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The Estimation of Age of Bloodstains by HPLC Analysis

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ABSTRACT: A new HPLC system is described for estimating the age of bloodstains deposited on clothing. In addition to the decomposition peak designated as "X" and reported earlier in the literature, several other ageing processes were studied and found suitable for estimation of age of the stains. The various processes can be used independently of each other for estimation of bloodstain age when the storing temperature is known. Moreover, the ratio between the different peaks formed by ageing is practically independent of temperature between 0°C and 37°C.

KEYWORDS: forensic science, criminalistics, high performance liquid chromatography, bloodstains, age estimation, hemoglobin

Being able to estimate the age of bloodstains is an important task in forensic science. The identity of the bloodstain may be established by DNA analysis but the determination of bloodstain age is desirable when the bloodstain has to be related to the time a particular crime was committed.

Several methods have been proposed for dating bloodstains. The first techniques for the estimation of bloodstain age were based on the decreased solubility of bloodstain in various aqueous solvents with age (see e.g., 1). It has been known for a long time, that bloodstain change in color from red to brown with increasing age, caused mainly by the oxidation of hemoglobin to methemoglobin. Several investigations were based on this fact (2–4). Patterson (5) measured the color changes with a reflectance colorimeter and was able to produce an objective method for color assessment. A non-destructive spectrophotometric method was developed by Kind et al. (6) and an analogous procedure was applied to ammoniacal extracts of bloodstains by Kind and Watson (7). Immunoelectrophoresis (8), surface absorption spectrophotometry (9,10), changes in enzyme activities of blood cells (11) and electron spin resonance technique (12–14) are some other methods applied for determination of age of bloodstains.

High performance liquid chromatography (HPLC) has been successfully used for examination of bloodstains, especially for species identification by an analysis of hemoglobin extracted from the bloodstains (15–17). Inoue et al. (18) reported a method based on a gradual decrease over time in the ratio of α-globin peak to the heme peak in human bloodstains. In the following work, Inoue et al. (19) described a new marker for estimation of bloodstain age by HPLC. The authors discovered a peak whose area increases with age of bloodstain. This peak was designated as "X" and was used to monitor the age of bloodstains stored at 4°C and at room temperature for time period up to about 1 year.

It is clear that many chemical changes occur when blood dries and ages. The disadvantage of chemical methods for dating is that the results are dependent on environmental influences, like temperature and humidity. Because nuclear decay methods can not be applied to age determination in the range of days or months, forensic scientists must search such methods and techniques in which the influence of the environmental influences is as weak as possible.

In this work two HPLC systems were used to study degradation products of hemoglobin in human bloodstains. Three of the many compounds formed on storing the bloodstains were studied quantitatively. The corresponding peaks, designated as "X," "Y," and "Z," were found useful for dating bloodstains on cloth, stored at different temperatures indoors. By relating peak areas of these peaks to each other, results practically independent of temperature were obtained.

Experimental Procedure

Bloodstain Samples

Bloodstain samples were obtained by dropping blood on pieces of white cotton cloth and in some experiments on filter paper. The stains were dried at the temperature of storage, if not otherwise stated. Three storage temperatures were used—0°C, room temperature (22–23°C), and 37°C. For HPLC analysis, a piece of bloodstain (about 2 by 2 mm in size) was cut out and immersed in 100 μL deionized water. The extraction was performed at 37°C for 20 min. Aliquots of 10 μL were taken for analysis.

Reagents—Trifluoroacetic acid (TFA, Uvasol®) was purchased from Merck. Acetonitrile was of HPLC grade and potassium perchlorate was of p.a. purity.

High Performance Liquid Chromatography

Two different HPLC systems were used in this study and utilized in parallel for analysis of the same blood samples.

System A—HPLC chromatograms were run on a Varian Model 5000 Liquid Chromatograph equipped with a variable wavelength detector (model 100). The detector wavelength was set at 220 nm. A fixed wavelength detector (Waters Model 440) operating at 254 nm was connected in series. The column used was a "protein" column—a Vydac 214TP54 (250 by 4.6 mm ID 5 μm particle size, 300 Å pore size, Scandinaviska GeneTech AB). A 2-cm Supelguard column with 5 μm Supelcosil LC-304 packing was used as guard column. The mobile phase consisted of two solvents—solvent A was a 95/5 mixture of acetonitrile/water with

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0.1% TFA added. Solvent B was a 40/60 mixture of acetonitrile/water with 0.1% TFA. The gradient was 10—65% solvent B from 0 to 30 min and 65–75% solvent B from 30 to 60 min. The flow rate was 0.7 mL/min and the system was operated at room temperature. The signals from the UV-detectors were monitored and recorded with an Omega Data system supplied by Perkin Elmer.

System B—This chromatographic system consisted of a Hewlett Packard series II 1090 Liquid Chromatograph connected to the HP's HPLC^{3D} ChemStation. The instrument was equipped with autosampler, auto-injector and diode array detector from Hewlett Packard. HPLC separations were performed using a 25 cm 5μm TSKgel ODS-120T (4.6 mm ID, Tosohaas Bioseparation Specialists) stainless steel column. The mobile phase consisted of two solvents. Solvent A was a mixture of 30% acetonitrile and 70% water containing 10 mM $KClO_4$, pH adjusted to 3.0 with perchloric acid. Solvent B was 100% acetonitrile. The gradient was linear from solvent A to solvent B in 30 min at a flow rate of 0.8 mL/min and operated at room temperature. The diode array detector was programed to record chromatograms at 220 ± 10 nm, 254 ± 10 nm and at 395 ± 15 nm. Full spectra were acquired on all significant peaks from 190 to 600 nm.

Results and Discussion

HPLC System A ("Protein" Column)

The chromatographic system A is useful for separation and detection of peaks corresponding to heme and α and β globin chains from human hemoglobin. In chromatograms of bloodstain extracts, a peak with a retention time of about 7 min was detected at 220 nm. This peak was not observed in extracts from fresh blood and increased with time of storage of the bloodstains as shown in Fig. 1. The peak was designated as "X" in agreement with observations reported by Inoue et al. (19). The increase of peak X with time of storage was measured at three different temperatures, 0°C, room temperature (about 23°C), and at 37°C. The bloodstains were dried at the temperature of storage. The X-peak was detected already after storage for one day at the lowest temperature (0°C).

Figure 2 shows the ratio of the peak area of the X-peak (detected at 220 nm) to that of the heme-peak (detected at 254 nm) as a function of time for bloodstains stored for up to one month. The increase in peak area of the X-peak is clearly non-linear and can approximately be described as an exponential increase ($Y = a \times (1\text{-}\exp(-b/t))$, where t is time and a, b are constants). No marked difference in peak areas was observed for bloodstains stored on cloth and filter paper, respectively (Fig. 2. the two curves recorded at room temperature).

Figure 3 shows changes in the X/heme ratios for bloodstains stored for longer time periods. After the initial non-linear increase for about the first 30 days the ratio increased linearly with increase in storage time.

HPLC System B (TSK ODS-120T Reversed Phase Column)

This chromatographic system uses a reversed phase C_{18} column with smaller pore diameter (120 Å compared with 300 Å for protein column). The column offers a better separation of smaller molecules, whereas it still makes possible the separation of hemoglobin molecule into heme-peak and two globin peaks.

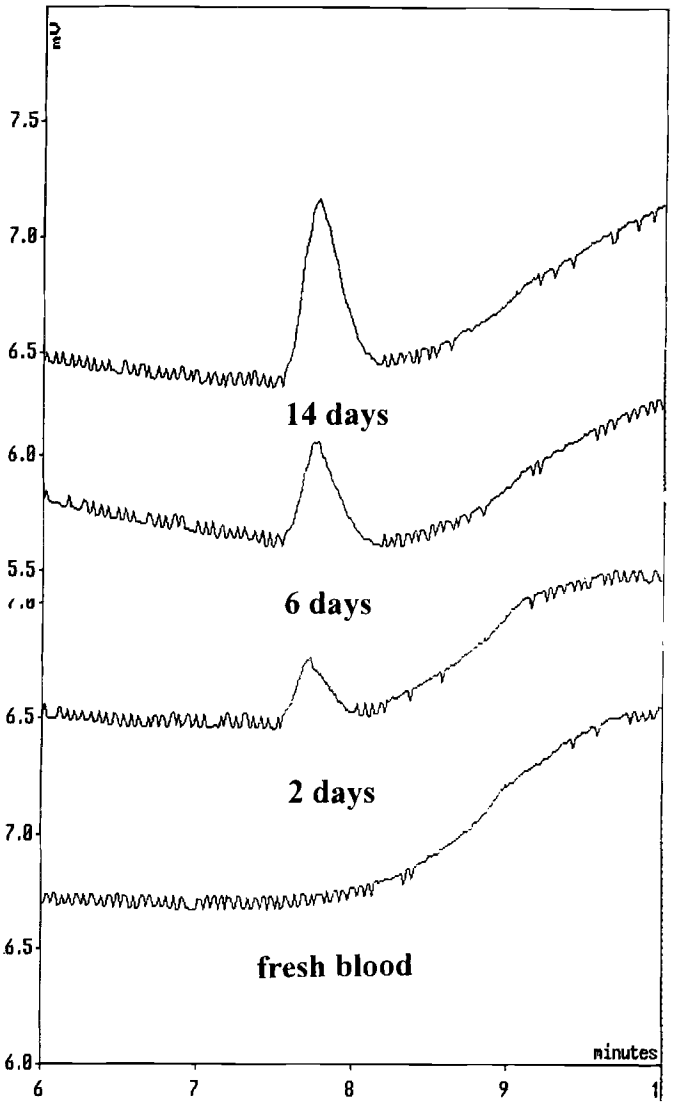

FIG. 1—*The appearance of the X peak detected by the HPLC system A for bloodstains stored on cloth at 37°C for different periods of time. The detection wavelength was 220 nm. It should be noted, that this peak is not present in extracts from fresh blood. The chromatograms in this figure were normalized to the same concentration of hemoglobin.*

Several processes were observed in chromatograms obtained for water extracts from aged bloodstains. Some peaks decreased whereas some increased with time of storage. Peaks that increase with time correspond to various decomposition products and these are the most suitable for age determination. In this study, we followed in detail three peaks designated as "X," "Y," and "Z." These peaks were not detected in extracts from fresh blood and increased with age of bloodstains. Figure 4 shows an initial part of the chromatogram obtained at 220 nm for extracts from fresh blood and from bloodstains stored at 37°C for 20 days, respectively. The peak observed at 4.9 min is the X-peak, the Y-peak has a retention time of 9.9 min. The Z-peak is detected at 395 nm with the retention time of 6.3 min (Fig. 5).

With a diode array detector UV-VIS absorption spectra of the compounds represented by the peaks X, Y, and Z were obtained (Fig. 6). The X-peak detected by the system B is identical with the X-peak observed in the system A. It exhibited absorbance only at short UV wavelengths, with maximum at about 223 nm (Fig. 6). The identity of the compounds represented by these peaks has

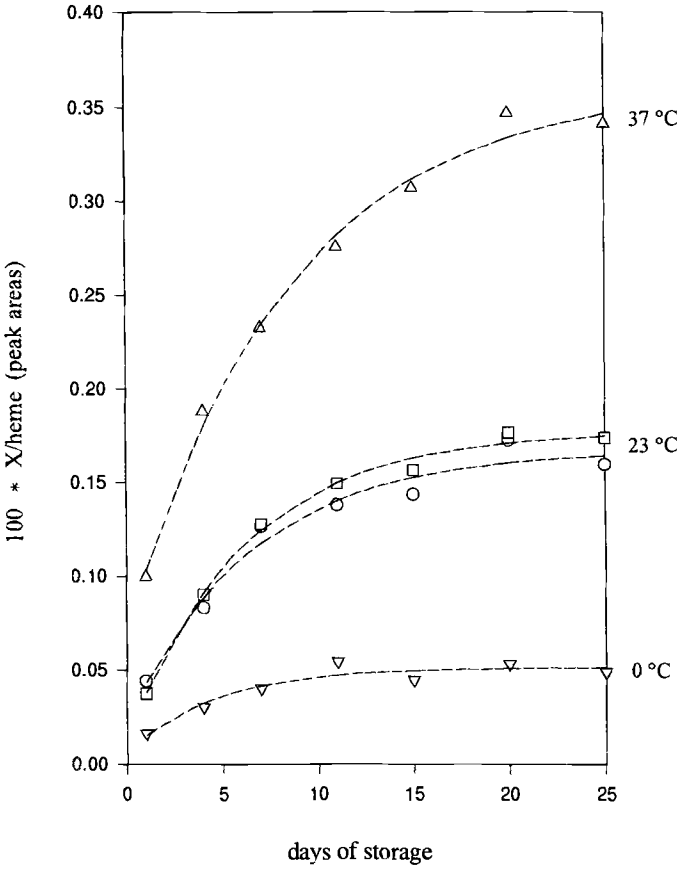

FIG. 2—*The increase of the X peak detected at 220 nm by the HPLC system A as a function of time for bloodstains stored on cloth at different temperatures. The X peak was related to the heme-peak of hemoglobin, detected at 254 nm. The two tracings for storage at room temperature were recorded for bloodstains on cotton cloth (○) and paper (□), respectively.*

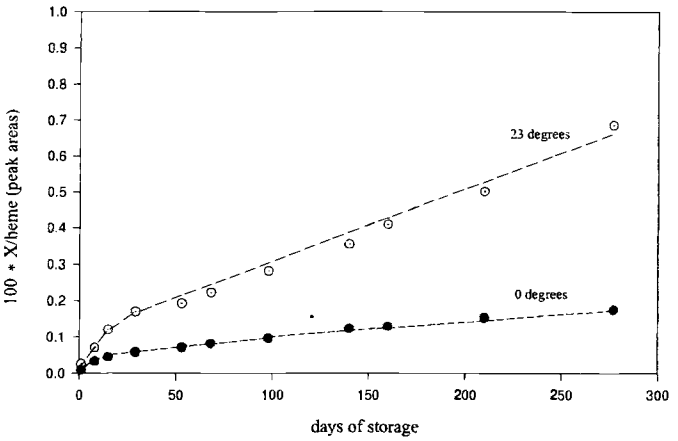

FIG. 3—*The X peak (220 nm) related to the heme-peak (254 nm) for bloodstains stored at 23°C and at 0°C for time periods up to almost 1 year.*

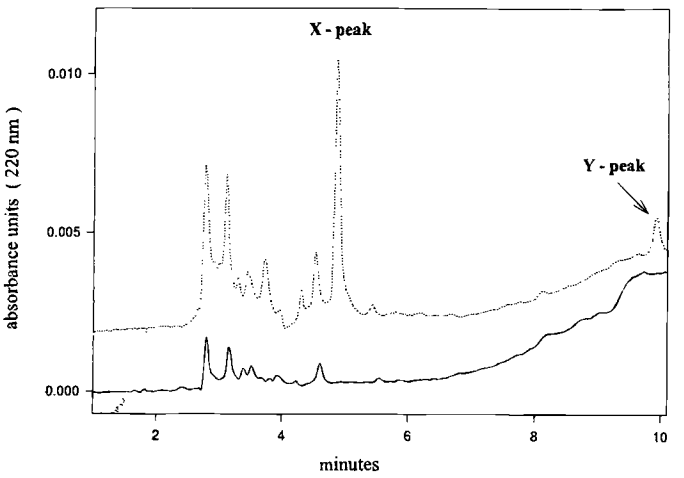

FIG. 4—*The appearance of the X and Y peaks detected at 220 nm in extracts from bloodstains on cloth stored at 37°C for 20 days (dotted line). For comparison, the analysis of fresh blood is shown (solid line). The Z peak is not detected at this wavelength, but is normally observed at 395 nm. Only the initial parts of the chromatograms obtained by the HPLC system B are shown.*

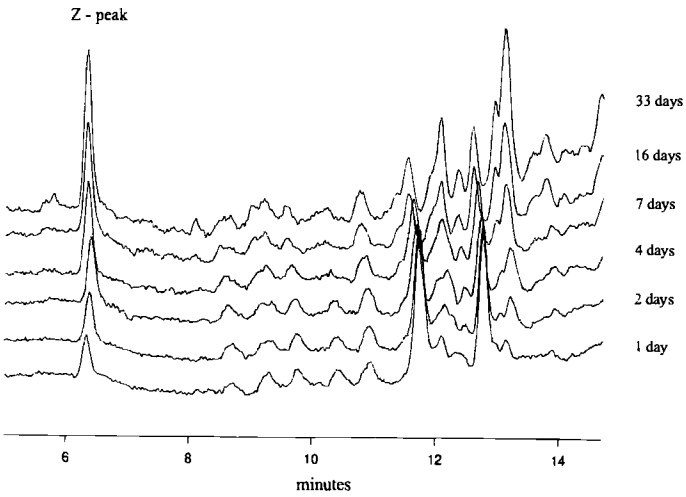

FIG. 5—*The appearance of the Z peak detected by the HPLC system B at 395 nm for bloodstains stored on cloth at 37°C for different periods of time. Other peaks decreasing or increasing with the time of storage can be seen in the chromatograms.*

not yet been solved. The Z-peak showed a spectrum similar to that of the heme-group in hemoglobin, with absorption maximum at 395 nm, and should thus be closely related to it. The spectrum of the heme-group is also included in Fig. 6.

The HPLC system B also detected the α and β globin peaks of human hemoglobin, eluted at about 15 and 16 min (detection wavelength 220 nm) and the heme-peak, eluted at about 19.5 min (detected at all three wavelengths with the maximum at 395 nm). The heme-peak detected at 254 nm was used as an internal standard (IS) and the decomposition's peaks were plotted in relation to this IS. Figure 7 shows the X/heme ratios for bloodstains stored at 0°C, room temperature, and at 37°C. The two tracings obtained for storage at room temperature were recorded for bloodstains on cotton cloth and paper, respectively, and there was no significant difference between them. Figure 8 depicts the Z/heme ratios obtained for the samples described in Fig. 7. Both Fig(s). 7 and 8 exhibit features similar to that of Fig(s). 2 and 3 (system A), namely a rapid increase in amount of degradation products during the first days of storage, followed by a slower, approximately linear increase. The increase of the X-peak in bloodstains stored up to one year was studied with system B and the results (the

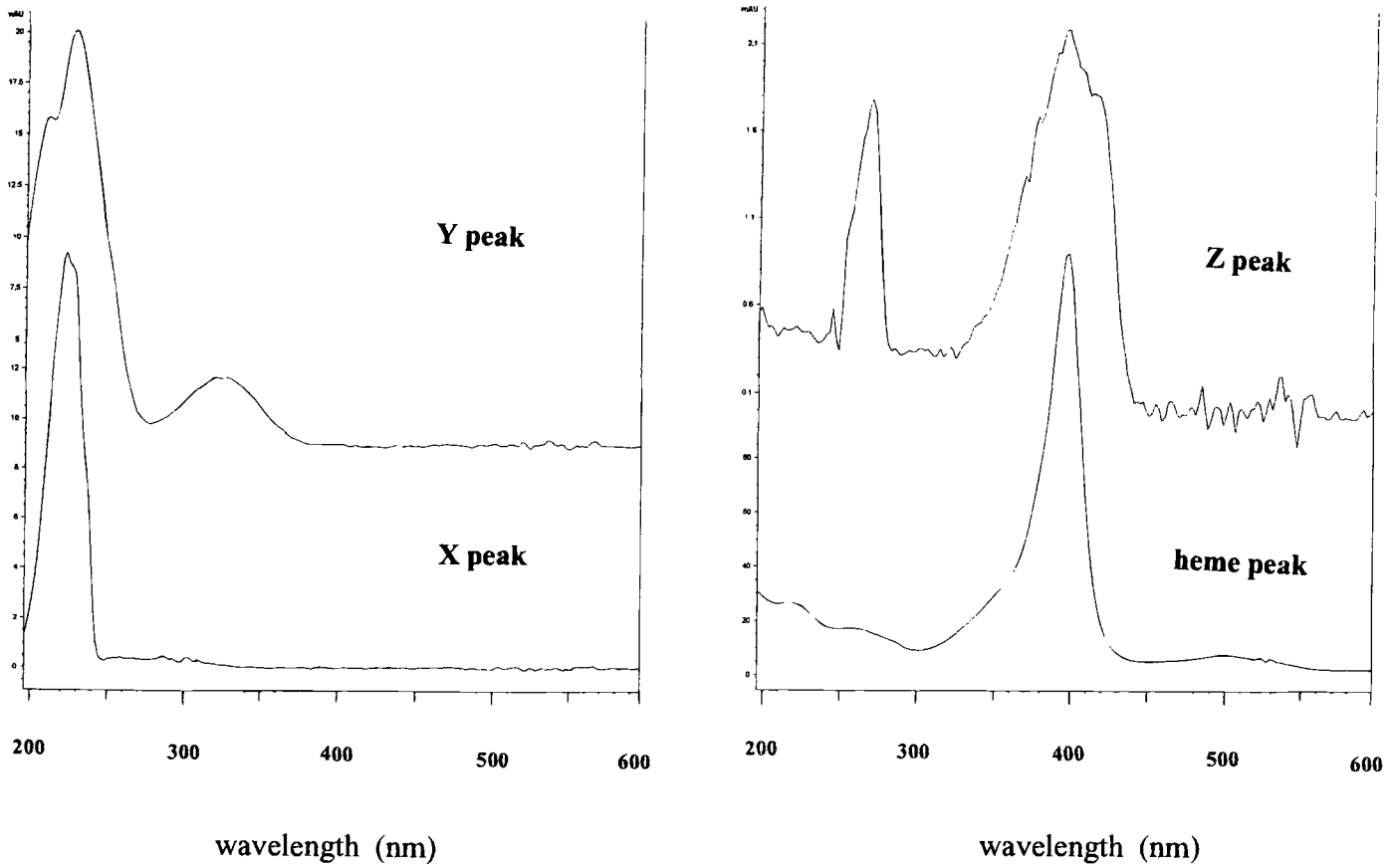

FIG. 6—*UV-VIS absorption spectra of the compounds represented by the peaks X, Y, and Z. For comparison, the spectrum of the heme-group originating from hemoglobin is also shown.*

appearance of the ageing curve) agreed well with those obtained with system A. The discrepancy in the peak ratio values between Fig(s). 2 and 7 depends on the fact that in system A two completely different kinds of detectors were used. The values in Fig. 7 were obtained with a diode array detector and should therefore be the correct ones for the instrumental settings used.

The complete statistical evaluation of precision in peak area determination was not carried out in this study. The mean standard deviation obtained for the X/heme and Z/heme ratios in repeated analyses of the same blood extract was between 5–10%, depending on storing time and temperature. For very short storing time and the lowest temperature investigated the precision was the lowest, because the X and Z peaks are very weak under these conditions.

The ageing of human bloodstains on clothing was studied in blood from three different individuals. The results for bloodstains stored for the same time period and under the same conditions were virtually indistinguishable, the differences between individuals being within 10%.

In some experiments, bloodstains were dried at one temperature and stored afterwards at another temperature. For example, a bloodstain was dried at 37°C (for about 1.5 h) and then stored at room temperature. Within experimental error of the measurements, the decomposition products followed the curve obtained for the temperature of storage. Thus, drying of the bloodstain at 37°C described above did not increase the concentration of the X and Z peaks compared with bloodstain dried and stored at room temperature.

The increase of the decomposition peaks X, Y, and Z is strongly temperature dependent. Thus the peak area of the X peak for bloodstains stored at 37°C is about 6 times larger than that at 0°C. For the Z peak the difference is smaller—the peak area at 37°C is about 4 times larger compared with 0°C. If the average temperature of storage is known, Fig(s). 7 and 8 can be used to estimate age of bloodstains by extrapolation. The ratios between the same peaks measured at different temperatures does not seem to vary much with time of storage. This simplifies the extrapolation for storage at any other temperature between 0°C and 37°C.

Efforts have been made to treat the data in a manner to decrease the influence of temperature. The various decomposition processes might be independent of each other and, using their ratio, a rough estimation of bloodstain age might be possible even when temperature of storing is unknown. Although X and Z peaks increased with age of bloodstains for long time periods, the Y peak showed a different behavior. This peak is formed during the initial period of storage (1–2 days) and then for a longer time period remains essentially unchanged. The peak area depends on the temperature of storage and can in this way serve as a kind of temperature indicator. We calculated peak area ratios X/Y and Z/Y from the results obtained by the system B at three different temperatures. Both ratios increased with time of storage. The X/Y ratio was within the experimental error independent of temperature of storage over the whole temperature range studied. The Z/Y ratio was approximately the same at room temperature and at 37°C, but was somewhat higher at 0°C. Figure(s) 9 and 10 show the calculated X/Y and Z/Y ratios. It should be noted here, that the results are based on single analyses of blood extracts. The precision can be improved by averaging results for repeated injections of the same sample.

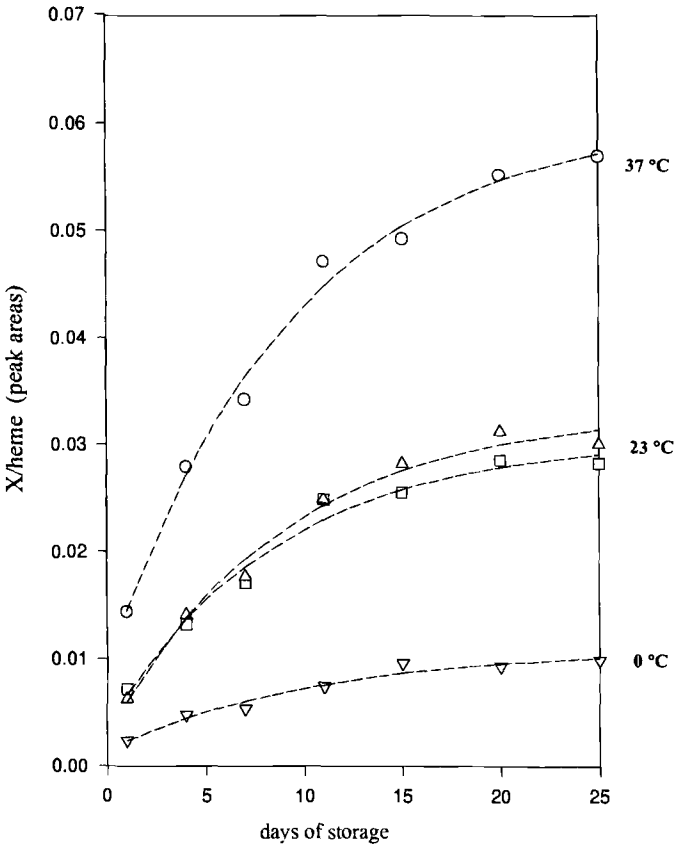

FIG. 7—*The increase of the X peak detected by the system B as a function of time for bloodstains stored on cloth at different temperatures. The X peak was related to the heme-peak of hemoglobin, which also was detected by this HPLC system and could actually be used as an internal standard (wavelength of detection—254 nm). The two tracings for storage at room temperature were recorded for bloodstains on cotton cloth (□) and paper (△), respectively.*

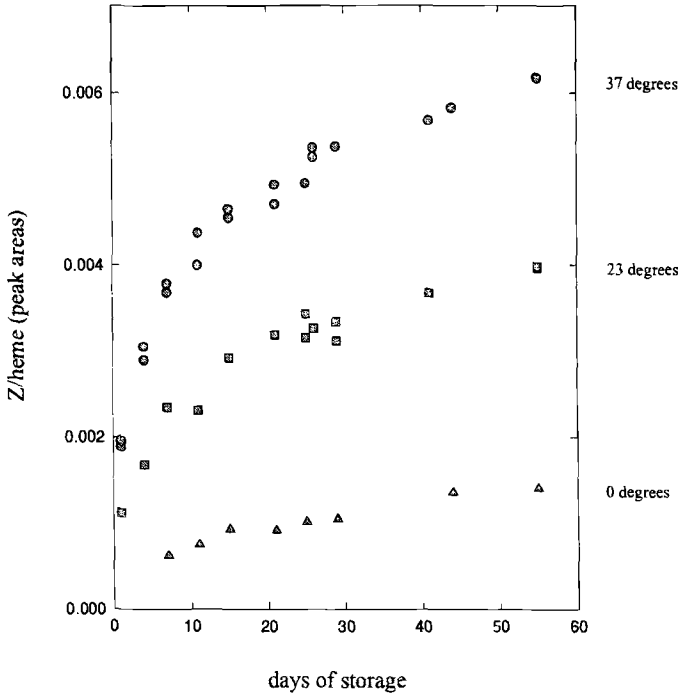

FIG. 8—*The Z peak (detected at 395 nm) related to the heme-peak (detected at 254 nm) for bloodstains stored on cloth at different temperatures. This peak is very weak and not detectable for the first few days of storage at 0°C.*

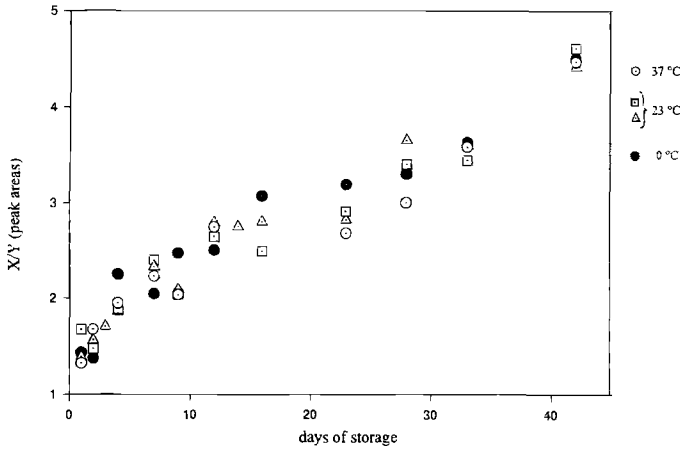

FIG. 9—*The ratio of the X to Y peak areas determined for bloodstains on cloth stored at three different temperatures, between 0°C and 37°C. Two different samples were stored at room temperature (23°C). The results represent single measurements of peak areas for water extracts from the bloodstains.*

The decomposition peaks X, Y, and Z are very small compared with the heme-peak, which can be noted from scale ranges of the Y-axes (see Fig(s). 7 and 8). The experimental errors in determination of peak ratios is therefore rather large. This is particularly important for the results shown in Fig(s). 9 and 10, where ratios between two small decomposition peaks are calculated. The repeated injections of the same sample give values which differ within about 20%. To improve the quantitative detection of peaks X, Y, and Z, larger sample amounts might be injected. There is, however, a limitation in sample size because of the nature of the samples, which contain large amounts of hemoglobin and even other proteins, that can influence the column separation and may contaminate the column. To improve the precision of measuring peak areas, two or more repeated analyses of the same sample should be carried out. We have performed some alternative experiments in attempts to improve the precision of peak area determination. Methanol was used to extract the bloodstains instead of water. The samples were extracted at 37°C for 1 h. Methanol caused denaturation of proteins; thus aliquots corresponding larger amounts of blood could be injected onto the HPLC column. The X and Y peaks were stronger compared with previous experiments. On the other hand, the Z-peak and the heme peaks were weaker. The extraction with methanol cannot be used when the X, Y, and Z peaks must be correlated to the heme group. The ratio of X/Y peak areas was calculated and plotted against time of storage (Fig. 11). As with water extracts, the X/Y ratio increased with age of bloodstains and was highly temperature independent in the temperature range used. Although reproducible, the X/Y ratio for methanol extracts was higher than for water extracts. A possible explanation is that the compound observed as the Y peak is reproducibly, but not quantitatively extracted from samples denatured with methanol.

It is known that the peak area of α globin chain to that of heme on chromatograms from bloodstain extracts gradually decreases with time (18). In our HPLC system B, we also observed this phenomenon. Additionally, the line width of the α and β globin

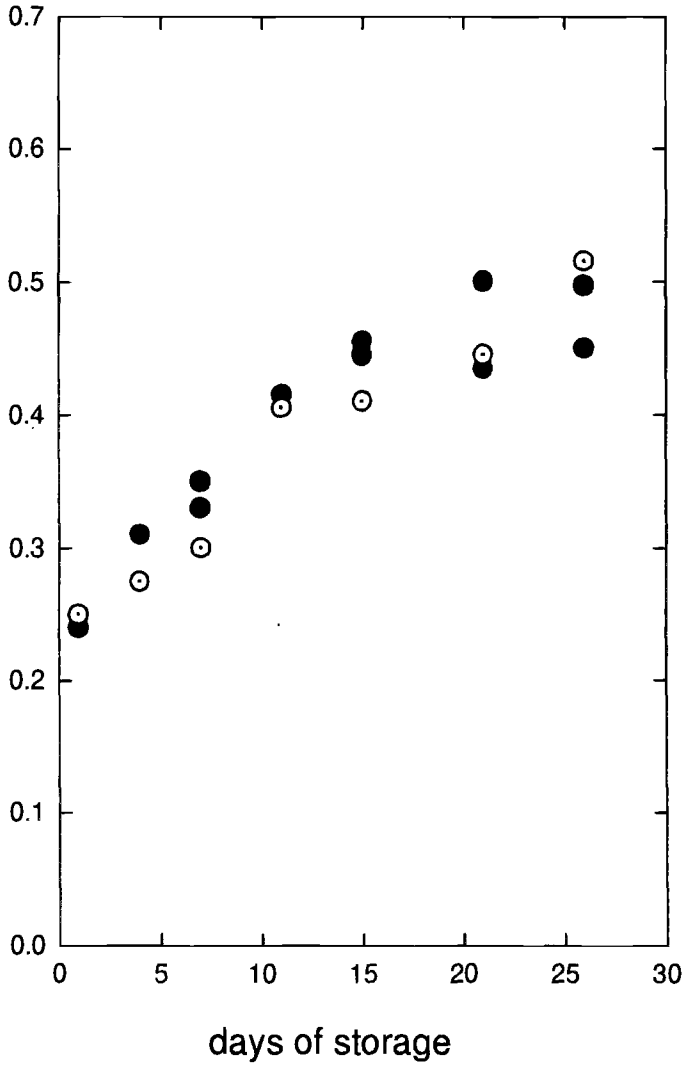

FIG. 10—*The Z/Y ratio (peak heights) measured for water extracts from bloodstains on cloth stored at 37°C and at room temperature (23°C). The values obtained at 0°C differed somewhat from those presented in this figure.*

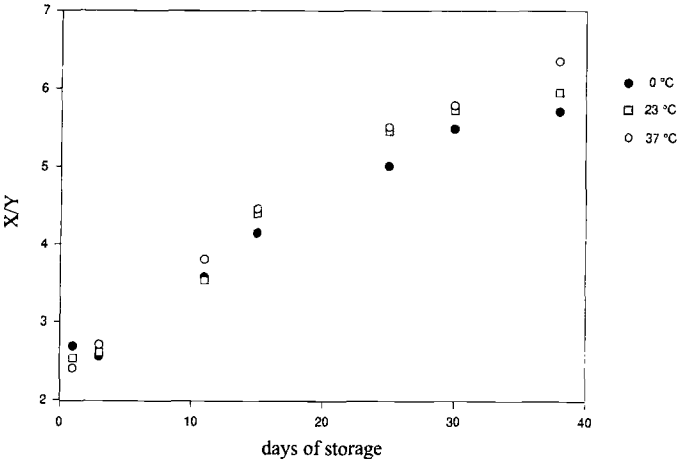

FIG. 11—*The X/Y ratio measured for methanol extracts from bloodstains on cloth stored at three different temperature. This ratio seems to be almost independent of the temperature of storage.*

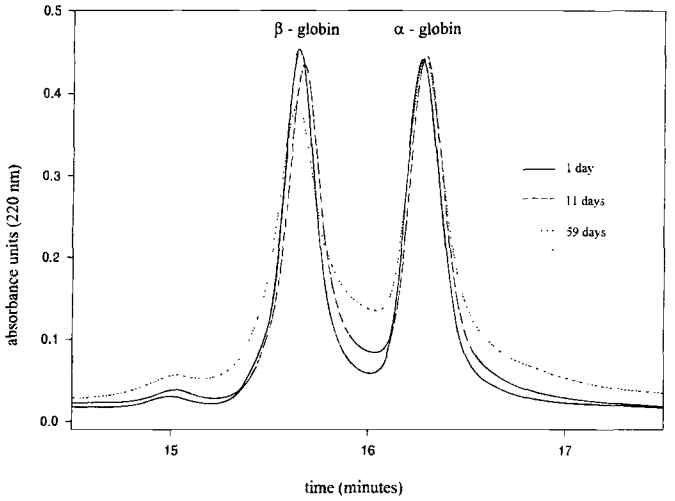

FIG. 12—*Changes in line forms of β- and α-globin peaks detected at 220 nm by the HPLC system B with time after storage at 37°C. Time of storage—1 day (connected line), 11 days (dashed line) and 59 days (dotted line). The valley position between the peaks and the peak width of both peaks increases and the height of the globin peaks related to that of the heme-peak decreases continuously with time of storage. This process is temperature dependent, being more pronounced at higher temperatures.*

chains increased with time of storage. As the globin peaks are eluted close to each other, the ageing process can be followed from the less efficient separation of these peaks and from the increasing valley position between them (Fig. 12). The reproducibility of the line forms for the globin peaks is good if the column is clean and a blank analysis is performed prior to the injection of blood sample. This process is, however, temperature dependent and not very suitable for extrapolation between different temperatures.

To help understand the degradation of hemoglobin in bloodstains, one should mention that the same degradation peaks were detected in water extracts of bloodstains from pig as well as human blood. The system B succeeded in separating pig hemoglobin into two globin peaks and one heme peak. The differences in the retention times observed for globin peaks of pig and human hemoglobin were much smaller compared with the system A.

Conclusions

Both HPLC systems, described in this study, are suitable for detection and analysis of peak "X," reported earlier in the literature as a marker for estimation of bloodstain age. The HPLC system B using a reversed phase TSK 120T endcapped column has several advantages compared with the system A using a 300 Å protein column. A number of ageing processes resulting in the formation of various decomposition peaks were observed by this system and appeared to be independent of each other.

These independent processes should improve the possibility of estimation of bloodstain age, because the results calculated from

X/heme respective Z/heme peak ratios can independently be used as control.

The results presented in this study consider bloodstains on cloth stored indoors. Samples were not exposed to extreme humidity and have not been wetted. The humidity range was 15–65%, which represents indoor humidity normally encountered in our country. If the average storage temperature is known, the age of bloodstains can be estimated from Fig(s). 7 and 8 by extrapolation. Duplicate analyses are recommended for better precision. The finding of X/Y peak ratios being practically independent of temperature of storage is the most interesting aspect of this study. It can be seen from Fig(s). 9 or 11, that the age of bloodstains stored at unknown temperature cannot be determined exactly. The uncertainty in age determination is several days and this uncertainty increases with the time of storage. However, there should not be any problem in distinguishing between bloodstains, e.g., 1 week respectively 3 weeks old, which is a task that should be solved when age of bloodstains is estimated in forensic science. We do not have data concerning the X/Y peak ratios for storage longer than 60 days.

This study should be considered as preliminary. The bloodstains investigated in this study were all stored at constant temperature and relatively low humidity. The ageing of bloodstains monitored by chemical methods can be influenced by many factors in addition to temperature and humidity. The problem is complex and many variables that may have an effect on the results should be investigated. Presently we are investigating other storing conditions, e.g., significant and frequent variations in temperature of storage. Ageing of bloodstains on non-porous materials like blades of knifes or glass will also be studied.

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