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RESEARCH ARTICLE

A follow-up on the analytical study of discolouration of the marble statues of Orsanmichele in Florence

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Abstract The research complements the complex study carried out to understand the source of brown discolourations of ten marble statues in the Church of Orsanmichele in Florence, Italy. Originally located in exterior niches, the statues were restored to reverse the extensive alterations they had undergone throughout the centuries. One of the major alterations was the application of a dark brown patina that dated just after 1789. After the statues were placed indoors, brownish discolourations started to appear on their surfaces. Cross sections were examined using FTIR mapping and immunological methods. In parallel, the pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) data already obtained from the statues' scrapings were compared with data from aged casein films applied to microscope glass slides and aged milk-treated marble. All the statues had been treated with milk-based substances before the time the bronze patina was applied. The values of temperature and illumination of the room were important factors in the ageing of organic substances and in the formation of calcium oxalates. It is likely that products of thermo-oxidation and photo-oxidation of the oils together with the oxalates caused the darkening. The marble samples corresponded to a Lunense provenance.

Keywords Marble statue discolouration · Oxalates · μ-FTIR-ATR mapping · Immunoassay technique · py-GC/MS · TMAH · Fluorescence · Microclimatic survey · Colour measurements

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Introduction

The research described in this paper follows and complements the complex study recently carried out to understand the source of brown discolourations of ten marble statues in the Church of Orsanmichele in Florence, Italy, dating back to the first half of the fifteenth century (Pinna et al. 2015). Although discolourations, and in particular those tending to brown, are a widespread phenomenon on marble, scientific literature on the subject has lagged behind. As reported in an extensive review in the earlier paper, only in certain instances technical investigations have resulted in a clear identification of the sources of the discolourations, while in many others, the causes are still subject to debate (Bams and Dewaele 2007; Garcia-Valles et al. 1997; Vazquez-Calvo et al. 2007; Doehne and Price 2010; Sai Prasad and Siano 2010).

The marble statues of Orsanmichele were originally located in exterior niches of the church and underwent a thorough restoration between the 1980s and 1990s to reverse the extensive alterations they had suffered throughout the centuries. One of the major alterations was the application of a dark brown patina (called 'bronzatura'), intended to make the statues look like bronzes, and that dated to interventions that occurred just after 1789. Only the *Madonna della Rosa*, attributed to Simone di Ferrucci, was spared from this treatment, having been placed indoors from 1628 until about 1925. In the 1990 restoration by conservators of the Opificio delle Pietre Dure, the dark patina was removed and it was decided to display the statues indoors in a museum created for them in the interior of Orsanmichele, placing replicas in the original outdoor settings. After their location indoors, the statues started to show brownish chromatic changes on their surfaces to the extent that some areas of the statues are now almost completely brown, strongly altering the clear tone and translucency characteristic of the marble (Fig. 1).

Previous research (Pinna et al. 2015) presented the results of non-invasive and invasive techniques used to address the question of the discolouration. In particular, the non-invasive techniques included photographs under ultraviolet light, fluorescence lifetime imaging (FLIM) and portable X-ray fluorescence spectroscopy. Invasive methods were SEM-EDX, ATR-FTIR and Raman spectroscopy on cross sections and pyrolysis gas chromatography/mass spectrometry (py-GC/MS) with (tetramethyl)ammonium hydroxide (TMAH) of scrapings from the surfaces and/or the bulk of samples. The results indicated that the discolouration is mainly a phenomenon affecting the marble structure up to 500 μm. Only in limited areas, very thin superficial films contributed to the brownish alteration. The paper also showed that the marble discoloured mainly on the statues that had been previously treated with the bronze-like patina and then cleaned. The statue of the *Madonna della Rosa*, which had never been treated with the dark patina but that was protected with microcrystalline wax after surface cleaning in the 1990s, had not discoloured. Calcium oxalates (mainly weddellite) and phosphates were detected on the surface and in the bulk of the marble, and specifically the oxalates extended within the marble on the statues previously subjected to the 'bronzatura', which consisted of an oil paint, pigmented with ochre pigments and Prussian blue. Comparative analysis of cross sections from various areas of the different statues revealed that the darker the marble, the deeper the calcium oxalate could be detected, while lighter coloured areas showed no evidence of calcium oxalate. The latter appears to correspond to areas where the bronze-like patina was either more superficial (e.g. *Sant'Eligio*) or not applied (*Madonna della Rosa*). Differences in discolouration of the marble and deeper formation of calcium oxalate in various areas of the statues and among the statues were interpreted as due to differential absorption of the oil from the bronze-like patina by the varied weathered marble surfaces, which also might have been cleaned prior to patination.

Mainly through chromatographic analysis of the marble scrapings, both from the bulk and close to the surface, it appeared that all the statues examined had been subjected to the same treatment in the past, which had occurred before the time the bronze patina was applied. The possibility that an early milk-based protective coating was used on the marble sculptures was suggested by the presence of nitrogen- and phosphorus-containing components and the relative distribution of specific fatty acids. The concomitant presence of these components was independent on whether or not the sculptures had been treated with the bronze-like patina, thus confirming that the treatment had been previously applied to all the sculpture examined. The question remained as to whether components of casein and sugars typical of dairy substances and/or their degradation products could be identified in the statues' marble samples as supporting evidence for a milk-based treatment. Another issue to explore further was the assessment of the colour changes of the statues in the context of the environmental conditions of display.

Thus, the present paper describes the results of analyses with the aim of addressing the abovementioned outstanding issues. In order to verify the presence of early milk-based treatment, the cross sections of the marble samples were further examined using FTIR mapping and immunological methods, to better localize contributing substances and to establish the source of the proteins present. In parallel, the py-GC/MS data already obtained from the Orsanmichele statues' marble scrapings were compared with data from aged casein films applied to microscope glass slides and aged milk-treated marble, analysed in the same analytical conditions. Since py-GC/MS with methylation (TMAH) is suited for the simultaneous detection of lipids, carbohydrates, proteins and phosphorous-containing compounds, all classes of materials present in dairy products could be detected in a single

Fig. 1 Attributed to Filippo Brunelleschi, *San Pietro*, ca. 1421. The statue covered by the bronze-like patina, immediately before restoration (**a**). The statue immediately after restoration in 1991 (**b**). A detail of the statue as it appeared in 2012, almost completely brown (**c**)

analysis. Microscopic samples of the marble were also taken to determine its mineralogical composition and its provenance, which might have also accounted for differences in its discolouration.

Then, the paper presents the results of colour monitoring performed on four statues over time, as well as the data of the environmental monitoring at the location of the statues, in order to highlight any interaction of environmental factors with the past conservation treatments.

Besides the new set of analysis on the samples of the previous study (vide infra), also two samples taken from the marble statue of *S. Giovanni Evangelista* were examined. This statue had a very different display history and was not treated with the bronze-like patina. Attributed to Simone Talenti and dated back to 1337, it adorned, like the other statues, an outside niche of the Orsanmichele Church until 1515, when it was moved to the courtyard of the Ospedale degli Innocenti in Florence, now museum. In the late nineteenth century, it was placed indoor in the Bargello Museum and in 1915 in the storerooms of the Museo degli Innocenti. Finally, in 2003, the statue was located in a cloister of that museum. A relevant information is that *S. Giovanni Evangelista* does not have any brownish discolourations on its surfaces with the exception of some localized mild yellow patinas (Fig. 2), thus providing an important term of comparison for the statues which had discoloured.

The research here described and discussed showed that the values of temperature and illumination of the room where the statues are placed have been important factors in the ageing over time of organic substances such as proteins, lipids and sugars and in the formation of calcium oxalates, either on the surface or in the bulk of the stone. Moreover, relative humidity could have affected the movements of the substances inside the stone. It is likely that products of thermo-oxidation and

Fig. 2 Statue of *S. Giovanni Evangelista*. Museo degli Innocenti, Florence, Italy

photo-oxidation of the oils together with the oxalates caused the darkening. Another possible causative factor of the darkening visual effect is the gradient of penetration of the organic substances inside the stone: the deeper they are, the darker the marble is. Immunoassay technique and py-GC/MS with TMAH clearly showed that all the statues but *S. Giovanni Evangelista* had been treated with milk-based substances.

The mineralogical, petrographic and isotopic characteristics of the examined marble samples corresponded to a Lunense provenance.

Methods

μ-FTIR-ATR mapping

A Thermo Nicolet (Thermo Fisher Scientific, Waltham, MA, USA), iN™10MX imaging microscope, fitted with a mercury-cadmium-telluride (MCT) detector cooled by liquid nitrogen was used for chemical mapping of four cross sections. The measurements were performed using a slide-on ATR objective, equipped with a conical germanium crystal, in the range 4000–675 cm^{-1}, at a spectral resolution of 4 cm^{-1}. A dedicated software, OMNIC Picta™ (Thermo Fisher Scientific, Waltham, MA, USA), was applied for a combined manipulation of the spectra dataset.

The experimental conditions were as follows: apertures 40 × 40, 60 × 60, and 100 × 100 μm; steps 8, 10 and 20 μm; and number of scans 64. The investigated areas varied from 58 × 18 to 150 × 170 μm and the number of collected spectra from 180 to 304.

Immunoassay procedure

The immunochemical procedure for the localization of casein in stone cross sections used a non-competitive sandwich-type immunoassay with chemiluminescence (CL) imaging detection (Dolci et al. 2008; Sciutto et al. 2011; Sciutto et al. 2013). Embedded samples were incubated under stirring for 1 h at room temperature in a non-specific binding blocking solution composed of soybean milk added with bovine serum albumin (BSA) to achieve a 5 % total protein concentration. Then, they were washed three times (3×) with 10 mM phosphate buffer saline solution (PBS), pH 7.4, containing 2 % of BSA (PBS/BSA). Subsequently, the cross sections were incubated overnight at 4 °C with the polyclonal rabbit anti-bovine casein antibody (primary antibody) diluted 1:500 (*v*/*v*) in PBS. The samples were then washed (3×) with PBS and incubated for 4 h at room temperature with horseradish peroxidase (HRP)-labelled goat anti-rabbit antibody (secondary antibody) diluted 1:2000 (*v*/*v*) in PBS. Samples were washed again (3×) with PBS, then the HRP CL detection reagent was added to cover the cross sections and the CL images were acquired using an integration time of 120 s. For each CL image, a live image of the sample was also acquired to assess the localization of the protein in the sample through comparison of CL and live images.

Chemiluminescence imaging microscopy experiments were carried out by a home-made imaging system composed by a BX 60 epifluorescence microscope (Olympus Optical, Tokyo, Japan) connected to a liquid nitrogen-cooled ultrasensitive CCD camera (LN/CCD Princeton Instruments, Roper Scientific, Trenton, NJ, USA). The microscope, which was enclosed in a dark box to avoid interference from ambient light, was also equipped with an OptiScan ES103 XYZ stage system (Prior Scientific Instruments Ltd., Fulbourn, England) to allow reproducible sample positioning and focusing without opening the dark box. Live images of the samples were obtained using the LN/CCD camera and a RGB filter (CRI Inc., Woburn, MA, USA): separate grayscale images for red, green and blue channels were acquired, and then they were merged in a single colour image. Image analysis software Metamorph v. 4.5 (Universal Imaging Corporation, Downingtown, PA, USA) performed image processing and quantitative analysis.

Pyrolysis gas chromatography/mass spectrometry with TMAH

Laboratory-prepared aged samples of marble treated with milk, aged casein films applied to microscope glass slides and D-(+)-galactose were analysed by py-GC/MS with methylation with TMAH.

The materials used to prepare the samples and the procedures of ageing were as follows:

- Naturally aged film prepared from non-pasteurized milk applied on a marble slab (20 × 20 × 2 cm) in 1995, aged outdoors (historical centre of Bologna) for 2 years and afterwards indoor in the laboratory
- Accelerated aged casein film applied on a glass slide (casein 1, Section Materials and chemicals): Casein concentration was 10 g of casein in 50 g of deionized water + 2 mL of 28 % aqueous ammonia solution. The accelerated photo-ageing consisted of four cycles (first, 100 h; second, 200 h; third, 250 h; and fourth, 250 h) at a constant temperature and irradiation (35 °C and 500 W/m^2) using a Xenon lamp equipped with an 'outdoor filter' ($\lambda > 310$ nm) for simulating outdoor exposure and an IR filter that eliminates IR radiation with $\lambda > 800$ nm (Solarbox 3000e, Co.Fo.Me.Gra., Milan, Italy)
- Naturally aged casein film (casein 2, Section Materials and chemicals) applied on a glass slide in 1976 and aged in the laboratory
- Naturally aged films, made of three types of commercially available caseins, applied on glass slides. Each casein was mixed with water as a 2 % solution (*w*/*v*) and swollen (3 h). Then, ammonia (10 % *v*/*v*) was added to each solution. The films were placed outdoor, with direct solar irradiation, for 140 days (February–June 2013). The three caseins used were the so-called French and those from Leuenberger and from BDH (casein 3, 4 and 5, respectively, Section Materials and chemicals). Until the 1966 flood of the Arno River in Florence, Italian restorers mainly used 'French' casein. Afterwards, it was realized that it yellowed over time. Casein from Leuenberger was preferred in the late 1980s, when it appeared on the market, as it contained a smaller amount of fats (circa 1 %) than the French casein. Finally, in the late 1990s, a new casein (biochemical) from BDH was sold, with a finer granulometry than the other two and a fat content lower than 0.1 % (Borgnis et al. 2014).

Samples on the order of 30–50 μg were accurately weighed on an Ultramicrobalance UMX2 (Mettler Toledo) in the pyrolysis cup (Eco-cup, Frontier lab). Three microliters of TMAH solution 25 % (*w*/*v*) in methanol were added to the samples prior to pyrolysis at 550 °C in the vertical micro-furnace of the double-shot 2020iD pyrolyzer (Frontier lab). The micro-furnace is interfaced to the gas chromatograph Agilent 6890 coupled with the Agilent 5973 Network Mass Selective Detector. The analysis was carried out in split mode 10/1, 20/1 or 30/1 according to the sample size. A J&W DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. The inlet was kept at 320 °C and the MS transfer line at 320 °C. Helium was used as the carrier gas, constant flow 1.5 ml/min. The GC oven temperature program was 40 °C for 1 min ramped to 320 at 10 °C /min, followed by 11 min isothermal period. Acquisition was performed in SCAN mode (*m*/*z* 29–550). Temperatures were at MS source 230 °C and at quadrupole 150 °C. A solvent delay of 1.5 min was used for most analyses. Data evaluation was performed using AMDIS, NIST mass spectral database and relevant literature.

Materials and chemicals

This paragraph provides information about the origin (when available) of the chemicals used. The chemicals are listed below:

- Polyclonal rabbit anti-bovine casein antibody, HRP-conjugated polyclonal goat anti-rabbit antibody and bovine serum albumin (BSA) (Sigma-Aldrich, USA)
- Soya milk (total protein content, 3.5 %): from a local drugstore
- Luminol-based HRP CL Supersignal ELISA Femto (Thermo Scientific Inc., USA)
- (Tetramethyl)ammonium hydroxide pentahydrate (≥97 %, Sigma-Aldrich, USA)
- Non-pasteurized milk (it is not possible to add further information)
- Casein 1 (Carlo Erba, Italy)
- Caseins 2 and 3 (Zecchi, Florence, Italy)
- Casein 4 (Leuenberger and C. SpA, Italy)
- Casein 5 (Casein biochemical, BDH, USA)
- D-(+)-galactose (≥99 %, Sigma-Aldrich, USA)
- Ammonia (Carlo Erba, Italy)

Analysed by py-GC/MS with TMAH, caseins 1–5 show comparable compositions, differing only in the proportion of lipids (Table 2).

Mineralogical, petrographic and isotopic analyses

For this study, we could take just a limited number of micro-samples from five statues on the back of the pedestals. Thin sections (thickness 30 μm) of the samples were examined at the optical polarizing microscope Zeiss Axioscope A1 by parallel and crossed nicols. The microscope was equipped with a camera (resolution 5 megapixel) and a dedicated image software (AxioVision). The observation allows highlighting microstructural characteristics, such as the maximum grain size, shape of grains' boundaries and type of microfabric, useful to identify marbles and their provenance. X-ray diffraction analysis (PANalytical X'Pert PRO diffractometer equipped with X' Celerator multidetector, Cu Kα_1 = 1545 Å operating at 40 KV, investigated range 2θ 3–70° at 30 mA) was carried out on powdered samples to examine the mineralogical composition. High score data acquisition and interpretation software were used to determine the mineralogical composition.

The C and O stable isotopic ratios were determined, to obtain information about any archaeometric differentiations of the samples. A mass spectrometer (Finningan MAT Delta Plus) coupled with Finningan Gas-Bench II was utilized. The samples along with standard mineral powders were treated with phosphoric acid for 1 h in sealed glass cups previously washed with helium. The CO_2 produced by the reaction entered the mass spectrometer. The analysis was conducted applying continuous-flow method with helium as carrier gas. A well-characterized CO_2 standard was used, whose isotope ratios were already known, as well as NBS-18 (international standard calcite material) for the samples' normalization on an international scale and internal standards as secondary references.

Colour measurements

Colour measurements were performed according to the procedure described in the European Standard EN15886 (EN 15886 2010) using the CIELAB 1976 method, with the standard illuminant D65 and observer 10°. The colour coordinates L^*, a^* and b^* were recorded over time for each selected area (Ø ~ 8 mm) using a KONICA MINOLTA CM2600d spectrophotometer. The areas were chosen among those of the most discoloured statues. Moreover, light-coloured areas of each chosen statue were selected for comparison with the discoloured ones. Dark and light areas of five statues (Table 4) were analysed measuring colour five times in the period 2005–2015. Four to six measurements were taken by repositioning the instrument on the same spot each time, and then they were averaged. The colorimeter was calibrated against SPECTRALON® prior to each measurement.

Microclimatic survey

Microclimatic measurements were performed for the periods of November 9, 2004 to June 16, 2005 and July 27, 2005 to September 8, 2006. Although the measurements were carried out about 10 years ago, the results are reported in this paper for two reasons. First, the microclimatic situation has not changed since that survey and, second, the long time was necessary to conduct the numerous and time-consuming analyses described, which have been discussed in the previous article and more thoroughly in the present one.

The overall survey started in November 2004 with sensor installation. Measurements were taken using cordless micro-data sensors. Their uncertainty was 0.1 °C for temperature and 2 % for relative humidity. They were located in the room to collect thermal and hygrometric data and set to measure and record the different values at every 10-min data acquisition interval. The sensors transmitted data to the system BABUC ABC (LSI-Lastem Company, Milano, Italy) for the acquisition, processing and registration. The software INFOGEN elaborates the data.

Sensors for relative humidity (RH), air temperature (T), surface temperature (TS) and light levels were positioned in eight locations of the room (Fig. 3). Sensors 4 and 5, measuring all the parameters, were placed on the shoulders of *San Marco* and of one of the group of *Quattro Santi Coronati*—one exposed to the north and the other to the south. Other sensors measuring RH, T and TS were located on central columns and near the entrance door at a height of about 3 m. In July 2005, three more sensors for RH and T were located in three corners of the room.

Results and discussion

μ-FTIR-ATR mapping and immunological methods

In the previous study, calcium oxalates had been detected either on the surfaces or in the bulk of cross section samples performing ATR-FTIR and Raman measurements on individual areas. In the attempt to visualize the localization of calcium oxalates in samples' bulk, μ-FTIR-ATR mapping was carried out on four cross sections (OR2, OR6, OR7, OR10 in Table 1) from two Orsanmichele statues (*Sant'Eligio* and *San Giacomo Maggiore*). Results successfully showed the localization of calcium oxalates either on the surfaces or inside the marble (Fig. 4) of three cross sections (OR6, OR7, OR10).

In the case of Orsanmichele statues, oxalates relate to the presence of organic substances used in restoration treatments, being the products of their transformation. Since μ-FTIR-ATR mapping confirmed and visualized the internal localization of oxalates, a further sophisticated technique, the immunological analysis, was applied to cross sections (Table 1) in order to identify and localize the possible dairy substances (in particular casein) that were previously suggested as present in the marble (Pinna et al. 2015). Remarkably, the technique identified and visualized the presence of casein on the surface and in the bulk (along the edge of the calcite grains) of many samples from brown discoloured but also from non-discoloured (white and yellow) areas (Table 1 and Figs. 5 and 6). In some of these areas, oxalates were not found even though it is not possible to exclude the presence of traces of calcium oxalates whose detection with spectroscopic techniques is hindered by carbonatic materials. However, the absence of oxalates could indicate that proteins and lipids did not undergo a chemical transformation there and, thus, they need particular conditions (temperature and/or light) to mineralize into calcium oxalates. In fact, the white area (sample OR3 in Table 1) is in a concavity of *Sant'Eligio* statue where the illumination striking the surface is not strong.

In addition, casein was located in the marble below the bronze-like patina (sample OR5 in Table 1). Interestingly, in

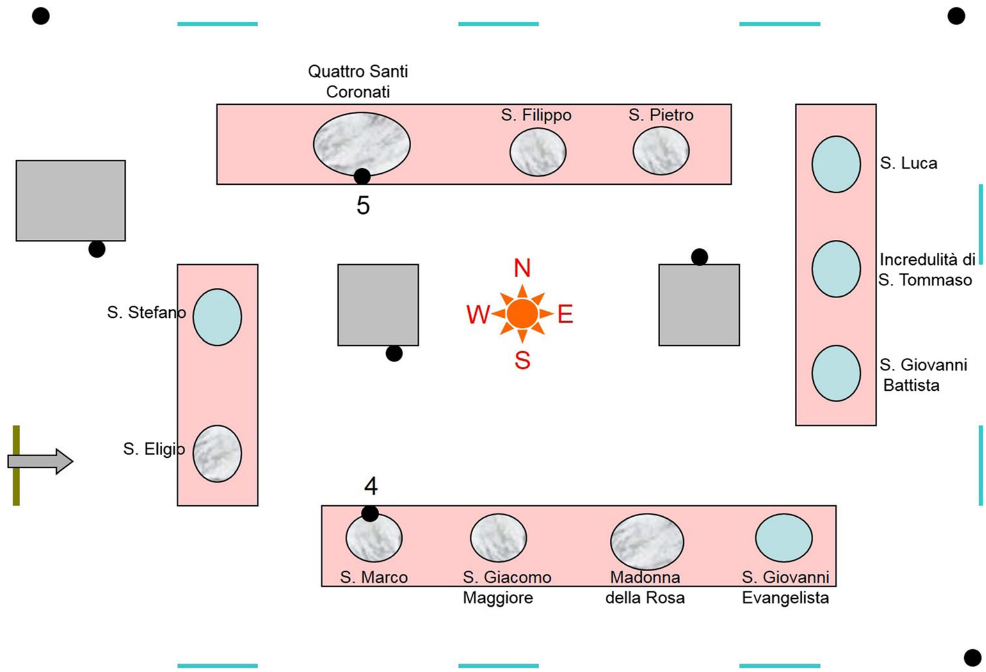


Fig. 3 Museum of Orsanmichele, plan, showing locations of climate sensors (*black dots*) and windows (*solid blue bars*) in relation to the sculptures

samples from *S. Giovanni Evangelista*, no casein was detected (Table 1). This result is very relevant to understand the conservation history of all the statues. Since *S. Giovanni Evangelista* was removed from Orsanmichele in 1515, it seems plausible to speculate that the remaining statues underwent a treatment with dairy substances in a period between 1515 and 1628. The latter is likely the *ante quem* date because *Madonna della Rosa* was placed indoors in that year. As the latter contains casein, it is likely that dairy treatments were applied to the surfaces within that period. It is possible that restorers had treated the outdoor statues first and the one indoor afterwards, namely until 1789 (*post quem* date of bronze-like patina application), but literature about stone treatments refer mainly to outdoor monuments. Such treatments were quite usual in the past as Marconi (1987) and Melucco Vaccaro (1996) quoted ancient treatises on the application of *scialbature* made of mixtures of water, lime and milk for the maintenance of Italian stone monuments outdoors. At times, these treatments contained also inorganic pigments. Other periodical maintenance practices used mixing water and lime with pozzolanic ash (pozzolana) and animal glue.

According to the restorers Danesi and Gambardella (2005), a well-established Italian past restoration practice was that described in detail by Orfeo Boselli) in his mid-seventeenth century treatise *Osservazioni de la scoltura antica* (Boselli 1663). Boselli, a renowned Roman sculptor and restorer at that time, describes a mimetic restoration criterion indispensable for imparting an ancient colour (*Dare il colore antico*) to marble sculptures. Besides that, such practice made the newly sculpted and whiter marble elements looked like the old ones. Boselli's recipe consists of a tint based on cheese, lime and water with addition of tuff powder, or otherwise stone or brick, making an impasto that is spread with a brush. Then, restorers sometimes applied a second layer, called a *velatura*, a more liquid wash made of soot boiled in water (in vinegar or better yet in urine), according to Raffaello Borghini who documented conservative iconographic issues raised by the Council of Trento, 1545–1563 (Borghini 1967, first ed. Firenze 1584).

In regards to other types of stone treatments, although original treatises are lacking, the technical literature of the early 1900s reported on the application of siccative oils on stones in the 1600s and 1700s (Torraca 1986). Torraca (1986) quoted some examples of survived original documentation on the practice. Among them was a document dated 1732 by the sculptor Pietro Bacci who applied oil on a marble head he made to restore and complete the Arch of Costantino in Rome. Even though to impart an aged appearance, this document proves that oil was used for treating marbles. Other information refers to a blend of natural resins and nut oil reported by a Venetian manuscript as used by the sculptor Antonio Sansovino (Rossi Manaresi 1972).

Pyrolysis gas chromatography/mass spectrometry with TMAH

The comparative analysis of data obtained from the scraping samples of the aged casein films on glass and the milk-treated marble, with that from the Orsanmichele statue samples, was most useful. This is evident from Table 2 and illustrated with an example in Fig. 7, which shows the striking similarity of the chromatograms obtained from the scraping sample from

Table 1 List of statues, samples analysed and results of spectroscopic and immunological methods

Statue	Bronze-like patina	Samples	Immunochemical detection of casein	FTIR, ATR-FTIR, SEM/EDS (selected results)	Py-GCMS-TMAH (lipids/wax/resins, S components)
Madonna della Rosa attributed to Simone di Ferrucci 1399	No	OR13 Yellow area	Positive for casein	Surface: traces of calcium oxalates Bulk: no oxalates	Bulk: fatty acids: C4:0, C12:0, C14:0, C15:0, C16:0, C17:0, little C18:0, C20:0, C22:0, C24:0 with dicarboxylic acids d-C4, d-C6, d-C8, d-C9. Microcrystalline wax, turpentine. (Methyl)methanesulfate, (dimethyl)sulphate
S. Eligio by Nanni di Banco ca. 1417–1421	Yes	OR2 Brown discolouration	Positive for casein in external and internal parts of the sample (ca. 300 mμ), along calcite grains' edge	Surface: no oxalates, no gypsum evident Bulk: no oxalates, no gypsum evident	Surface: fatty acids: C12:0, C14:0, C16:0, C18:0, C18:1 with dicarboxylic acids acid-C4, d-C8. (Methyl)methanesulfate, (dimethyl)sulphate. Bulk: fatty acids: C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1. (Methyl)methanesulphate, (dimethyl)sulphate
		OR3 White area	Positive for casein in internal part of the sample (ca. 200 mμ)	Surface black crust: gypsum Bulk: no oxalates	Surface black crust: fatty acids: C8:0, C12:0, C14:0, C15:0, C16:0, C18:0, C18, with dicarboxylic acids d-C4, d-C9. (Methyl)methanesulphate, (dimethyl)sulphate Bulk: low amounts of fatty acids: mainly C16:0, C18:0
		OR5 Bronze-like patina	Positive for casein within fragments of marble below the bronze-like patina	Patina: ochre pigments, Prussian blue, calcium oxalates, gypsum Bulk: calcium oxalates, gypsum	Patina: fatty acids: C15:0, C16:0, C17:0, C18:0, with dicarboxylic acids d-C7, d-C9. (Dimethyl)sulphate, (methyl)methanesulphate
		OR6 Brown discolouration	Positive for casein in external and internal parts of the sample (ca. 400 mμ), along calcite grains' edge	Surface and bulk: calcium oxalates Surface: SEM/EDS traces of phosphorus	Bulk: fatty acids: C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C18:1, low d-C9
San Giacomo Maggiore by Niccolò di Pietro Lamberti ca. 1414	Yes	OR7 Brown discolouration	Positive for casein in external parts of the sample	Surface and bulk: calcium oxalates, gypsum Surface: SEM/EDS traces of phosphorus	Whole: fatty acids: C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0. (Methyl)methanesulphate, (dimethyl)sulphate
		OR8 Yellow discolouration	Positive for casein in internal part of the sample (ca. 350 mμ), along calcite grains' edge	Bulk: gypsum. No oxalates	–
		OR9 No discolouration, very damaged area	Negative for casein	–	–
		OR10	Positive for casein in external parts of the sample	Bulk: calcium oxalates, gypsum	Bulk: low amounts of fatty acids: C12:0, C14:0, C16:0, C18:0. (Methyl)methanesulphate, (dimethyl)sulphate

Table 1 (continued)

Statue	Bronze-like patina	Samples	Immunochemical detection of casein	FTIR, ATR-FTIR, SEM/EDS (selected results)	Py-GCMS-TMAH (lipids/wax/resins, S components)
		Back, not carved, yellow area			
Quattro Santi Coronati (also referred as Santi Quattro Coronati) by Nanni di Banco ca. 1409–1417 (group of four statues)	Yes	OR14 Yellowish discolouration	Positive for casein in external parts of the sample	Surface (orange accumulation): calcium oxalates Bulk: no oxalates	Surface: fatty acids: C12:0, C14:0, C15:0, C16:0, C18:1, C18:0, C20:0, C22:0, C24:0 with dicarboxylic acids d-C9 (low), d-C4. (Dimethyl)sulphate. Bulk: fatty acids: C12:0, C14:0, C15:0, C16:0, C17:0, C18:1, C18:0
S. Giovanni Evangelista by Simone Talenti 1337	No	No discolouration	Negative for casein	–	–

The information about the presence or absence of the bronze-like patina have been reported in an article describing the restoration of the statues (Giusti 2012)

the marble of one of the *Quattro Santi Coronati* (OR14) and that of the aged milk-treated marble.

The fatty acid distribution and the presence of sulphate and phosphate derivatives are the most evident common features, but also small peaks derived from galactose are seen in both.

The lab-prepared films showed the distribution of fatty acids typical of dairy products, already noted in the Orsanmichele samples (Pinna et al. 2015). In particular, even carbon number fatty acids, with relative high amounts of lauric acid (C12:0), and lower amounts of pentadecanoic (C15:0) and heptadecanoic acids (C17:0) are present (Lindmark Månsson 2008; Reis et al. 2011). In addition, glutaric acid (d-C5) is present in discrete amounts in most samples and particularly in the casein and milk-treated marble. Interestingly, low amounts of azelaic acid (d-C9) and lower amounts of suberic acid (d-C8) were detected in the aged milk-treated marble, suggesting an origin from the oxidation of unsaturated fatty acids in the lipid component of the milk (Chiavari et al. 1998). This explains their presence in the sample of the *Madonna della Rosa* and their lower amounts in the casein films, where the lipid content of the milk is primarily removed. Nitrogen-containing components in the lab-prepared films find correspondence in the marble scrapings from the statues but are not diagnostic for dairy proteins; therefore, they have not been reported in Table 2. The presence of (methyl)phosphate in the films and Orsanmichele samples confirms that it is a pyrolysis and methylation product from phosphorous-containing components, such as phosphoproteins, phospholipids and calcium phosphates, of dairy substances (Corbridge 2013). Furthermore, the Orsanmichele samples show markers for galactose previously reported as derived from thermochemolysis of monosaccharides (base peak m/z 129) (Fabbri and Helleur 1999) that was confirmed by data from the galactose reference and from the aged milk-treated marble. Other unidentified markers from galactose and casein are present consistently in the reference milk-treated marble and the Orsanmichele samples. Additional reported components of galactose such as trimethoxybenzenes and 1,2,3,4-tetramethoxybenzene (the latter reported in Table 2 and Fig. 7) are recognizable in the samples but are not diagnostically useful. The milk-treated marble also shares the same sulphate-derivatives from the environment-induced transformation of the carbonate of the marble into sulphate (e.g. gypsum), as already reported in the Orsanmichele samples (Pinna et al. 2015). Moreover, as discussed and detailed in the early paper (Pinna et al. 2015), the source of the organic substances detected did not indicate a biological origin.

Marble provenance

Table 3 reports the main mineralogical, petrographic and isotopic characteristics of the marbles of the five sculptures sampled. All the marbles are calcitic, medium fine grained

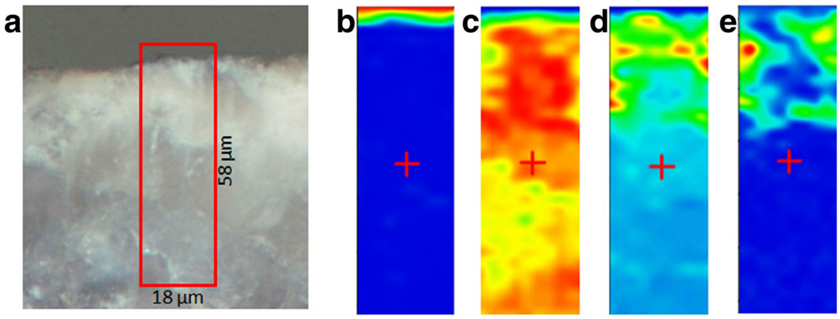


Fig. 4 Localization of calcium oxalates in the cross section from *San Giacomo Maggiore* statue. µ-FTIR-ATR mapping. Polished cross section of the sample. The *red rectangle* indicates the analysed area. Visible illumination, objective ×10 (**a**). False colour images showing the embedding resin (peak area = 1726 cm^{-1}) (**b**), calcium carbonate (peak area = 875 cm^{-1}) (**c**), calcium oxalates (peak area = 1324 cm^{-1}) (**d**) and gypsum (peak area = 3393 cm^{-1}) (**e**)

(maximum grain size <500 µm) with well-formed (euhedral) calcite crystals and exhibit straight grain boundaries without shape orientation. The fabric is almost homeoblastic. XRD analyses registered the presence of calcite in all the samples and traces of gypsum only in samples from *Madonna della Rosa* and *San Marco*.

The isotopic ratios of samples from *Quattro Santi Coronati*, *Sant'Eligio* and *San Marco* show δ^{13}C values 1.84 to 2.11 and δ^{18}O values −1.27 to −1.71. Only sample from the *Madonna della Rosa* has a very low value of δ^{13}C, 0.72. According to the published databases (Gorgoni et al. 2002; Attanasio 2003; Lazzarini 2004; Attanasio et al. 2015), isotopic ratios (Fig. 8) and the petrographic characteristics correspond to a Lunense provenance. Although the isotopic ratio of the sample from *Madonna della Rosa* fell out of the variability range of Lunense marbles, Prof. Lorenzo Lazzarini confirmed that provenance on the basis of correlation with an unpublished database (personal communication). The quarry of *Madonna della Rosa*'s marble is different from the quarries of the other marbles.

Colour measurements

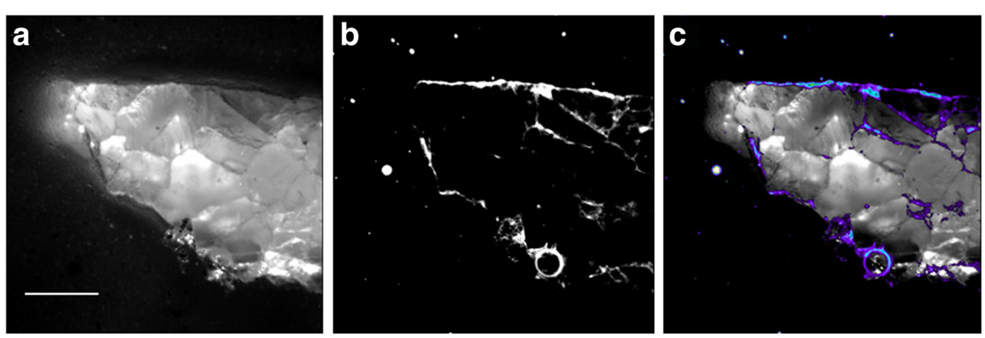
As mentioned above, the measurements started in January 2005. On this date, the sculptures already showed advanced discolouration, yet an actual t_0 was not measured because the statues did not show discolouration on the surfaces when they were restored in the 1990s and the conservators did not plan any monitoring of the colour. A quantitative information on the colour changes over time is provided by the comparison of colour values between the light and the brownish areas of the five monitored statues (Table 4).

Regarding the measurements of the *L** (lightness), *a** (red/green) and *b** (yellow/blue) parameters of four statues (*San Pietro*, *San Filippo*, one of the *Quattro Santi Coronati*, *Sant'Eligio*), the major changes of the colorimetric parameters in the overall period are relatively low, 2 or 3 units. Many *L**, *a** and *b** values slightly changed. Some exceptions are three *L** values, one of *San Filippo* (dark area 1 in Table 4) and two of the *Quattro Santi Coronati* (dark area 1 and light area 1 in Table 4), as they decreased 4.65, 3.72 and 5.24 units, respectively, indicating a significant darkening. Another exception is the *b** value of *Sant'Eligio* light area, which decreased 4.65 units (Table 4). Moreover, the last *L**, *a** and *b** values (November 2012) of one of the dark area 1 of this statue were not considered in this discussion because in September 2012 the area underwent a cleaning trial.

Over the 10-year monitoring, most measuring spots (11 out of 16) darkened while some spots seemingly getting lighter (4) or stable (3). The parameter *a** was mostly stable or had slight insignificant variations. As an example, the trend for *L**, *a** and *b** values of the *Quattro Santi Coronati* is illustrated in Fig. 9a–c.

Overall variations in *L** and *b** parameters are those more reliable and significant because they provide a useful guide for the evaluation of the colour changes over time. As previously

Fig. 5 Chemiluminescence immunolocalization of casein in the cross section from *Sant'Eligio*. Live image (**a**). Localization of the chemiluminescence signal (**b**). Overlay of the live image of the cross section with the pseudo-coloured chemiluminescence signal (**c**). *Bar* = 200 µm



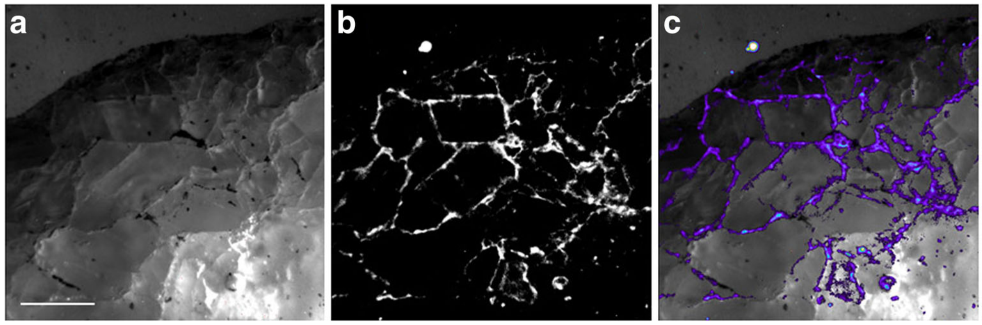


Fig. 6 Chemiluminescence immunolocalization of casein in the cross section from *San Giacomo Maggiore*. Live image (**a**). Localization of the chemiluminescence signal (**b**). Overlay of the live image of the cross section with the pseudo-coloured chemiluminescence signal (**c**). *Bar* = 200 μm

mentioned, *L** diminished in many spots indicating that the surfaces are getting darker, with the exception of four spots where *L** increased (*S. Pietro* dark area 1 and light area 1, *S. Filippo* light area 2, *S. Eligio* light area). As two of these values are associated with a lowering of the parameter *b**, one of the possible causes of the lightening of the stone relates to a reduction of the yellowing, a phenomenon worth monitoring in the future for a better understanding.

The parameter *b** was quite variable. In fact, in some of the areas that darkened, it increased (more yellowing, *S. Pietro* dark area 2, *S. Filippo* dark area 1, *S. Eligio* dark area 2) or diminished around 2 units (increase in the blue component, *Quattro Santi Coronati* dark areas 1 and 2, light area 1). Whatever the causes of the brownish discolouration are in both cases, the variations are consistent with a darkening of the stone.

The *Madonna della Rosa* measurements are discussed separately because this statue had never been treated with the dark patina. The present research showed that, similarly to the other statues, it had been treated with a dairy substance, but it was

Table 2 Relevant components from py-GC/MS with TMAH of Orsanmichele marble samples, galactose, casein and milk on marble

Peak	Rt (min)	Relevant *m/z* (decreasing intensity)	MW	Structural assignment	Occurrence (reference samples)	Occurrence (Orsanmichele samples)
1	5.02	59 89 45 58 102	134	Glycerol trimethylether	Ma, Ca	2S, 2B, 3S, 3B, 5, 6, 7, 10B, 13B, 14
2	5.20	58 45 43 75 74	N.A.	Glycerol derivative	Ma, Ca	2S, 2B, 3S, 3B, 5, 6, 13B
3	5.56	110 79 109 95	140	Methylphosphate	Ma, Ca	2S, 2B, 3S, 3B, 5, 6 (traces), 10B, 13B, 14
4	7.68	45 103 71 104	N.A.	Unidentified	Ma, Ga, Ca	10, 14
5	8.65	74 87 43 55	158	Caprylic acid ME (C8:0)	Ma, Ca	2B, 3S, 6, 14
6	8.82	100 59 129 101	160	Glutaric acid di-ME	Ma, Ca	2S (traces), 3S, 6, 10B, 13 (traces), 14
7	10.03	127 42 142 56 57	N.A.	Unidentified	Ma (impure of 127), Ca	2S, 2B (traces), 3S, 6 (traces), 10B, 13, 14
8	10.28	156 141 53 95	N.A.	Unidentified	Ma, Ga	3S, 10B, 14
9	11.02	154 53 125 83 139	N.A.	Unidentified	Ma, Ga	3S, 14 (impure)
10	11.14	129 75 115 101 161	N.A.	Monosaccharide derivative	Ma, Ga	3S, 5 (traces), 10, 13 (traces), 14
11	11.35	74 87 143 155	186	Capric acid ME (C10:0)	Ma, Ca	2B, 6, 7, 14
12	13.05	198 183 140 69	198	1,2,3,4-Tetramethoxybenzene	Ma, Ga	10B, 2S (traces), 3S, 7, 14
13	13.12	74 129 171 97	202	Suberic acid di-ME (d-C8)	Ma, Ca	2B, 3S, 5, 13B, 14
14	13.54	129 75 101 161 191	N.A.	Monosaccharide derivative	Ma, Ga	2S traces, 3S, 7 traces, 10B, 13, 14
15	13.86	129 75 101 45 161 220	220	Monosaccharide derivative	Ma, Ga	2S (traces), 3S, 10B, 14
16	14.11	74 87 143 214	214	Lauric acid ME (C12:0)	Ma, Ca	2S, 2B, 3S, 6, 7, 10B, 13B, 14
17	14.37	152 55 74 185	216	Azelaic acid di-ME (d-C9)	Ma, Ca	3S, 5, 6, 13B, 14
18	16.43	74 87 199 242	242	Myristic acid ME (C14:0)	Ma, Ca	2S, 2B, 3S, 6, 7, 10B, 13B, 14
19	17.50	74 87 213 256	256	Pentadecanoic acid ME (C15:0)	Ma, Ca	2B, 5, 6, 7, 13B, 14
20	18.53	74 87 227 270	270	Palmitic acid ME (C16:0)	Ma, Ca	2S, 2B, 3, 5, 6, 7, 10B, 13B, 14
21	19.53	74 87 241 284	284	Heptadecanoic acid ME (C17:0)	Ma, Ca	2B, 5, 6, 7, 13B, 14
22	20.21	55 69 74 264	264	Octadecenoic acid ME	Ca, Ma (traces)	2S, 2B, 3S, 6, 10B, 14
23	20.44	74 87 255 298	298	Stearic acid ME (C18:0)	Ma, Ca	2S, 2B, 3, 6, 7, 13B, 14

Representative of all five casein samples, since they show comparable compositions, differing only in the proportion of lipids. Sample numbers not qualified as S or B refer to those analysed as a whole

ME methyl ester, *N.A.* not assigned, *Ma* milk on marble, *Ca* casein, *Ga* galactose, *S* surface, *B* bulk

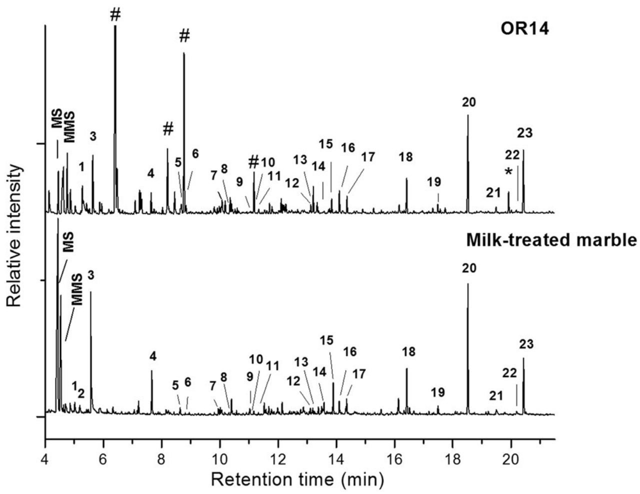


Fig. 7 Total ion chromatogram (*TIC*) detail from py-GC/MS with TMAH of the scraping marble sample OR14 (*Quattro Santi Coronati*) (*top*) compared with a scraping of the aged milk-treated marble (*bottom*). Numbered peaks correspond to components listed in Table 2

the only one that was protected with microcrystalline wax after surface cleaning in the 1990s. The statue had not discoloured. Moreover, in 2014, it was cleaned again. The three measuring areas darkened as parameter L^* decreased remarkably (3 to 8 units) over time, also after cleaning (Table 4). The parameter a^* had been quite stable with the exception of the last values after the 2014 cleaning. Here, a^* values increased 2–3 units indicating the stone colour turned towards red. Also, the parameter b^* had been quite stable excluding again the last measurements. In fact, after the cleaning, it increased 3 to 4.5 units. It is likely that dust and atmospheric particulates adhered to the surfaces of the *Madonna della Rosa* more than to other statues because of the wax coating. Therefore, the shift towards red and yellow of a^* and b^*, respectively, could be attributed to the cleaned wax surfaces from which a superficial layer of microcrystalline wax mixed with atmospheric particles was removed. The cleaning effects with reduction of the layer of wax can also partially explain the lowering of L^* values.

Microclimatic survey

The room in which the statues are located is at the second floor of the building. Along the four walls, there are large windows, which had no UV filters during the period of monitoring. Those are partially screened by curtains but still let in a lot of light. There is also one door leading directly to the outside (Fig. 3). The sensors 4 and 5 placed on the shoulders of *San Marco* and one of the *Quattro Santi Coronati* (Fig. 3) gave relevant information about what happened on the stone surfaces with changes in the environmental conditions. RH values for the two sensors are similar, ranging from 70 to 80 % in many periods (December, January, February and March), while the minimum values are in the range 18–30 % mainly in the summer but also in November 2005. In fact, in the summer (June–September), lower RH values are recorded (20–55 %). Relative humidity had frequent fluctuations from 30 to 80 % in a few days. This phenomenon occurred in many periods: November and December 2004; February, April and November 2005; and March and August 2006. However, almost every month high variations (≥10 %) of RH occurred within a single day for 3–5 days.

During the entire monitoring period, *San Marco*'s maximum values were 5 to 10 % higher and minimum values slightly higher (3–5 %) than those on the *Quattro Santi*. This discrepancy is likely due to *San Marco*'s location, closer to entrance.

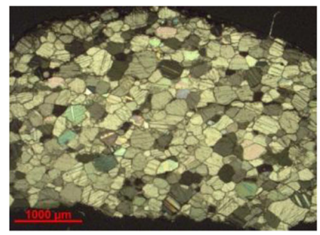
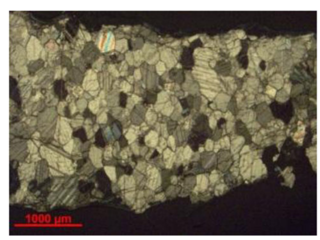
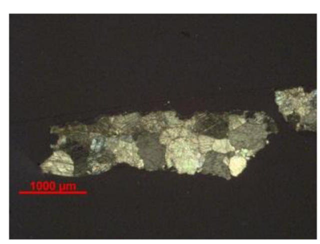
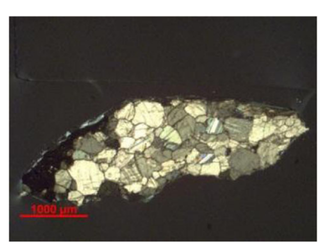
Air temperatures were the same in the two positions. They varied greatly with seasonal changes (2.3–2.8 to 30.6–30.8 °C), but they fluctuated less than relative humidity. *San Marco*'s sensor showed overall less fluctuation than that of the *Quattro Santi Coronati*. The variations of the first sensor occurred mainly in March–May and August 2005 (ΔT = 4 to 5 °C). The same situation occurred to a smaller extent in the same months of 2006. The main variations of the second sensor (ΔT = 4 to 6.5 °C) instead occurred in March, April, August and October 2005 and January and March 2006. The surface temperatures at both positions followed the same trend and were just slightly higher than air temperatures. Interestingly, when the sunlight strokes the statues, surface temperatures increased 5° more in comparison with air temperature.

Even though the dew point of the *Quattro Santi Coronati* sensor reached high values (15–20 °C) in the period July, August and September 2006, it did not rise above the surface temperature, indicating that superficial condensation was unlikely to have occurred. Unfortunately, we do not have the measurements of the other sensors, including that of *San Marco*, but we can presume they followed a similar trend, since T and RH of the two sensors were comparable.

Direct solar radiation affects the values of the light levels measured by the two sensors (*San Marco* exposed to the north with sensor on the neck, the *Quattro Santi Coronati* exposed to the south with sensor on the back), with differences due to the exposure of the statues and the positioning of the sensors. The light levels reached very high values, up to 5000 lx when the statues were subjected to direct irradiation. Values around 5000 lx were reached in many periods within the monitoring time (Fig. 10). Values around 1000 lx were reported 53 times a year. The data demonstrates that even though partially screened by curtains, the windows have a significant effect on the museum's climatic conditions and the light and heat through them cause stone surfaces' overheating during the warmer months.

The trend and values of the other sensors for RH and T are quite similar to sensors 4 and 5. RH varied between 17 and 93 %, with the highest values corresponding to sensors close to the walls. RH values could vary dramatically even over short periods (Fig. 11). Over a few days, variations were between 20 and 80 %. There are differences of 10–15 % between sensors placed in the middle of the room and sensors close to the walls. The air temperature also varied greatly (from 2 to 30 °C), but it was much more constant than the relative humidity. The highest fluctuations occur in summer. The comparison of our results with data obtained by the Osservatorio Ximeniano—an independent measurement and research institution specialized in meteorology and geophysics based in

Table 3 Mineralogical, petrographic and isotopic characteristics of marble samples

Statue	Mineralogical composition	Maximum grain size (μm)	Microfrabric	Thin section	Isotopic ratios
Quattro Santi Coronati	Calcite	250–300	Homeoblastic		δ^{13}C 1,84; δ^{18} −1,53
San Filippo	Calcite	300	Homeoblastic		Not determined
Madonna della Rosa	Calcite, traces of gypsum	400	Homeoblastic/ heteroblastic		δ^{13}C 0,72; δ^{18} −1,71
Sant'Eligio	Calcite	500	Homeoblastic/ heteroblastic		δ^{13}C 2,06; δ^{18} −1,27
San Marco	Calcite, traces of gypsum	350–400	Homeoblastic/ heteroblastic		δ^{13}C 2,11; δ^{18} −1,38

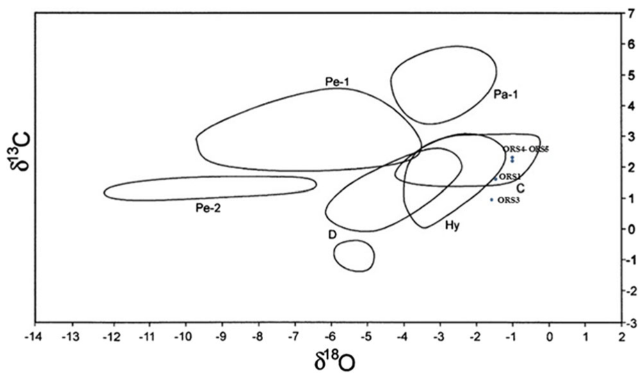


Fig. 8 Global isotopic diagram (including quarry and artefact data) of ancient marbles with maximum grain size (*MGS*) < 2 mm. *C* = Lunense; *D* = Docimaenian; *Hy* = Hymettian; *Pa-1* = Parian lychnites; *Pa-3* = Parian from Karavos; *Pe-1 and 2* = Pentelic (modified from Lazzarini 2004)

Florence—demonstrated that, as expected, temperature and RH conditions within the room followed the external conditions closely, due to the free air exchange that took place through the entrance.

It is well known that values and fluctuations of temperature and relative humidity in the environment are one of the most important causes of deterioration of cultural heritage. Sudden and frequent variations of air temperature and relative humidity can induce stresses to several materials. This in turn creates cumulative and irreversible alterations of the physical and chemical properties that could accelerate the deterioration process depending on the materials (Camuffo 2013). This climatic survey clearly shows that the current climatic conditions of the room of Orsanmichele, which have persisted for a number of years now, are not adequate for the conservation of the stone statues. High light levels and strong fluctuations of temperature and relative humidity reaching worrisome values have likely played a role in the darkening of the marble surfaces, by affecting substances, whose change is responsible for the current brownish alterations.

The statues monitored were treated in the past with a dairy coating, milk-based (vide infra), and, most of them were also covered with a now-removed pigmented bronze-like patina, bound in a drying oil. Therefore, it is worth correlating the results of the climatic survey with the extant literature on the light and thermal ageing of proteins and oils. Indeed, several articles report on the ageing of these substances since they have been most often used as binding media for paintings (Colombini et al. 2000; Mallégol et al. 2001; Boyatzis et al. 2002; Manzano et al. 2009; Matteini et al. 2009). The studies mostly used model samples subjected to thermal- and photo-chemical accelerated ageing. Xenon lamps were generally used to simulate outdoor solar exposure, and temperature and relative humidity setups were mostly in the range of 20–70 °C and 15–80 %, respectively, thus similar to the values measured in the Orsanmichele room.

UV-light ageing, even prolonged, does not significantly affect the amino acid profile of protein binders, meaning that proteins are quite stable (Colombini et al. 2000; Manzano et al. 2009; Vagnini et al. 2009). Although amino acids lysine and tyrosine have been reported as being more affected

Table 4 Colour measurements of brownish and light areas of the five monitored statues

Statues	January 2005			January 2007			March 2008			November 2012			January 2015		
	*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
S. Pietro dark area 1	46.02	8.49	17.78	46.24	8.15	17.49	45.83	8.36	17.40	47.54	8.17	17.93	48.35	8.39	18.82
S. Pietro dark area 2	46.91	7.21	15.56	45.66	8.24	17.57	45.58	8.55	18.07	46.58	7.03	13.99	44.72	8.40	17.63
S. Pietro light area 1	61.09	4.70	20.17	59.76	4.09	18.77	62.97	4.28	19.27	61.12	4.38	16.90	59.12	4.22	16.98
S. Pietro light area 2	58.04	2.38	12.73	57.45	2.35	12.49	58.47	2.91	13.14	60.05	2.32	11.46	57.94	2.42	11.93
S. Filippo dark area 1	54.57	5.95	13.10	53.07	5.92	12.03	52.26	6.26	12.75	51.66	5.94	13.27	49.92	6.91	13.37
S. Filippo dark area 2	51.63	5.39	12.62	53.21	5.06	11.98	52.29	5.38	12.55	56.04	5.49	12.23	52.33	4.81	11.79
S. Filippo light area 1	74.23	4.56	19.20	74.80	4.53	19.21	74.81	4.58	19.63	74.65	4.84	18.27	74.76	4.42	18.51
S. Filippo light area 2	71.31	5.34	19.82	73.50	5.07	19.76	73.05	5.00	19.73	73.13	5.04	19.04	73.13	5.03	19.48
Quattro Santi Coronati dark area 1	58.08	5.61	16.09	57.98	5.66	16.09	57.56	5.91	16.45	56.76	5.69	15.18	52.84	5.88	14.77
Quattro Santi Coronati dark area 2	56.18	5.02	15.29	57.58	5.22	14.77	57.40	4.02	14.24	55.40	3.61	13.16	53.04	3.55	12.72
Quattro Santi Coronati light area 1	72.60	2.59	15.95	74.35	2.37	16.12	74.09	2.26	16.63	70.27	2.45	15.95	68.88	2.13	15.20
Quattro Santi Coronati light area 2	68.68	3.15	15.99	67.20	3.46	16.71	66.84	3.48	17.25	64.92	3.68	16.81	65.56	3.54	16.24
S. Eligio dark area 1	53.50	5.90	12.64	53.37	5.61	12.49	51.15	6.48	13.16	62.14	3.11	5.41	–	–	–
S. Eligio dark area 1	52.21	4.19	10.08	52.11	4.69	11.60	50.91	4.96	12.42	52.51	3.67	8.46	50.47	4.69	11.52
S. Eligio light area	68.35	4.35	17.19	70.75	3.50	14.09	71.02	3.46	14.15	75.19	2.71	8.66	70.12	2.99	12.54
Madonna dark area 1	63.15	3.27	12.79	60.81	3.80	13.34	60.70	3.98	13.25	–	–	–	56.98	5.00	16.02
Madonna dark area 2	50.37	5.48	16.97	47.43	4.72	11.46	–	–	–	–	–	–	–	–	–
Madonna light area	71.12	1.70	14.58	70.67	1.73	14.68	70.52	1.80	14.74	–	–	–	63.15	4.27	19.11

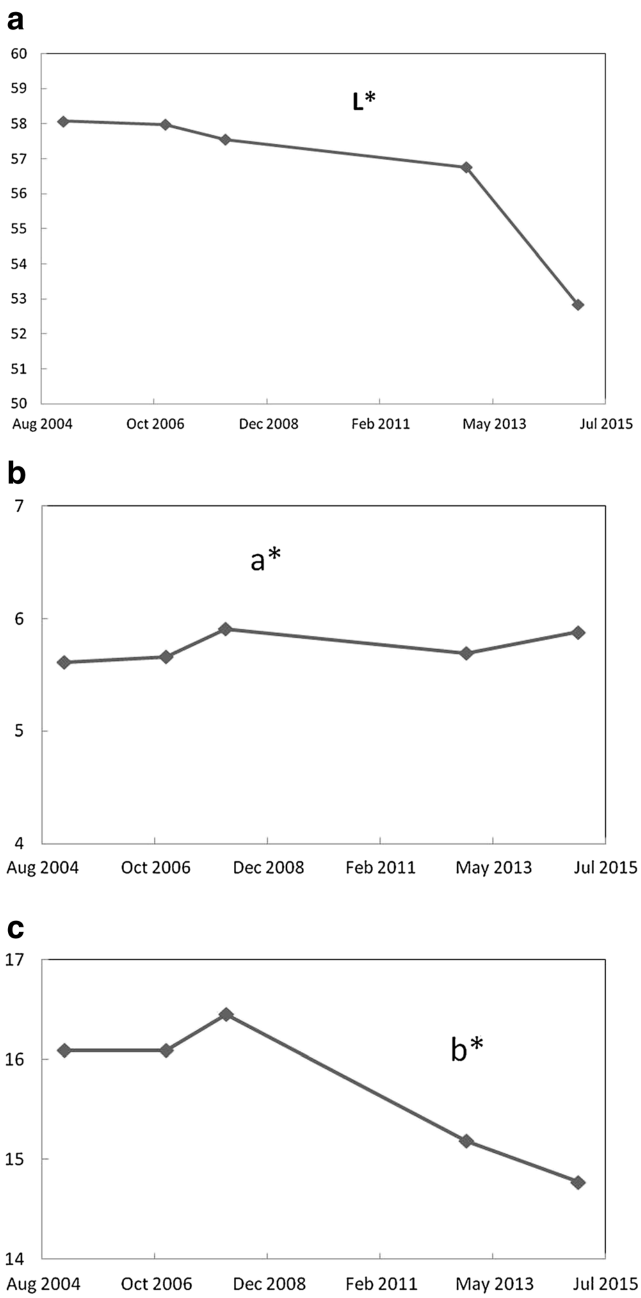


Fig. 9 **a**–**c** Trend of L^*, a^* and b^* colour values of dark area 1 (Table 4) of the *Quattro Santi Coronati*

(Colombini et al. 2000; Matteini et al. 2009), the amino group of lysine may react with lipid hydro-peroxides to form imines (Colombini et al. 2000), and/or condensation of the amino groups with carbonyl groups of carbohydrates may occur, according to the Maillard reaction. This process may take place in milk and may lead to the formation of melanoidines and of brown-coloured polymers related to humic substances. In addition, tyrosine is sensitive to photo-oxidation with formation of dityrosine as its by-product (Matteini et al. 2009). These substance, however, were not detected in the samples from the statues.

Unlike proteins, siccative oils are much more affected by oxidative degradation. Linseed oil, for example, possesses an elevated percentage of conjugated bonds within its fatty acid chains that promote either polymer curing or ageing by forming low-molecular-weight acids and aldehydes (Matteini et al. 2009). These compounds can react with metal ions of pigments to form metal carboxylates and/or with some nitrogen compounds to generate imines, as mentioned above (Colombini et al. 2000; Matteini et al. 2009). Thermo-oxidation phenomena have been reported in linseed oil films aged at 40 °C for more than 1 year (Vagnini et al. 2009). However, thermal curing of oil films at 60 and 100 °C did not yield significant yellowing (Boyatzis et al. 2002). UV irradiation substantially affects drying oil binders, such as linseed oil, leading to the formation of azelaic acid and other dicarboxylic acids including oxalic acid (Colombini et al. 2000). The formation of oxalic acid from pure unsaturated fatty acids supports the chemical origin of calcium oxalate patinas on marble statues treated with oil, including the statues examined.

Correlation of the strong and widespread fluorescence in the cross sections of discoloured areas from the statues with specific organic substances or their transformation products is difficult. Literature exists on the autofluorescence of pigmented and unpigmented mixtures of linseed oil and proteins, submitted to artificial photo-ageing (Matteini et al. 2009). In addition, photo-oxidation of tryptophan, present in discrete amount in milk, produces derivatives fluorescing in the 430-480 nm range, and tyrosine oxidation products also fluoresce in the blue green. Other substances possibly involved in the fluorescence response of protein-based media include the products of Maillard reaction. The possible presence of these substances in the marble of Orsanmichele statues would be very difficult to establish.

However, it is undeniable that, as demonstrated by the microclimatic survey, temperature and illumination have been important factors in the ageing of the organic materials on the surface and in the bulk of the stone, over time. These organic substances likely underwent thermal- and light-induced transformations leading to the formation of calcium oxalates, and other possible oxidation products, contributing to brown discolouration of Orsanmichele statues. Since the fluorescence is a phenomenon specific to the discoloured areas, and, as seen in the cross sections, it extends into channels within the bulk of the marble, it is likely that the texture of the surface, as well as the environmental conditions, affected the ageing and the movements of the substances inside the stone.

Conclusions

The discussion on 'the why and the how' dark brown discolouration formed on Lunense marble statues can be summarized below.

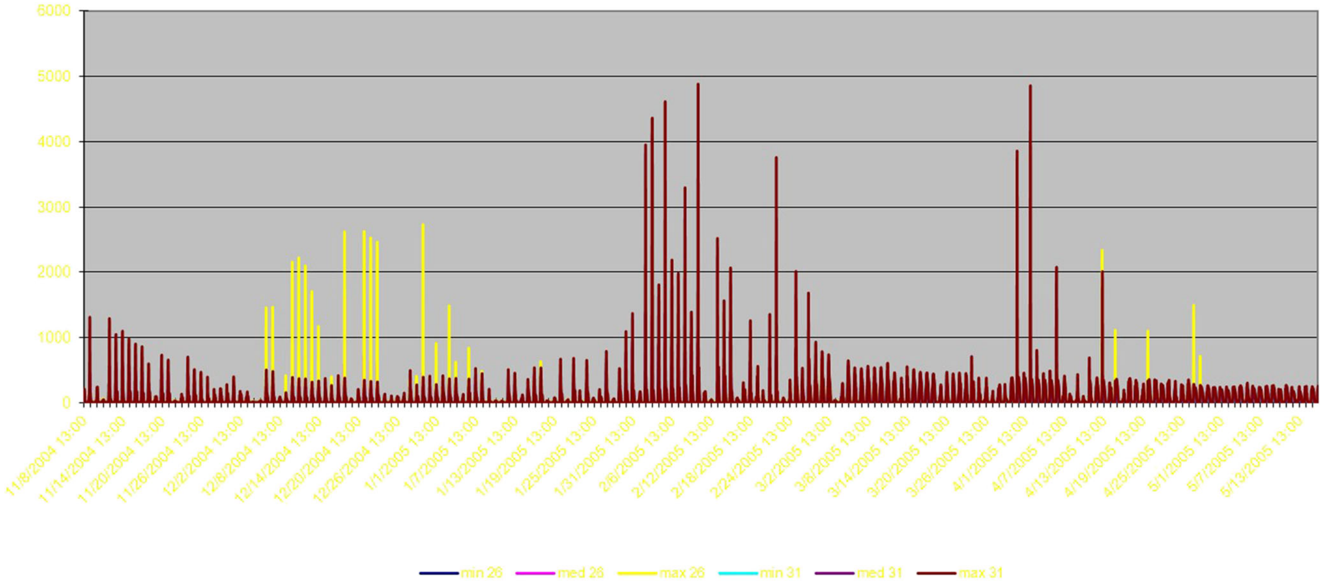


Fig. 10 Values of the light levels measured by the two sensors 4 (*yellow lines*) and 5 (*brown lines*)

Immunoassay technique clearly showed that all the statues but *S. Giovanni Evangelista* had been treated with milk-based substances at a time after 1515 and, likely, before 1628. These results were corroborated by data obtained from samples of the statues analysed by py-GC/MS with methylation, with the exception of *S. Giovanni Evangelista* and OR9 *San Giacomo Maggiore*, which were not analysed by this technique. The former had not been further sampled at the time of analysis, and no sufficient sample from the very damaged and non-discoloured area was available from the latter. Among the corpus of statues analysed, the *Madonna della Rosa* and *S. Giovanni Evangelista* are the only ones which had not been coated with the bronze-like patina and are also the least discoloured. This means that the dairy-based treatment alone was not responsible for the discolouration, since the *Madonna della Rosa* would have also discoloured. Given that the *Madonna della Rosa* and *S. Giovanni Evangelista* were kept indoors for longer period, their surfaces were probably also less worn than those of the other statues that remained outdoors.

Photographic documentation of these outdoor statues prior to their 1990s restoration showed that they had many areas, mainly those jutting out from the niches, where the bronze-like patina was worn away (Fig. 12). Therefore, in each statue,

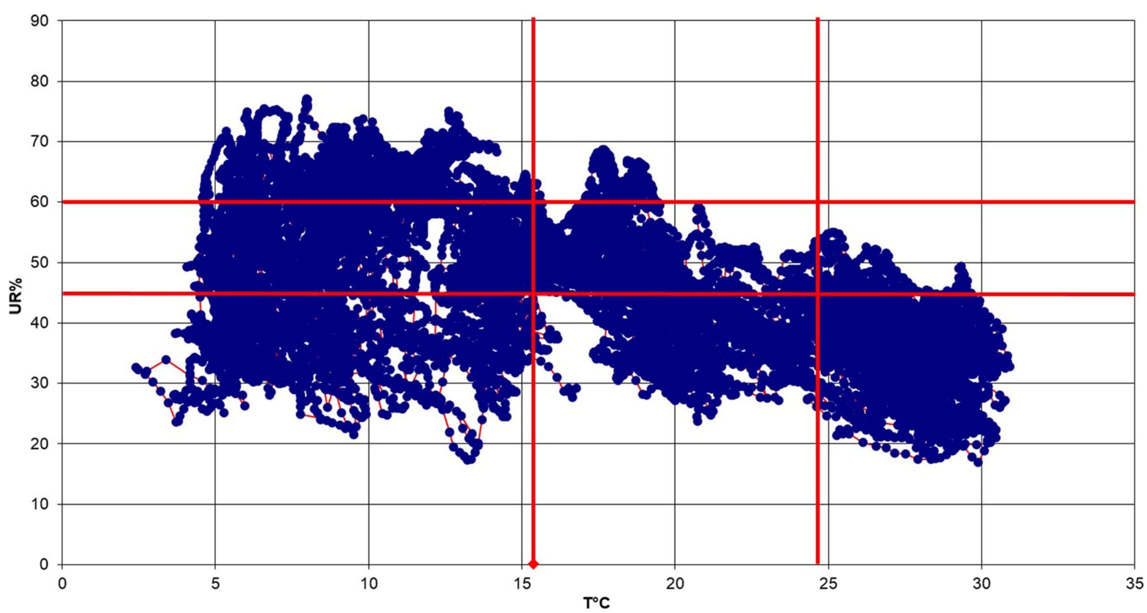


Fig. 11 Graph showing the correlation between temperature and relative humidity for sensor 4 (average hourly data of the whole period). *Red lines* mark the recommended values for RH and *T*. Most of the *dots* are outside the *square* that marks the best combination of the values

Fig. 12 *Quattro Santi Coronati* by Nanni di Banco ca. 1409–1417. Past photographic documentation prior to their 1990 restoration. In many areas, mainly those jutting out from the niche, the bronze-like patina was worn away

there are parts strongly weathered and damaged close to parts that remained protected over time.

The discolouration started to appear on the surfaces of these statues gradually after the restorers removed the dark oil patina in the 1990s, and over the 10-year-long colour-monitoring period, most measuring spots have further darkened, confirming that the discolouration has worsened over time. We can speculate that once the restorers removed the bronze-like patina, the transformation of the substances in the marble started or progressed faster. Hence, the dark patina might have behaved as a sort of barrier to oxidation. Since the statues which once featured the bronze-like patina are those which have undergone the most intense discolouration, it is plausible that the oil binder of that paint and its degradation products had contributed to the formation of calcium oxalates and other by-products, perhaps also by reaction with the original dairy components and/or their transformation substances, causing darkening of the stone. It is thus possible that the areas where the discolouration is now more intense are those where the content of these substances in the bulk of the stone is greater. In fact, when the statues were treated with the dark patina, the condition of their surfaces differed. Thin superficial calcium oxalate films (Pinna et al. 2015) may have protected some areas while others weathered more, as shown from the changes in the marble structure in some of the cross sections studied. Therefore, localized variations in granulometry and porosity at the surface may have resulted in preferential absorption of the oil from the bronze-like patina. The same could have happened with the previous milk-based treatment.

This discolouration therefore depends not only on the nature but also on the extent and depth of penetration of the organic substances within the marble which would have varied according to differential weathering and/or natural

Fig. 13 *Madonna della Rosa* attributed to Simone di Ferrucci 1399

anomalies, thus accounting for a more random localized discolouration. Indeed, in the previous paper (Pinna et al. 2015), FLIM showed that the discolouration is affecting the marble structure, a phenomenon also observed in cross sections.

The cases of the *Madonna della Rosa* and *San Giovanni Evangelista*, which had not discoloured, have been very useful in interpreting the discolouration on the other statues. *S. Giovanni Evangelista*, which remained indoor longer than *Madonna della Rosa*, only shows localized mild yellow patinas (Fig. 2). Likely it was not treated, or substances within the treatments did not penetrate and transform deep inside the marble causing obvious discolouration. The *Madonna della Rosa*, with only a few confined brown-coloured areas (Fig. 13), shows a marble less weathered from its reduced outdoor exposure and less discoloured, since the treatment with microcrystalline wax likely protected it from oxidation under the climate conditions indoors. Indeed, analysis showed no evidence of calcium oxalate either on the surface or in the bulk of the stone. Continuing colour monitoring will provide further insight into the causes of darkening of this statue after the recent cleaning.

The microclimatic survey (period 2004–2006) showed that the relative humidity in the room where the statues are displayed had frequent fluctuations from 30 to 80 % in a few days and in many periods. The temperature varied greatly (circa 3 to 31 °C), and light values can be as high as ca. 5000 lx on the marble surfaces in many periods regardless of the season. The values of temperature and illumination of the room where the statues are placed have been important factors in the ageing over time of organic substances such as proteins, lipids, sugars as well as the formation of calcium oxalates either on the surface or in the bulk of the stone. Moreover, relative humidity could have affected the migration of the substances inside the stone.

In conclusion, this study demonstrates the complexity of the issue of darkening of marble statues, which depends on the nature of the materials applied to the stone, conservation treatments, condition and texture of the marble and, last but not least, environmental parameters which can greatly contribute to transformation processes and need to be controlled to limit further discolouration phenomena.

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