was: flow 0,8ml/min, gradient of eluent B from 0% to 46% at 13 minutes. There was also rinse time of 13–16 minutes at 100% B at flow of 1,5 ml/min to avoid baseline disturbances in the ensuing analyses.

The amino acid similarity pattern between two kinds of proteins (experimental and standard data) was calculated with the correlation coefficient (equation 1), a useful statistical tool to determine the degree of association between data sets. In the equation 1, *X* and *Y* are the relative composition for each amino acid, *n* is the number of amino acid measured, and *r* is the correlation coefficient.

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{n}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{n}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{n}\right)}} \qquad (1)$$

Accelerated Aging of the temper films

The protein temper films were subjected to various types of artificial aging to examine the influence of light, temperature and moisture on protein binders. The samples were divided into 4 groups depending on the type of aging considered:

Aging at room temperature

The samples were placed in front of a window, being exposed to conditions of natural light, temperature and moisture environments.

Aging in the UV-Visible chamber

For this aging we used the UV radiation chamber, Solarbox ® 3000E, equipped with a Xenon lamp and a filter that removes all the radiation below 280 nm, which simulates exposure to more critical sunlight condition. The chamber was placed in operation with an irradiation of 400 W/m^2 and a blackbody temperature of 55 ℃.

The aim was to submit the samples to an artificial aging equivalent to 100 years of natural exposure. Thus, knowing that the level of exposure in a museum for a year is $36{,}5 \times 10^6$ h.lux and that 1W/m^2=97,037 lux[10], to an 400 W/m^2 irradiation 940 h of artificial aging is calculated in this chamber.

To follow this aging over time, samples were taken at 0, 68, 160, 348, 514, 705, 824 and 940 h, which is equivalent to 0, 7, 17, 37, 55, 75 , 88 and 100 years of natural aging.

As the dimensions of the chamber were not sufficient to test aging with all films at the same time, they were divided into two lots, I and II. However, in the test of lot II, the xenon lamp was no longer able to stabilize in the desired irradiance. Thus, it was impossible to know which aging lot II was indeed submitted to. Therefore, these samples were excluded from the study. These were gelatin grounds, gelatin paint with white lead, bindheimite, hematite, orpiment, vermilion, azurite, ultramarin, malachite, green earth and crisocola pigments, and casein paint with all the pigments.

Aging in the climate chamber

In this artificial aging, the Climate Fitoclima 150 EDTU chamber was used. Similarly, it was intended that the samples were submitted to a natural aging of 100 years. Knowing that θ=60 ºC, φ=55 % e t=90 days correspond to 64 years of natural aging[11] and based on the principle that every 5ºC higher than room temperature (20ºC) doubles the reaction speed[11], θ=70 ºC φ= 55 % and t=864 h were used.

To follow this aging with time, samples were taken at 0, 67, 161, 329, 665 and 864 h, which is equivalent to 0, 8, 19, 38, 77 and 100 years of natural aging.

Aging in UV-Visible and climate chambers

The samples were subjected to aging in the climate chamber, after undergoing 940 h of aging in the UV-Visible chamber.

Colorimetric analysis

To develop the colorimetric analysis, a Mercury spectrophotometer (International DataColor) was used. In this device, the light source is a xenon lamp and the detector is photodiode sensitive to the 360-750 nm spectrum region.

One of the limitations of this technique is that it is necessary a minimum area sample, with about 2 mm radius, to carry out this analysis. Because it was not always possible to collect samples of this size, since they fragmented into smaller pieces, we could not get the color data for all samples.

We, therefore, measured the parameters L* (lightness), a* (red-green axis), b* (yellow-blue axis), c* (tone) and h (saturation).

Use of EDTA to inhibit the effect of pigment over proteinaceous binding media identification

The characterization of protein binding media using the technique described above may be influenced by the pigments in the sample, which react with amino acids from the hydrolysis of proteins, particularly those pigments containing lead, copper, calcium, iron and manganese.[12]

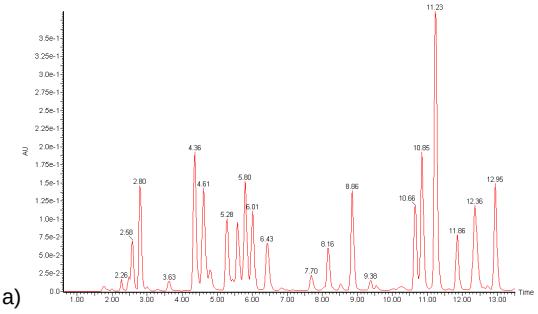
Indeed, as a first approach, it was found that the samples containing azurite, malachite and crisocola (copper based pigments) had a completely different and random chromatographic profiles.

Thus, EDTA was used as a chelating agent to form metal ions-EDTA complexes before the derivatization step, avoiding the formation of metal ions-amino acids complex.[12] So, a 0,2 M Na_2EDTA solution (ammonia/ammonium chloride buffer) was added [12] before the step of drying the sample. Several tests were done using the amino acid standard solution mixed with ca. 1 mg of pigment (white lead, as control, and azurite). The solution of EDTA was added after the amino acid hydrolysis step and before the drying step.

Results and Discussion

Using of Picotag+HPLC technique to proteinaceous binding media identification

Initially, the study began with the analysis of pure protein tempers, not artificially aged, using the Picotag+HPLC technique. Some of the results obtained are depicted in Figure 1.



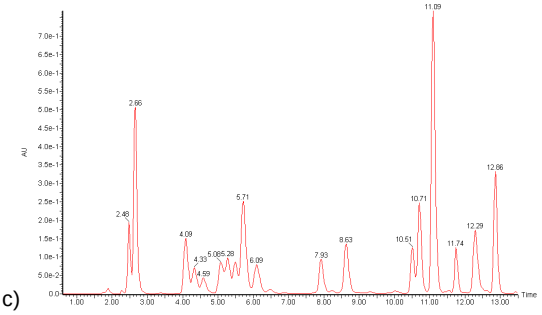


Figure 1 – Samples chromatograms: a) Egg yolk temper, b) Gelatin temper, and c) Casein temper.

It was possible to verify that hydroxyproline (marked as “OH-Pro” on Figure 1 b.) appeared only in the gelatin temper, making the identification of paint-based gelatin or glue grounds easy and quick. The distinction between egg and casein temper was not so easy, but was possible with tools such as the correlation coefficient, mentioned above.

Thus, the correlation between these samples (whose composition was determined in LCR) and those whose composition is published[3] was made. As expected, the correlation of data was better when the same type of temper was considered: the correlation for the glue temper was excellent (r> 0.97), but the correlations for the egg yolk and casein tempers, although reasonable, were not as good., Probably this is due to the not very good resolution of some peaks, including Arg, Thr, Ala and Pro, which disturbs the determination of the relative amount of amino acids and therefore the values of the correlation coefficients.

After the implementation of this technique using the pure protein tempers, the ideal would be to apply this method on real samples, taken from paintings. However, most of samples taken from paintings came from green and blue tones, containing malachite and azurite, pigments that contain copper. This metal (and others) strongly interferes with the identification of protein binders implemented by the method, so we were not able to test this method on real samples. However, in LCR there are a number of films, that were prepared in 1976 by Isabel Ribeiro, whose composition is known, and that do not contain any copper pigments. Therefore it was decided to test the Picotag+HPLC technique in this set of samples. The analysis of this data showed that in all cases there is a correlation between the temper used in the film and the one identified by HPLC.

Study of temper films aging

During the different types of aging, the variation of color and amino acids relative amount for several films were

analyzed: i) aging of pure temper ii) aging of gypsum and chalk grounds, and iii) aging of paint.

Colorimetric

As the analysis of the parameters (L*,a*,b*) is not very intuitive, the coordinates of each sample were input in the *Adobe Photoshop CS2* program, obtaining the corresponding color. In Table 1 the colors obtained for each film are presented.

The colorimetric data in Table 1 was analyzed and revealed that: i) the color remained virtually unchanged over the natural aging, as it was expected, since short time elapsed; ii) in the films made from gelatin and casein, there was no major change in color, regardless of the artificial aging type (although expected, these data are not representative because few tests were developed due to the fact that the area available was insufficient for testing, and because accelerated aging in the UV-Vis chamber for some gelatin and casein films was not made); iii) in egg yolk films, there was a change of color, especially in the absence of pigment (in the UV-Visible chamber there was a color lighting - the L* grows over time, especially at the beginning of aging; in the climatic chamber there was a darkening of the color with L* being dramatically reduced at the beginning of aging; in the accelerated aging using the two chambers consecutively there was a darkening further behind, as if the two chambers were acting synergistically). These color changes were less pronounced in the case of egg yolk temper mixed with pigments, as if the pigment protects in some way the temper degradation.

Picotag+HPLC

To study the protein degradation it would be ideal to do a quantitative analysis of each amino acid, weighting the samples and using an internal standard. However, due to the non-existence of a micro-balance in the LCR this was impossible. Thus, only the relative amount of each amino acid was determined (based on the total area of all amino acids).

Egg yolk temper

Whilst analyzing the data of the egg yolk temper film it was possible to observe that the correlation between the experimental and standard values was always higher when the correlation was made with standard "egg yolk temper", as would be expected. However, the values of correlation dropped slightly when the samples were submitted to aging in the climate chamber, especially in the early hours of aging. In the case of subsequent use of two cameras, UV-Visible and climate, it appears that the values of correlation with "egg yolk temper" had fallen considerably, especially in the early hours of accelerated aging. This suggests that the more the sample aged, the more difficult it is to identify it.

The variation of the amino acid relative amount along with each type of aging for the films of egg yolk temper can be seen in Figure 1 a.-c. In these charts there is not a uniform change in the relative amount of any of the amino acids, in any kind of aging. However, in a detailed analysis it is found that there is a gradual reduction of the relative amount of some amino acids such as Lys, and in other cases, the relative amount is constant over aging (Val, Leu and Phe). This is, probably, due to the fact that the sample is very complex and the egg tempera has a low amount of stable amino acids (Pro, OH-Pro, Ala, Val)[3].

Gelatin temper

In these films, during the climate aging, the correlation between the experimental and the standard "gelatin temper" data is excellent (r> 0.96), regardless of the elapsed time. This fact indicates that this tempera is very stable, as shown by the values of the variation of the amino acid relative amount (Figure 1 d.).

Casein temper

Again, for these samples, during the climate aging the correlation to the standard "casein temper" data is good (r> 0.98), regardless the elapsed time. The change in the amino acid relative amount along the aging (Figure 1 e.) indicates that this temper is also very stable, because the values being almost constant.

Gypsum glue ground

The results for the films gypsum glue ground, indicates that, as expected (after the results obtained for the films of gelatin temper), the correlation is very good to the standard “Gelatin temper," regardless aging length. This and the fact that there was practically no change in the composition of amino acids along the aging (Figure 1 f.) indicate that this preparation is very stable. This is confirmed by the use this kind of ground has had over several centuries in painting, being confirmed that it is a very stable media.

Paints

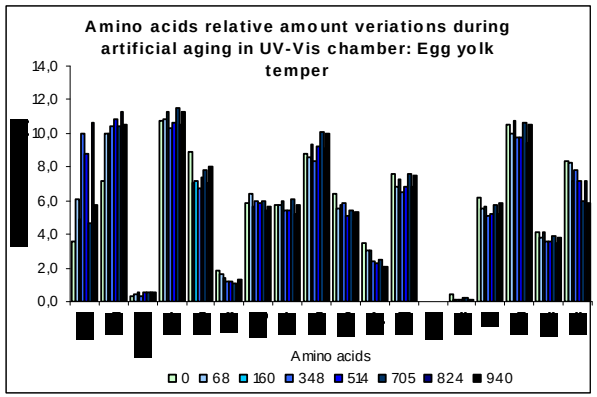
In an initial assessment, a sample of each paint was analyzed. The analysis of these chromatograms (e.g. Fig. 3) showed that some pigments interfere in the analysis, particularly those made from copper (azurite, malachite and crisocola). This had been previously described[2, 6], but it was not considered to fully prevent the identification of the temper, which proved to be verifiable. Then, two pigments were chosen to study their influence on the aging of protein tempers: white lead and realgar.

Table 1 – *Photoshop* color of (L*,a*,b*) parameters obtained by appying DataColor to the paint films during natural and artificial aging.

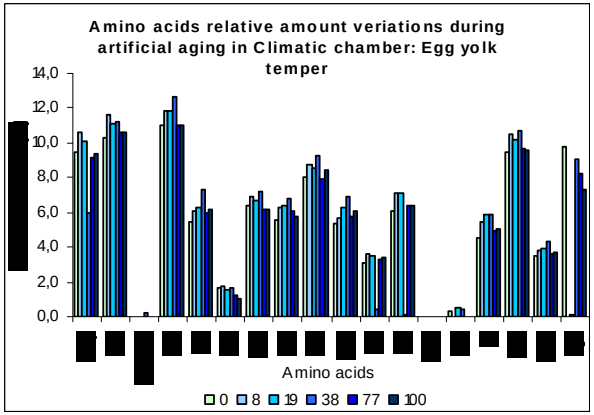
Paint Films	Natural aging (days)							UV-Vis Chamber (correspondence in years)								Climate chamber (correspondence in years)						UV-Vis + Climate chamber (correspondence in years)					
	0	8	22	42	64	89	180	0	7	17	37	55	75	88	100	0	7	17	37	75	100	0	7	17	37	75	100
Egg yolk																											
Egg yolk temper																											
Glue + Gypsum																						-	-	-	-	-	-
Glue + Chalk																						-	-	-	-	-	-
White lead + ET					-	-	-									-	-	-	-	-	-	-	-	-	-	-	-
White lead + GT					-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bindheimite + ET					-	-	-									-	-	-	-	-	-	-	-	-	-	-	-
Bindheimite + GT						-		*	*	*	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*
Hematite + ET					-	-	-															-	-	-	-	-	-
Hematite + GT						-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*
Orpiment + ET					-	-										-	-	-	-	-	-	-	-	-	-	-	-
Orpiment + GT						-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*
Goethite + ET					-	-											-		-		-	-	-	-	-	-	-
Goethite + GT						-										-	-	-	-	-	-	-	-	-	-	-	-
Realgar + ET					-	-	-									-	-	-	-	-	-						-
Realgar + GT						-	-									-	-	-	-	-	-	-	-	-	-	-	-
Vermillion + ET					-																	-	-	-	-	-	-
Vermillion + GT						-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*
Azurite + ET					-	-										-	-	-	-	-	-					-	-
Azurite + GT					-	-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*
Azurite + CT								*	*	*	*	*	*	*	*							*	*	*	*	*	*
Ultramarine + ET					-	-	-									-	-	-	-	-	-	-	-	-	-	-	-
Ultramarine + GT						-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Ultramarine + CT								*	*	*	*	*	*	*	*							*	*	*	*	*	*
Malaquite + ET					-	-	-									-	-	-	-	-	-	-	-	-	-	-	-
Malaquite + GT					-	-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Malaquite + CT								*	*	*	*	*	*	*	*							*	*	*	*	*	*
Green Earth + ET							-											-							-		
Green Earth + GT					-	-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Green Earth + CT								*	*	*	*	*	*	*	*							*	*	*	*	*	*
Crisocola + ET																											
Crisocola + GT					-	-	-	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Crisocola + CT								*	*	*	*	*	*	*	*							*	*	*	*	*	*

ET - Egg Yolk Temper; TG – Gelatin Temper; TC – Casein Temper, "-" - Insufficient area for analysis; * - Aging in the UV-Vis chamber not made

Note - The egg yolk temper is transparent, and it acquires a grayish white tone by the influence of the location where the sample is placed (a white sheet of paper) when examining the DataColor.



a)



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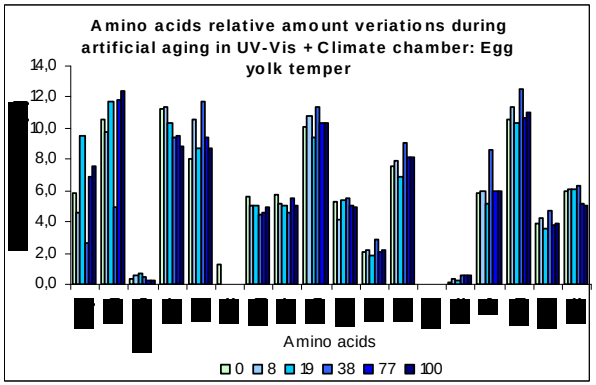

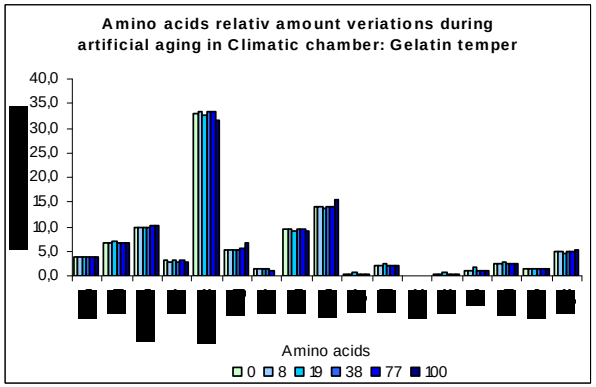

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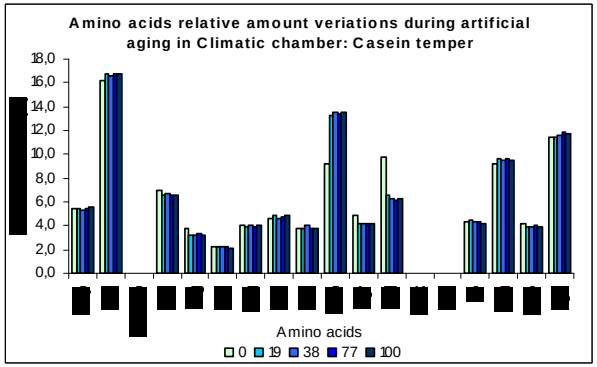

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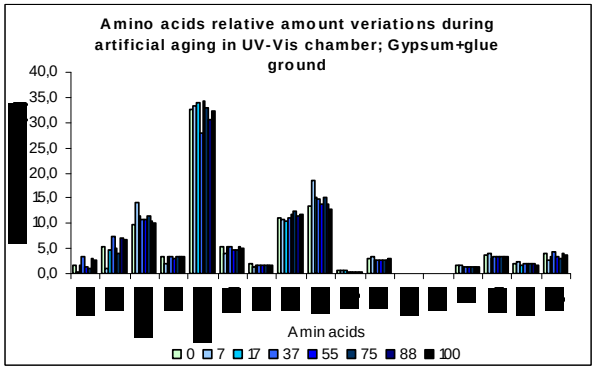

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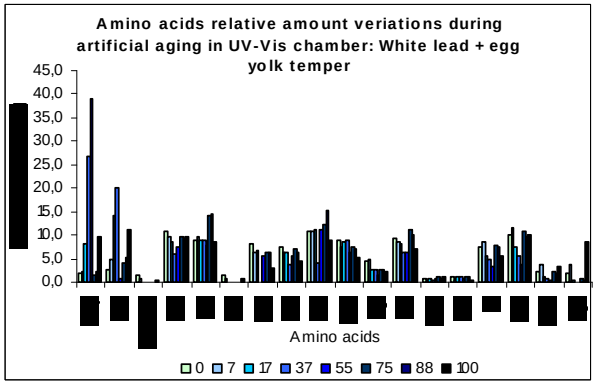
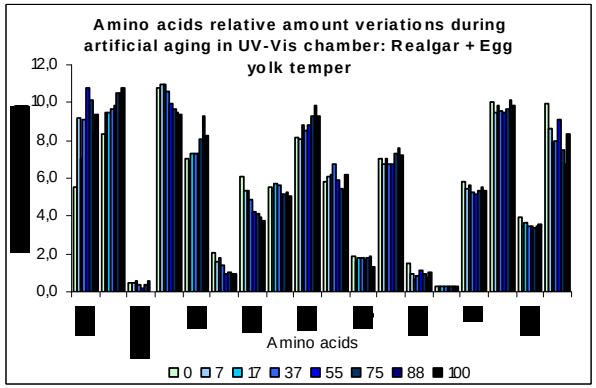


g)



h)

Figure 2 – Amino acid relative amount variation during the different artificial aging: a) Egg yolk temper in UV-Vis chamber; b) Egg yolk temper in Climatic chamber; c) Egg yolk temper in UV-Vis an Climatic Chambers; d) Gelatin temper in Climatic chamber; e) Casein temper in Climatic chamber; f) Glue and Gypsum ground in UV-Vis chamber; g) Paint “White lead + egg yolk temper” in UV-Vis chamber; and h) Paint “Realgar + egg yolk temper” in UV-Vis chamber.

It was observed that the identification of the proteinaceous binding media in the paint “white lead + egg yolk temper” became problematic with the aging in the UV-Visible chamber and it may even become impossible to distinguish from casein temper. This is probably due to the quantities of Glu and Asp which vary considerably, as shown in Figure 1 g.

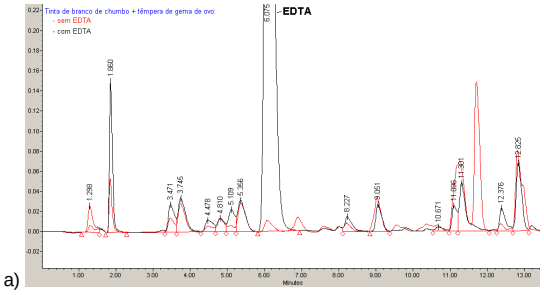
In the analysis of the paint “realgar + egg yolk temper”, it appears that the realgar does not influence the identification of egg yolk temper, as the correlation with the standard "egg yolk temper" is good. Over the aging of the egg yolk temper in the presence of realgar, however, a slight decrease in the amount of Ser, Gly, His and Arg occurs (Figure 1 h.).

Use of EDTA to inhibit the effect of pigment over proteinaceous binding media identification

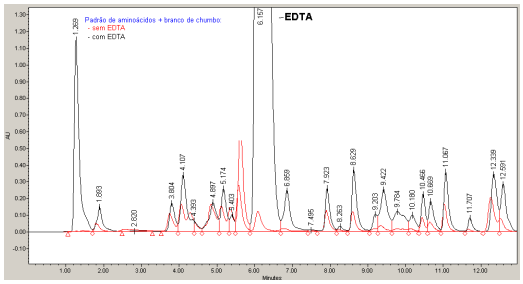
To analyze the possibility to use EDTA as complexing agent for copper, the addition of 0,2 M Na_2EDTA was made. This solution contains an ammonia/ammonium chloride buffer, which provides a pH of ~9, that favors the formation of metal ion-EDTA complexes.[12]

The films “white lead + egg yolk temper” and “azurite + egg yolk temper” were selected. These two films were compared because the EDTA does not have great influence in the film white lead, which is just to compare. We also analyzed the effect on the amino acid standard mixture (20 µl) to which ca. 1 mg of each of the pigments (white lead and azurite) was added.

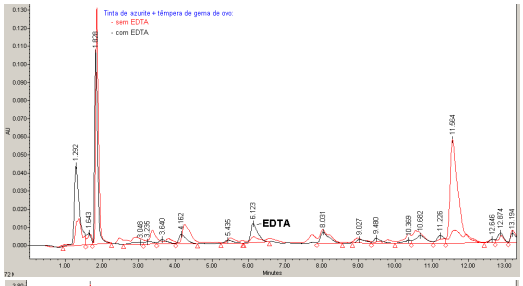
To these four samples (“white lead + egg yolk temper”, “azurite + egg yolk temper”, “white lead + amino acid standard” and “azurite + amino acid standard”) various quantities of 0,2 M Na_2EDTA solution were added: 0 µl, 10 µl, 20µl, 50µl, 75 µl e 100 µl. The addition of EDTA in the various steps was also tried: before hydrolysis, after hydrolysis and before the drying step, after the drying step and before the derivatization step. In Figure 3 some of the results obtained are shown.



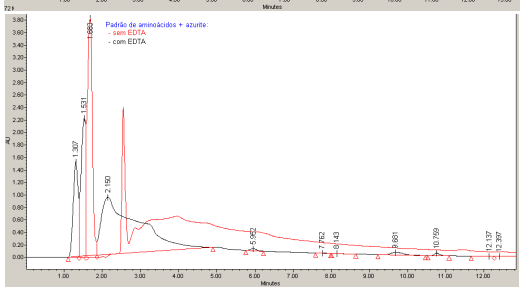
a)



b)



c)



d)

Figure 3 – Chromatogram without EDTA addition (red) and with 20 µl of 0,2 M Na_2EDTA in a buffer at pH 9 (black) of the samples: a) white lead + egg yolk temper; b) “1mg white lead + 20 µl amino acid standard”; c) azurite + egg yolk temper; and d) “1mg azurite+ 20 µl amino acid standard.

By analyzing the chromatograms (Figure 3) we can see that, in the presence of white lead pigment, EDTA improves the resolution of some peaks, making it easier to identify the type of temper. However, using azurite, there has been no change upon addition of EDTA, and the temper identification continued impossible. The amount of EDTA added was also increased up to 1 ml, as well as the amount of pigment used decreased with no improvement on results of the effect of suppressing the influence of pigment in the analysis.

The results suggest that the interference of metal ions, including the Cu(II), occurs at the hydrolysis step and the EDTA has no effect to prevent this reaction. Thus, an option would be to use a much faster method of hydrolysis, as for example hydrolysis by micro-wave radiation[13], which requires ca. 5 min., unlike the method used that requires 24 hours. It is possible that with a much shorter hydrolysis time the breakdown of amino acids does not occur so extensively, allowing the identification of proteins.

Conclusions

The Picotag+HPLC technique implemented in LCR was shown to distinguish the different types of proteinaceous binding media. However it has some limitations related to the sample preparation method, especially since the quantities of sample available are very small: the protein hydrolysis can be incomplete, as well the drying and derivatization step; in addition, the drying can be unsuccessful. This technique is not yet refined to protein tempers that contain pigments or other substances containing copper or a few other metal ions.

The evaluation of changes in different tempers under different conditions of artificial aging allowed the detection of:

- Some chromatic changes in tempers along the aging, such as: (i) color lighting on exposure to UV-Visible light, (ii) yellowing and darkening of the color in the climatic chamber, and (iii) synergistic effect between the UV-Visible and climate chambers, with a darker color than in aging only in the climate chamber. These color changes are especially visible in the egg yolk films, in the absence of pigment.

- Casein and gelatin tempers seem to be more stable than egg yolk temper, under identical conditions of accelerated aging. The difference may be due, in part, to the fact that glue has a high content of “stable” amino acids, and low levels of amino acids easily photo-oxidized (such as histidine, tyrosine and sulfur containing amino acids like methionine and cysteine). On the other hand, the egg yolk promotes photo-oxidation and decomposition induced by radicals.[3] In addition, the egg yolk temper has a high content in oil, not present in the gelatin and casein tempers. The change of color in films may therefore be due to change of oil and not of proteins. This is reinforced by the fact that there are no significant changes with aging in the amino acid composition in the egg yolk temper films.

- In gelatin and casein tempers there were no significant changes in the amino acid relative amount over time. In the egg yolk temper films there are slight changes, but these are not significant. This highlighs the fact that the proteinaceous binding media are excellent binders and as such they have been used for a long time. In fact, the glue temper is back in vogue because of its stability.

- Some pigments, such as white lead, made the temper identification difficult, especially in aged paints, because the pigment accelerates the degradation of some amino acids such as Glu and Asp; yet others, such as realgar, have no influence in the proteinaceous binding media identification.

The suppressive effect of EDTA in pigment interference, coordinating to metal ions has not been confirmed. The results indicate that the amino acid degradation catalyzed by pigments as copper occurs during the hydrolysis, and that the addition of EDTA did not prevent this reaction, Further studies need to be developed in this field.

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