TECHNICAL NOTE

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The Use of Microspectrophotometry to Characterize Microscopic Amounts of Blood

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ABSTRACT: The use of a Nanospec 10 S microspectrophotometer to examine minute bloodstains is discussed. To satisfy criteria for chemical identity, absorption spectra were recorded before and after treatment of the blood with Takayama reagent. The technique is of value where only tiny particulate bloodstains are available for testing and in other specialized instances.

KEYWORDS: forensic science, spectroscopic analyses, blood, microspectrophotometry

The testing of items for bloodstaining in forensic science laboratories follows well-established and documented patterns [*1-3*].

Examination of the results of successive proficiency tests carried out by the National Bureau of Standards/Collaborative Testing Services [*4*] show that the phenolphthalein (Kastle-Meyer) test is the most widely used preliminary color test to locate potential bloodstains and that the Takayama crystal test [*5*] is the preferred confirmatory test. In our laboratory, a microspectroscope is also used to observe the characteristic spectral lines of pyridine hemochromogen produced when Takayama reagent is added to a bloodstain.

These methods have the disadvantage that when dealing with minute stains the entire sample might be consumed either during the preliminary test or when conducting the confirmatory test.

A single test to prove the presence of blood in microscopic amounts would be a great advantage, particularly if it used such a small amount of stained material as to be essentially nondestructive, thus leaving more material for further testing.

Fiori [*1*] states that "absorption spectroscopy of hemoglobin and its derivatives is generally considered the best among the methods for identification of bloodstains with 'absolute certainty'." He further states that "microspectrophotometry may be recognized as an important means for the precise and reliable identification of small bloodstains."

An initial literature search showed Refs *1, 2, 6,* and *7* referring to the use of ultraviolet-visual spectrophotometry" (UV-VIS) in blood examinations. However, in all of them the

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bloodstain had to be extracted in some chemical solution. None of these mentioned the spectra being obtained directly from plain untreated dried blood, or any details on the use of microspectrophotometry. An "accidental" observation made during the examination of some fibers recovered from a bloody knife blade seemed to offer the potential of providing a simple and sensitive test that may encourage the use of microspectrophotometry to recognize minute bloodstains, or stains that have proved unresponsive to other tests.

Method

A Nanospec 10 S microspectrophotometer system fitted to a Leitz Ortholux microscope with a ×25 objective was used. The spectrophotometer was set in the absorption mode with the following parameters: scanning range: 390 to 650 nm; scan speed: 200 nm/min; and time base 10 s/cm. Full scale and slit width were adjusted individually for each sample. Sample preparation is minimal: using a low power stereoscope a single bloodstained fiber or a few fragments of blood crust can be teased out onto a clean microscope slide. Samples can be run dry with or without a coverslip, or mounted in a permanent medium such as a XAM Improved Neutral White (obtainable from Searle Diagnostic, P.O. Box 53, Lane End Rd., High Wycombe, Bucks, England HP12 4HL) used routinely in this laboratory for fiber examinations.

Results and Discussion

Figure 1*a* shows the characteristic absorption curve for human blood, in this case a 10-min-old blood smear. It has a major peak at 421 nm (known as the Soret band) [7] and two

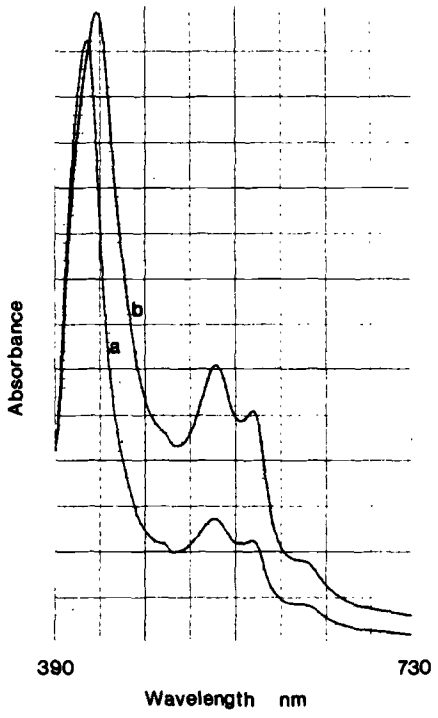

FIG. 1—*Absorption spectrum of blood (oxyhemoglobin) showing the effect of increasing sample thickness/full scale on absorption maxima:* (a) *full scale 135 and* (b) *full scale 150.*

smaller peaks at approximately 545 and 581 nm known as the α and β bands. Sample size presents no limit to the examination. Spectra have been recorded from crusts measuring for example only 10 by 20 μm; indeed we have recorded excellent curves (Fig. 2) from a single red blood cell in a fresh smear (15 min old).

It was quickly apparent that the thickness and irregularity of the sample surface would affect the quality of the spectrum obtained. Best results were obtained from a thin even area, relatively pale in color (normally found at the edge of the crust). Recording from a thicker area with a large slit width will cause noise and shifting of the main peak position to the right towards 433 nm (Fig. 1*b*), although the curve does retain its characteristic appearance.

Further examinations were undertaken to investigate the effects of age, abnormal hemoglobins, and most important of all, the specificity for blood.

Effect of Aging

We were still able to record an identifiable spectrum from an 18-year-old bloodstain which had been stored at room temperature (Fig. 3).

The only consistent feature in connection with aging of bloodstains is a progressive tendency for the intensity of the peaks at 545 and 581 nm to diminish, with the β-band becoming progressively smaller and shallower. It moves back to around 574 nm indicating the formation of methemoglobin [*2*].

It is well known, however, that bloodstains change from red to brown as aging occurs. This means that there should be a corresponding progressive change in the values of the chromaticity coordinates x and y and the luminance value Y [*8*]. Measurement of complementary chromaticity coordinates x'. y' by recording absorption instead of transmission

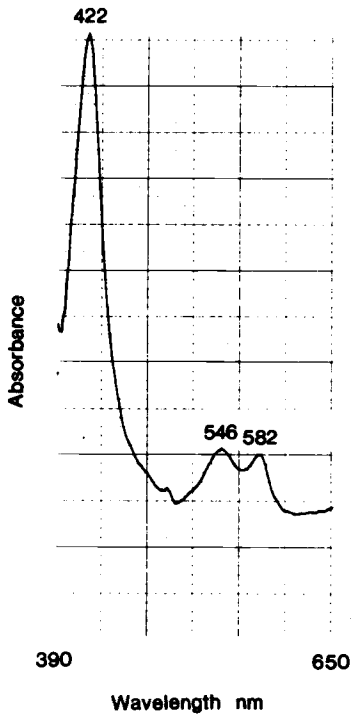

FIG. 2—*Spectrum from a single red blood cell without coverslip (15 min old).*

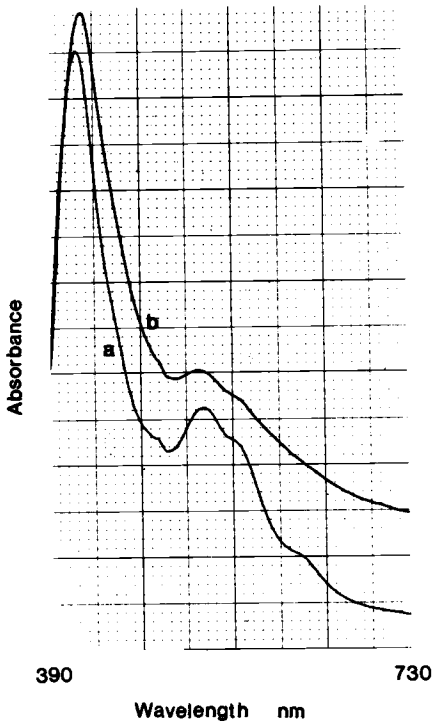

FIG. 3—*Spectrum recorded from* (a) *four-month-old stain preserved at room temperature, full scale 113 and* (b) *stain made in 1967 and preserved at room temperature, full scale 100.*

spectra will eliminate any variance as a result of concentration. A preliminary investigation made using a Commodore PET 4032 computer interfaced to the Microspectrophotometer SDP 2000 by an Anaspec IEE 488 analogue to digital converter, showed that there is indeed variance of x', y' as aging occurs. Further studies on this very complex subject are in progress.

Abnormal, Hemoglobins and Animal Bloods

Stains from other animal bloods were tested (dog, rabbit, ape, chicken, and sheep). All produced the same characteristic curve. No difference was found between the hemoglobin types Hb-A, AC, AS, CC, F, and Sb (Thalassemia). The spectrum obtained from menstrual blood was identical to that obtained from normal venous blood.

Specificity

If the absorption curve obtained from bloodstains can be shown to be specific for blood, the amount of material required for a confirmatory test is dramatically reduced. Accordingly, we examined absorption spectra recorded from numerous red substances (see Table 1). In no case did the curve match that obtained from blood. In the majority of instances, the microscopic appearance of the substance also bore no resemblance to that of blood.

Lemberg and Legge [9] stated that "while it is certainly true that under identical conditions the same substance cannot have different absorption spectra, spectra which are appar-

TABLE 1—*Red colored substances from which absorption spectra were recorded.*

Bile
Lipstick [*2*]
Blusher
Cake icing decorator
Cherry fruit chewy sweets
Tomato sauce
Paprika sauce
Grease pencil
Red plastic sliver [*2*]
Nail polish [*4*]
Xmas tree stain (red)
Nuclear fast red stain
Red newsprint dye
Rust
Felt tip ink pen
Strawberry juice
Raspberry juice
Acrylic paint: Ford Venetian Red
Shoe creme (dark red-brown)
Cherry juice
Carmine

ently identical are insufficient evidence for chemical identify" and further "it is always necessary to demonstrate identical changes in spectra when chemical reactions are performed before the identity of the two substances may be assumed." The absorption spectrum of carmine in ammonia is quoted as an example of a substance that has bands near to that of oxyhemoglobin but does not show modification with acetic acid or reducing compounds [*10*]. Our spectrum recorded from carmine [Sigma Chemical Co.] in ammonia bears no resemblance to that of oxyhemoglobin. However, if this statement leads one to believe that it is essential that the sample under test has to be treated with a chemical in order to obtain a second spectrum, this criterion can be easily fulfilled by the addition of a coverslip and a little Takayama reagent [*5*] to the dry sample. The addition of the Takayama reagent alters the spectrum considerably: although the large peak at about 421 nm remains, the two small peaks at 545 and 581 nm vanish and two other peaks appear—one at 530 nm and a characteristic sharp one at 560 nm [*6*] (Fig. 4*a*). The longer the reagent is in contact with the sample the larger the two peaks become. These peaks are caused by the formation of hemochromogen in this test and correspond to the two dark bands lying between the sodium D and E lines (527 to 590 nm) which are seen in the yellow and green areas of the spectrum in the spectroscopic examination used in some laboratories.

Once the Takayama crystals are formed it is also possible to record a spectrum directly from a crystal of pyridine hemochromogen (Fig. 4*b*), however, this appears to be less satisfactory because of problems caused by recording from the uneven crystal surface, and also quite often the small crystals will drift away from the slit opening. The recording of dual spectra, before and after the addition of the Takayama reagent, should satisfy the criterion proposed by Lemberg and Legge [*9*] and quoted by Fiori [*1*] and show that these absorption spectra are specific for blood. It is also possible to obtain a spectrum from blood adhering to a colored fiber, even if the bloodstaining does not overlap the edge of the fiber. Care has to be taken to obtain a proper background spectrum from the dye of the fiber before running the spectrum of the suspected blood on the fiber. Figure 5 shows a composite of the spectra obtained from a single colored fiber with blood, from the dye only and from the blood after subtraction of the dye spectrum.

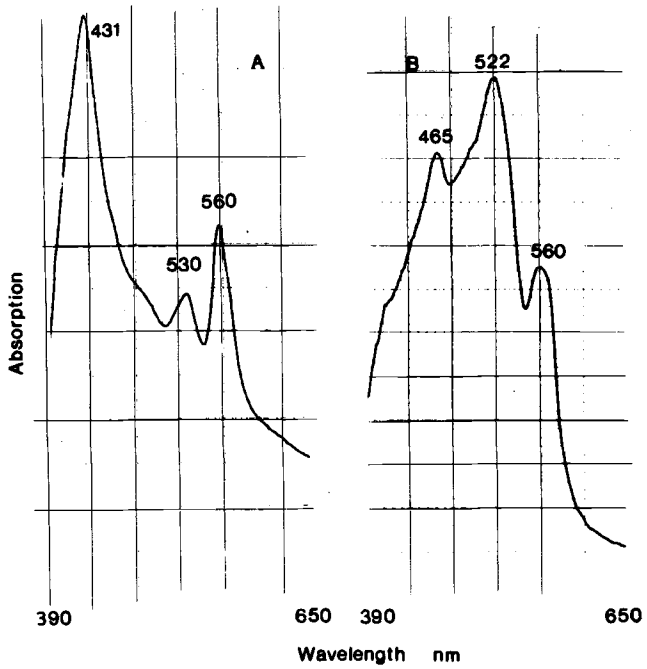

FIG. 4—*Spectrum of* (a) *pyridine-hemochromogen formed on addition of Takayama reagent to a bloodstain and* (b) *spectrum recorded from a single Takayama crystal.*

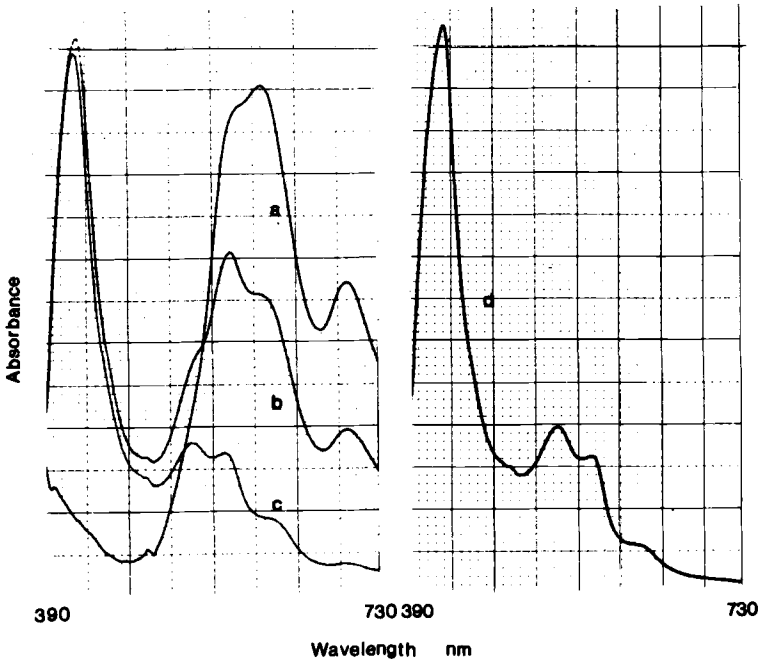

FIG. 5—*Spectrum from* (a) *unstained area of blue polypropylene fiber,* (b) *bloodstained area of the same fiber,* (c) *spectrum of blood produced by subtracting* a *from* b, *and* (d) *spectrum from control bloodstain.*

Summary

The authors do not envisage that this method will replace established techniques for the recognition of bloodstaining, but that it may provide a helpful alternative in specialized instances, that is, when dealing with tiny, particulate bloodstains found for example on weapons, or on tapings from clothing or other fabrics. It may also be useful where dealing with very dilute or washed-out bloodstains.

1. It saves material, using only a single fiber or a very small blood crust instead of a whole thread, and it eliminates the need for a confirmatory test. On material with an open weave pattern, it is possible to run the test by placing the bloodstained area of the material directly on the microscope stage. The saving in material may allow a successful species determination to be carried out, which might not otherwise be possible.
2. It provides a permanent record suitable for court presentation.
3. It may help to associate transferred fibers with bloodstaining or to associate bloodstaining with a particular area of the fabric (by virtue of the fiber type).

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