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CHARACTERIZATION OF BIOPOLYMERS BY PYROLYSIS GAS CHROMATOGRAPHY AND MULTIDIMENSIONAL ANALYSIS

APPLICATION TO SYNTHETIC MELANINS

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SUMMARY

A method for characterization of the melanin biopolymer has been developed and validated by the use of synthetic melanins derived from tyrosine, dopamine or hydroquinone. The technique involved pyrolysis gas–liquid chromatography with capillary columns. A back-flushing technique is described which improves pyrogram reproducibility such that closely related melanins can be distinguished with the aid of principal components analysis and non-metric multidimensional scaling.

INTRODUCTION

For many years researchers have been looking for techniques that can reliably characterize the polymeric pigment, melanin[1,2]. Degradative[1] and various spectroscopic techniques[3–6] have been investigated, but they lack the ability to differentiate between similar melanin types.

Pyrolysis of biopolymers results in thermal fragmentation, forming a pyrolysate that is characteristic of the original molecule. The pyrolysate can then be analysed by mass spectroscopy[7,8] or gas–liquid chromatography[9,10]. Previous applications have involved the characterization of bacteria[11,12], fungi[13] and carbohydrates[11].

This paper describes how pyrolysis gas–liquid chromatography (Py-GLC) provides a method for differentiation between synthetic melanin polymers. Characteristic, reproducible pyrograms are readily obtained. This is due, in part, to reproducible sample pyrolysis and capillary column–pyrolysis interface back flushing. A new method for modelling pyrograms in multidimensional hyperspace is described that enables all characteristic reproducible peaks (data) appearing in the pyrograms to be used in the modelling procedure. Pattern recognition methods such as principal com-

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ponents analysis[14] and non-metric multidimensional scaling[15] are then applied to the modelled hyperspace in order to make some qualitative assessments about the similarities between synthetic melanins.

EXPERIMENTAL

Apparatus

A Packard 430 gas chromatograph (Packard Instrument Company, Downers Grove, IL, U.S.A.) with a flame ionization detector was used. The chromatograph was fitted with a temperature-controlled horizontal injection port interface (Chemical Data Systems, Oxford, PA, U.S.A.) designed to accept a platinum ribbon (35 mm × 1.5 mm × 0.0127 mm) Pyroprobe®, controlled by a Chemical Data Systems 100 series solids pyrolyzer. A GISS-4AK inlet splitter system (SGE, Melbourne, Australia) was used to set splitting ratios. A 28 mm × 0.23 mm I.D. SP2100 wall-coated open tubular (WCOT) glass capillary column was used for chromatographic separations. Helium was used as carrier gas ($\bar{v}$ = 28 cm/s) and the column head pressure controlled by a PR8286 pressure regulator (SGE).

For optimal detector sensitivity, detector make-up gas (helium) was introduced via a three-way zero dead volume fitting connected at the end of the capillary column. A column back-flushing system was designed, which consisted of two tri-directional valves (B-41XS2; Whitey, Highland Heights, OH, U.S.A.) and a MNVU micro needle valve (SGE) that was positioned between the end of column and the detector. The plumbing for the back-flushing system is shown in Fig. 1. All connective plumbing utilized glass-lined steel tubing.

A Dick Smith System 80® microcomputer (Dick Smith Electronics, Australia) was interfaced with the printer circuitry of the gas chromatograph. This allowed automated data handling of the integrated peak height values calculated by the microprocessor of the gas chromatograph. A listing of the circuitry and programs is available from the authors on request.

Synthetic melanins

Melanin was prepared from dopamine hydrochloride (Sigma, St. Louis, MO, U.S.A.) and hydroquinone (Sigma) by the method of Das *et al.*[5]. Commercially available, synthetically prepared tyrosine-derived melanins were purchased from Sigma and from Koch-Light Laboratories (Colnbrook, U.K.). These melanins were used without further treatment or purification.

Procedure

Synthetic melanins were ultrasonically suspended (Bransonic Model 220, 125 W) in methanol (25 μg/μl) for 15 min. A 20-μl aliquot was then uniformly layered in the middle 3-cm region of the pyrolyzer ribbon. Residual methanol was evaporated by heating the ribbon to 100°C. The probe was then inserted and sealed into the heated injection port interface (250°C) and the complete system purged with carrier gas for 3 min to ensure anaerobic pyrolysis. The pyrolysis ribbon was heated to give a temperature rise of 20°C/ms to a final temperature of 1000°C, which was maintained for 20 s. A sample splitting ratio of 10:1 was used. Pyrolysis and the oven temperature program were initiated simultaneously. The oven temperature program consisted of

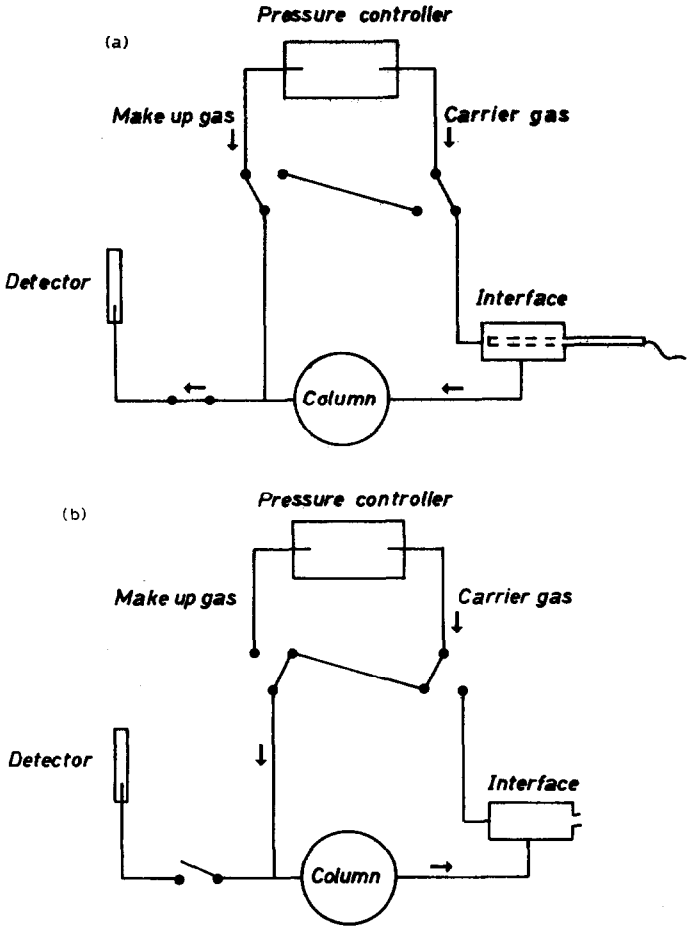

Fig. 1. Diagrammatic representation of the plumbing and the direction of gas flow in the gas chromatograph: (a) run mode; (b) back-flush mode.

a 5-min isothermal period at 50°C, a 15°C/min ramp for 5 min to 125°C, followed by a 2°C/min ramp for 5 min to a final temperature of 135°C. The pyrolysis ribbon was cleaned by flashing at 1400°C for 2 s between analyses. Column back-flushing at 165°C for 20 min was performed after every second pyrolytic analysis. Whenever the column was not being used for analysis, it was left in the back-flushing mode at 165°C. At least four pyrograms were obtained for each melanin type so as to allow a statistical basis for pyrogram comparisons.

Pyrogram comparison

Multidimensional hyperspace models were used to describe all pyrograms. Characteristic reproducible peaks from at least four pyrograms were selected by means of a learning set[16]. The mean and standard deviation of the retention time were calculated for each peak. Peak heights were normalized on an intra-pyrogram

basis to sum 100. When the retention times of separate peaks in replicate analyses were within twice the standard deviation of the mean of another peak, only the peak with the largest normalized peak height value was retained. Statistical equivalence of the retention times of the reproducible peaks in the differing melanin samples was then determined using the previously computed mean and standard deviation values. Single *t*-tests, the Tukey or Scheffé methods of statistical analyses were used where appropriate[17]. Independence was assumed for all comparisons and the level of significance was $p \leqslant 0.05$. A "dimension equivalence table" was constructed grouping statistically equivalent retention times (dimensions) between melanin types. Normalized peak height values for all pyrograms are matched with their respective dimension. Pyrograms that do not have a peak height value at a retention time that appears in another melanin group are given a value of zero.

Principal components analysis[14,18] and non-metric multidimensional scaling[10,15] were applied separately to the pyrograms modelled in the multidimensional hyperspace. The normalized peak height values, matched with their respective dimensions as indicated from the dimension equivalence table, formed the data input matrix for the principal components analysis. This was performed using the technique of principal factoring without iteration (PAI) and optimized rotation (Varimax) by the computer package SPSS[18] on a Cyber 73-16 computer. Non-metric multidimensional scaling (non-linear mapping) was performed on a triangular data matrix of euclidean distances, calculated between the pyrograms modelled in hyperspace. The euclidean distance in a multidimensional hyperspace of k dimensions, d_{ij}, is a measure of the similarity between two points, x_i and x_j:

$$d_{ij} = \left[\sum_{k=1}^{n} (x_{ik} - x_{jk})^2 \right]^{\frac{1}{2}}$$

Non-metric multidimensional scaling and co-ordinate mapping was performed by the computer package ALSCAL[19] on a Cyber 76 computer.

RESULTS AND DISCUSSION

Pyrogram reproducibility

The four types of synthetic melanins characterized by Py-GLC were the synthetically prepared dopamine and hydroquinone derived melanins, and the commercially available Sigma and Koch-Light melanins. Replicate pyrograms of these melanins that were obtained on different days of analysis are shown in Figs. 2–5. The replicate pyrograms are shown in order to demonstrate the reproducibility of the Py-GLC system. The reproducible pattern of peaks, and the consistency of peak heights indicate the reproducibility of the ribbon probe pyrolysis. The broad band of apparently unresolved material is due to column bleed that occurs when the oven heating profile is 15°C/min. Reproducibility of retention time in this region was unaffected by the column bleed, and the resolution of the pyrolysate was not impaired by utilizing this heating profile. The advantage gained by using this temperature program was the short analysis time of 15 min.

The retention times for the ten reproducible peaks of four pyrograms of Sigma

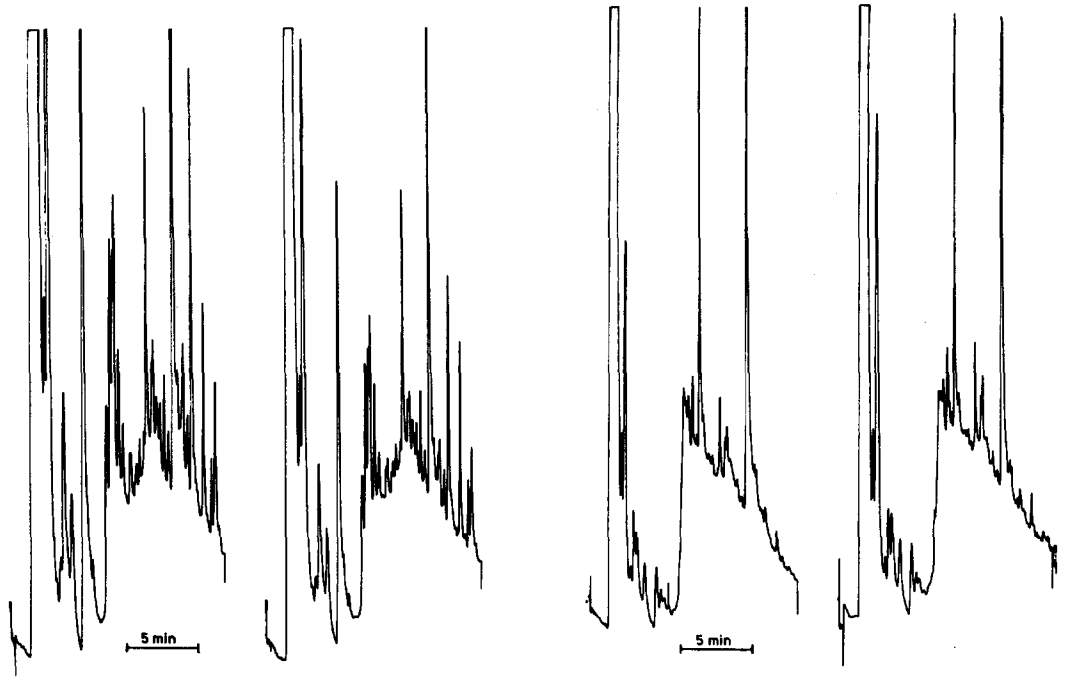

Fig. 2. Replicate pyrograms obtained from dopamine-derived melanin.

Fig. 3. Replicate pyrograms obtained from hydroquinone-derived melanin.

melanin are given in Table I. A number of factors contributed to the obvious reproducibility of retention time. A column head pressure controller was incorporated in the carrier gas line. This assumes importance during temperature programming of complex mixtures. Column contamination due to high-molecular-weight and/or poorly volatile substances is a common problem when analysing complex biopolymers by Py-GLC[11], as it will decrease retention time reproducibility. After continual pyrolytic analysis, spurious or "ghost" peaks often elute unless a column preservation procedure is used[11,12]. The back-flushing procedure described has preserved the integrity of the capillary column after more than 300 samples have been analysed. The back-flushing procedure is simple to operate and has the advantage that the detector gas flows are not altered during the back-flushing. After back-flushing is complete, the appropriate valves are switched, and as the sensitivity of the detector has been maintained, analysis can recommence immediately.

Multidimensional modelling

Once reproducible pyrograms are obtained, methods for their comparison are required which will then allow qualitative assessment of the polymeric similarity of the original melanins. The pyrograms were modelled in multidimensional hyperspace which was then analysed by two separate pattern recognition techniques. Kowalski and Bender[20] made a pertinent observation when they noted, "The true value of pattern recognition is realised when several methods are used in combination as a

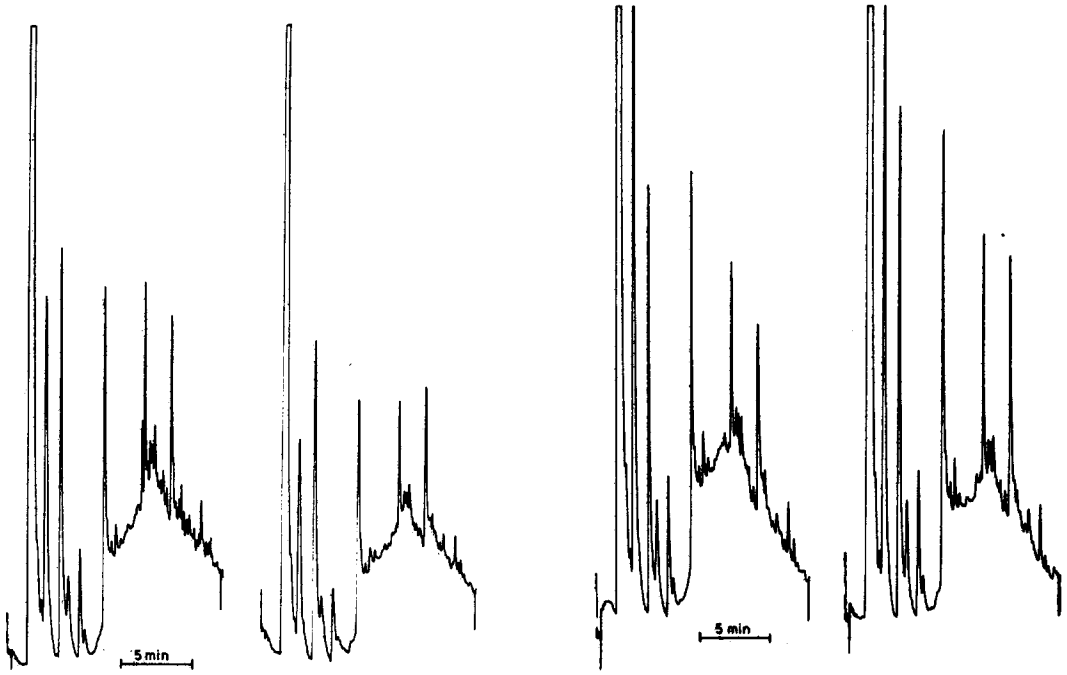

Fig. 4. Replicate pyrograms obtained from Sigma melanin.

Fig. 5. Replicate pyrograms obtained from Koch-Light melanin.

system." Both principal components analysis and non-linear mapping are used in this laboratory. If the same qualitative inter-relationships are exhibited in the multidimensionally modelled pyrograms following analysis by both data reduction techniques (one method using a linear and the other a non-linear mathematical model), one can be confident of the qualitative nature of the results. Previous methods of pyrogram modelling[12,13] have used what is best described as the selective window approach. Reproducible peaks (retention times) in a pyrogram of the standard polymer are identified, and the occurrence of these peaks (retention times) in pyrograms of other types of polymers noted. The problem with using this approach to define

TABLE I

RETENTION TIMES OF THE TEN REPRODUCIBLE PEAKS OBTAINED BY PYROLYSIS OF SIGMA MELANIN

Each of the replicates was run on a different day.

Replicate	*Retention times (min)*									
1	2.65	3.90	6.75	8.10	9.00	9.80	10.35	10.75	11.20	12.60
2	3.00	4.00	7.05	8.20	9.10	9.95	10.45	10.95	11.40	12.70
3	2.80	4.00	7.00	8.35	9.00	9.85	10.40	10.80	11.30	12.50
4	2.85	3.95	7.00	8.15	8.60	9.80	10.45	10.90	11.35	12.65

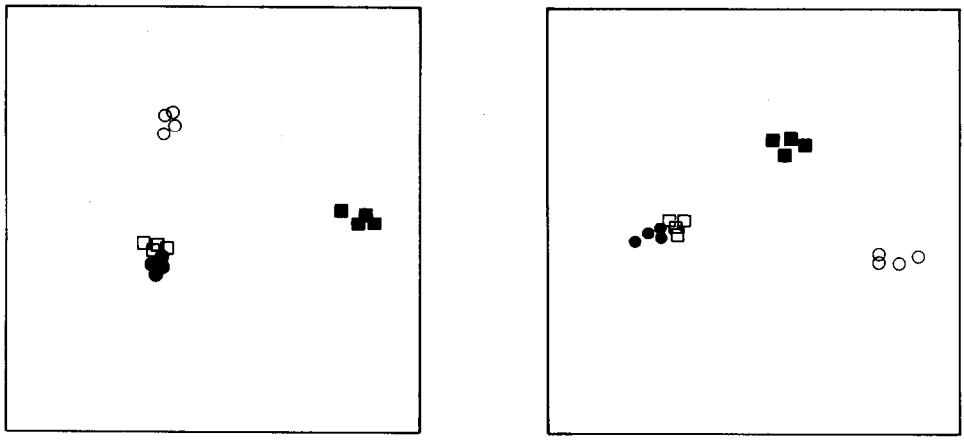

Fig. 6. Plot of the facscores associated with the first principal component *versus* the facscores associated with the second principal component of four replicate pyrograms of each melanin type: ○, hydroquinone-derived melanin; □, Sigma melanin; ●, Koch-Light melanin; ■, dopamine-derived melanin. Variation explained by the first two principal components is 86% of total variation in 36 dimensions.

Fig. 7. Two-dimensional map produced by non-linear multidimensional scaling of four replicate pyrograms of each melanin type. Symbols as in Fig. 6. SStress (= stress index derived from squared distances) = 2.7%, RSQ = 99.7%; for explanation see text.

the dimensions in the hyperspace is that reproducible peaks in the test pyrograms may be discarded if they do not correspond with the retention time windows that are set by the standard pyrogram. This procedure introduces an artificial similarity in the pyrograms when they are modelled in hyperspace. The approach taken in this work has been to retain all reproducible peak data from all samples, and after appropriate statistical comparison of retention time equivalence, model the complete pyrograms in multidimensional space.

Pyrogram comparison

Fig. 6 shows a principal components plot, and Fig. 7 shows a non-linear map, of a set of pyrograms. Each symbol on these plots represents one complete pyrogram. In both cases the plots are two-dimensional representations of the spatial arrangement of the points in the multidimensional hyperspace. A similar arrangement is evident in the two different plots. Thirty-six-dimensional space was required to model the pyrograms for this analysis.

The Koch-Light and Sigma melanins have plotted close together, inferring similarity in their polymeric structure as analysed by Py-GLC. This is not unexpected as they are both tyrosine-based melanins. The dopamine and hydroquinone melanins have plotted separately from the Koch-Light and Sigma melanins and this is indicative of differences in their structure. Visual analysis of the pyrograms supports these groupings. The principal components analysis has described 86% of the variation in the data in two dimensions and the squared correlation index (RSQ) and the squared stress index (SStress) values for non-linear mapping are 99.7% and 2.7% respectively[19]. These values indicate that these projections in two dimensions are a good rep-

resentation of the similarities exhibited in the data. Both methods of pyrogram comparison exhibit the same relationships in the data, so these plots can be viewed with confidence.

Pyrolysis GLC has had limited use as an analytical tool due to problems associated with pyrogram reproducibility and subsequent analysis of the pyrograms. Many researchers use pyrolysis mass spectroscopy[7,8] to overcome these problems associated with Py-GLC, however, access to mass spectrometry is often limited and contamination of the instrumentation due to the pyrolytic procedure is of major concern. This paper has shown that reproducible Py-GLC of complex polymers can be obtained, provided adequate steps are taken.

The techniques described have the ability to characterize or "fingerprint" synthetic melanin polymers. Clear discrimination of synthetic melanin types is now possible by application of the technology described. Studies are currently underway to characterize some naturally occurring phenolic polymers.

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