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REFERENCE MATERIALS FOR THE ANALYSIS OF ORGANIC COMPOUNDS OF ENVIRONMENTAL AND OCCUPATIONAL CONCERN

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Summary: The requirements, preparation, purification, characterisation, determination and certification of purity, utilisation and handling of reference materials for the analysis of organic pollutants found in the environment and workplace are reviewed in relation to specific groups of organic compounds.

I PRODUCTION, CERTIFICATION AND APPLICATION

1. Introduction: Problems and Needs

Monitoring the presence and concentrations of the many organic pollutants occurring in the environment and in the workplace poses problems not only in their identification but also in their accurate and reproducible analysis by the numerous national and international agencies and organisations involved in such monitoring. Harmonisation of the results obtained by the various laboratories is therefore an important consideration.

Contamination of the biosphere by polycyclic aromatic compounds (PACs) including polycyclic aromatic hydrocarbons (PAHs), their related heterocyclic analogues, containing oxygen, sulphur or nitrogen in one or more rings, and nitro-PAHs has been well established[1-6]. Similarly global pollution by organochlorine compounds (OCs) including organochlorine pesticides and polychlorinated biphenyls (PCBs) has been established by a number of environmental surveys[7,8].

PACs are known to pass into the environment during the combustion of fossil fuels in power stations, and in domestic houses, as well as from industrial plants, car exhaust gases[9,10] and directly through the use of manufactured materials such

as lubricants, detergents, dyes, plastics and solvents, or indirectly by their degradation[11]. PACs may also contaminate the workplace[12].

Because PCBs are very stable to biodegradation and have passed into the environment due to their widespread use in past decades for industrial applications, they have become persistent pollutants of the biosphere. Numerous studies of their levels and their regional and global distribution have been published (e.g. see [13-17]).

Many PACs and OCs are biologically active and PACs with four or more fused rings are known to be carcinogenic[4]. Their wide occurrence in the environment creates long-term health risks to all living organisms. If it is assumed that such compounds may be released into the environment at an annually increasing rate of between 2 and 3%, contamination levels could rise by one or more magnitudes within the next century[18].

Because of the health risks, and because many countries have issued official or legislative controls with respect to the concentration levels of a number of these organic pollutants in food and drinking water, in the atmosphere, and in other environmental matrices, there is a clear requirement for the regulatory control of such pollutants. This in turn requires the identification and accurate analysis of the component pollutants at trace levels (μg/kg to μg/g). The availability of pure certified reference materials (CRMs) of representative organic pollutants greatly facilitates this, and the use of CRMs plays an important role in the normalisation of the quantitative measurement of PACs and OCs in the environment when measurements are carried out in independent monitoring laboratories[19]. The provision of suitably pure CRMs for this purpose has been the aim of a series of cooperative projects developed by the Community Bureau of Reference (now renamed the Measurements and Testing Programme (M & TP)) of the Commission of the European Communities, and at present 74 CRMs of this type are available from the M & TP.

2. Preparation of Pure Reference Materials

2.1 Selection and Purity Criteria

In making a choice of the most suitable or representative compounds to be

prepared as potential CRMs for issuance, a number of considerations were taken into account.

Thus for the PACs the following points were assessed:

- Occurrence of the compound as an environmental and occupational pollutant.
- Carcinogenic and toxicological aspects.
- Nonavailability of the pure compound from commercial or other sources.
- National and international regulations or recommendations published by organisations such as the European Economic Community (EEC), World Health Organisation (WHO), and the U.S. Environmental Protection Agency (EPA).
- Analytical requirements.

In the case of the PCBs the additional points considered were;

- The RMs should be key representatives of environmentally found PCBs.
- There should be at least one high-purity isomer representing each of the six isomeric groups (di- to hepta-chlorobiphenyls) available.

Additional requirements were that the chosen compounds could be prepared homogeneously to the required purity level, that sufficiently reliable and reproducible methods of analysis existed, and that the prepared compounds were stable for a prolonged period of time (a five year period was envisaged).

The minimum purity level requirement was set at a mass fraction of 0.99 g/g with the additional requirement that any major impurity remaining in the purified material at levels of more than 0.0001 g/g should be identified by mass spectrometry, nuclear magnetic resonance spectrometry, or other applicable method.

At the outset of the programme a pilot study was undertaken in order to establish that these technical points could be met and that the analytical methods applied were reliable.

For use as practical CRMs for the calibration of analytical instruments or analytical procedures both PACs and PCBs can be prepared in various forms:

1. As solid compounds of certified purity.
2. As solutions containing a certified or specified concentration of the reference material.

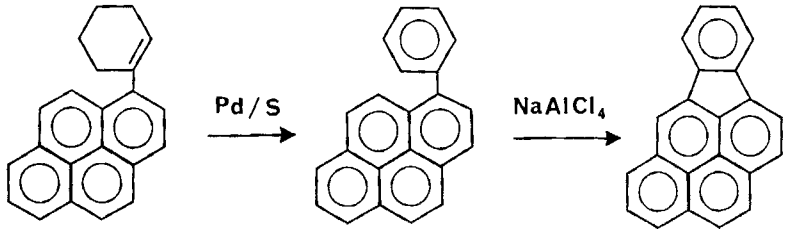


FIGURE 1
Synthesis of Indeno(1,2,3-cd)pyrene

3. As a matrix material containing certified concentrations of the pollutants.

The preparation of pure reference materials as solids can proceed by two possible routes: by multistage synthesis of the compound followed by purification of the product, or by the purification of a commercial sample. In general synthesis was the preferred route since the cost was lower, but in some instances the purification route was utilised, particularly when the commercial sample was already at a level of purity appropriate for further purification. Certain RMs falling within the categories 2 and 3 above have also been produced under the M & T programme, and are briefly considered in sub-section 9.

2.2 Synthesis

In the majority of cases the most readily applicable synthetic procedure described in the literature was found to give, by scaling up, acceptable yields of the required compound. The target amount was usually in the range 5 to 50 g. In a few instances alternative synthetic routes were developed where the literature method provided very low yields, or where unwanted by-products were formed which were not easily separated during the purification process. Examples of improved or developed syntheses are those for indeno(1,2,3-cd)pyrene[20] (Figure 1) and for cyclopenta(cd)pyrene[21].

The synthetic procedures of condensation, addition, cyclisation and reduction were generally applied in the synthesis of the polycyclic aromatic compounds including heterocyclic analogues. Two examples are illustrated by the structural equations given below (Figures 2 and 3).

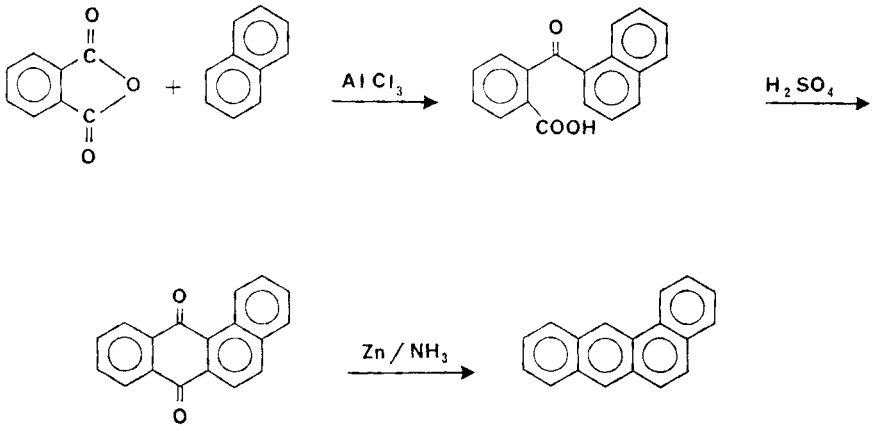


FIGURE 2
Synthesis of Benz(a)anthracene

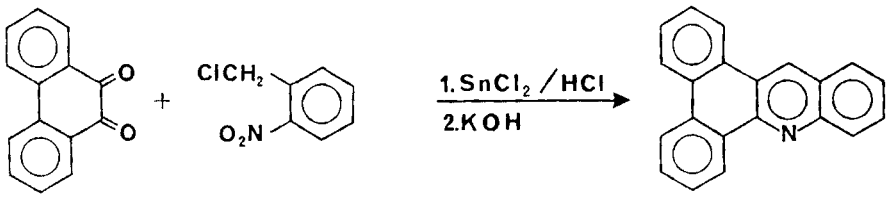


FIGURE 3
Synthesis of Dibenz(a,c)acridine

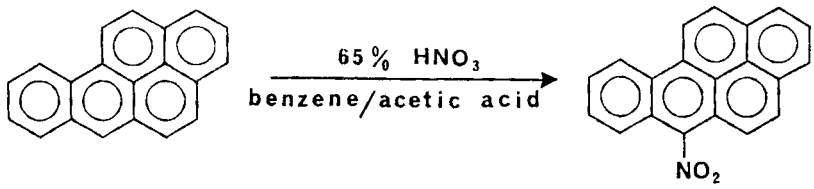


FIGURE 4
Nitration of Benzo(a)pyrene

With the exception of 2-nitro-7-methoxynaphtho-(2,1-b)furan, the synthesis of which required a condensation reaction, and of 2-nitronaphthalene, the precursor of which was 2-aminonaphthalene, the nitro-PACs were synthesised by nitration of the corresponding polycyclic aromatic hydrocarbon (Figure 4).

In the case of the polychlorinated biphenyls the precursors were generally suitably chloro-substituted anilines which could be diazotised and coupled to provide the required biphenyl. Two of the compounds were synthesised by coupling a suitable polychloro-iodobenzene by means of an Ullmann type reaction.

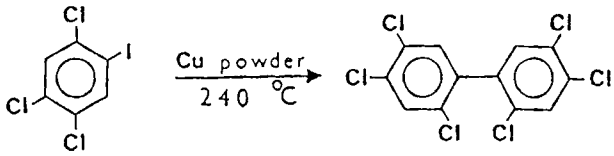


FIGURE 5
Synthesis of 2,2′,4,4′5,5′-Hexachlorobiphenyl

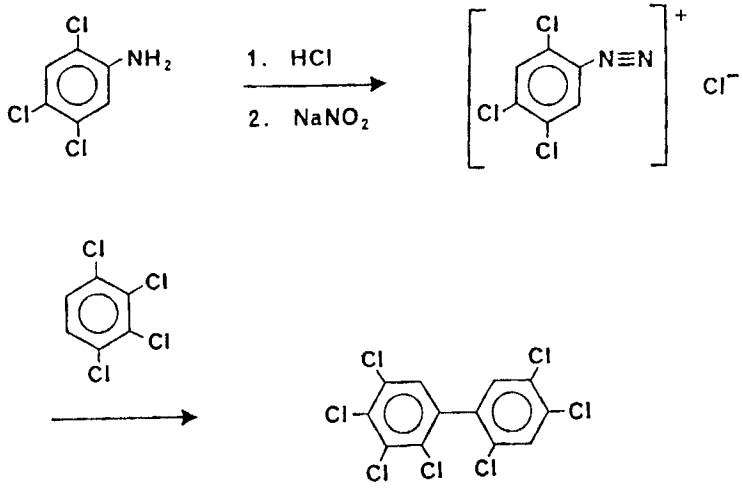


FIGURE 6
Synthesis of 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl

The reactions did not yield a unique single product and generally a mixture of at least two isomers was obtained. The by-products were removed in the purification process. Two examples of the syntheses are shown below (Figures 5 and 6).

3. Purification

3.1 General Procedure

Purification of the compounds required as reference materials was carried out by a variety of techniques. The most important of these were:

a) Recrystallisation

b) Sublimation

c) Column chromatography

d) Preparative high-performance liquid chromatography (HPLC)

e) Zone melting

f) Vacuum distillation

Recrystallisation from pure solvents was the most generally applied method[22]. A suitable solvent for the recrystallisation was identified by preliminary trials and in addition to the commonly applied solvents such as cyclohexane, benzene and chloroform, binary mixtures were also used.

Sublimation[22] was used to separate the main component, when it was stable at its melting point, and where its vapour pressure at or near the melting point was sufficiently high and differed from that of the impurities. The sublimation procedure also had the advantage of eliminating any inorganic impurities that might be present in the original sample. Direct sublimation was achieved by heating the substance to its melting point under vacuum and collecting the sublimate on a "cold finger", this procedure being most suited for small amounts of sample (<1 g). Fractional sublimation involved heating the sample at a temperature just below that of its melting point, in a glass tube, along which a decreasing temperature gradient was maintained, and to which a vacuum was applied to reduce the internal pressure below that of the vapour pressure of the main compound in the sample. The tube was demountable, consisting of short sections connected by standard taper ground joints, thereby facilitating retrieval of the sublimate fractions condensing along the tube. Generally this method required

large samples (>1 g), was time consuming, of low efficiency and somewhat hazardous for carcinogenic compounds, and consequently it was only adopted when necessary, for a preliminary purification stage.

For some PAC samples separation of impurities from the main component was made by column chromatography using the recycling solvent apparatus described by Depaus [23]. Typically, about 5 g of the impure sample was intimately mixed with a suitable amount of bauxite which formed an upper layer on a silica gel column, and the eluant (hexane containing 3 to 5% benzene) was continuously recycled through the column by means of reflux distillation. Advancement of the bands was followed by fluorescence UV. This procedure permitted the main component to be purified to a purity exceeding 99.5% in a period of about 10 hours, and the separation of the impurities present in sufficient amount to permit structural identification by NMR. This procedure greatly reduced solvent consumption and had the considerable benefit of limiting the exposure of the operator to these hazardous materials.

In suitable cases, preparative HPLC was employed [23,24] using a commercial instrument with a UV detector operating at 254 nm. In a typical run 30 g of the material (purity ~95%) was taken and purified to the 99-99.5% level using silica as the column packing and hexane or hexane/chloroform as the eluant. In 40 hours a yield of 70 to 75% was achieved. Oxygenated solvents such as acetone were avoided since photosensitive PACs appeared to be less stable in such solvents compared to pure hydrocarbons. Similarly, to avoid photodecomposition of sensitive PACs, direct sunlight was excluded during the HPLC operations.

Organic solids which are stable above their melting point can be purified by zone melting[25,26,27]. The compound is usually packed into a glass or polytetrafluoroethylene tube and zone melting achieved by an external travelling ring heater. Purification of the main component is achieved if the distribution coefficient of the impurity between the solid and molten phases is greater than or is less than unity. The impurities become concentrated in the upper or lower region of the tube, the purified compound being recovered from the central section. To be effective many passes of the heater are required. Polycyclic

aromatic compounds of low molecular mass were more amenable to this method of purification than those of high molecular mass.

Where the polycyclic aromatic compound was thermally stable a preliminary purification was attained by distillation under application of a high vacuum, as described for indeno[1,2,3-cd]pyrene[20].

In general more than one of these purification techniques were applied to each batch of the candidate reference compound. The combination of techniques was chosen so as to achieve the optimum purity without undue expenditure of time. The experimental approach selected greatly depended on the batch size and the degree of purity of the unpurified material. Consequently the methods adopted for the purification of a custom-synthesised product containing only minor impurities differed markedly from those applied to the purification of a commercial batch of material, which often was contaminated with major impurities. In the first case recrystallisation together with sublimation or column chromatography were appropriate, whereas the presence of major impurities necessitated their initial removal by chromatographic separation followed by recrystallisation and/or sublimation.

3.2 Purification of Custom-Synthesised Compounds

Since in this case only a limited batch of material was generally available the yield of purified material was of paramount importance. Where no major impurities were known to be present a combination of recrystallisation and sublimation provided a suitable means of attaining the requisite standard of purity. Good results could normally be achieved in the case of the PACs by a presublimation of the synthesised product, followed by one or more recrystallisation steps and then a final sublimation of the end product to remove non-volatile impurities such as inorganics. Optimum sublimation conditions were best established by preliminary experiment. A suitable sublimation rate was approximately 1g/min. For those few compounds which were unstable under sublimation conditions, column chromatography followed by recrystallisation was applied. In those cases where impurities were present which were difficult to

remove from the main component by crystallisation only column chromatoghraphy was applied.

3.3 Purification of Commercial Materials

Where the starting material was an impure commercial batch, column chromatography and/or preparative HPLC were usually applied in the first instance, with subsequent recrystallisation and/or sublimation. A number of the impurities isolated by these procedures from the purification of PACs, were identified and commonly found to be heterocyclic analogues which possessed the same number of rings as the principal component, one of which had been replaced by a thiophene or pyrrole ring[28]. Some PACs, such as benzo(a)pyrene and 9-nitroanthracene, are subject to photooxidation when in solution, and therefore in these cases during purification it was important to protect the solution from direct sunlight.

4. Characterisation and Identification

Prior to certification the solid candidate reference materials were subjected to identity checks and to test regimes to establish their homogeneity and stability. A preliminary measurement of the purity was also made.

For the majority of compounds the synthetic route chosen was sufficiently proven to allow the unambiguous preparation of the individual compound. In those cases where there was some measure of doubt the identity of the compound was confirmed by applying a variety of physical measurements, which included determination of the relative molecular mass from mass spectrometric data, electron impact mass spectrum, infrared spectrum, nuclear magnetic resonance spectrum (proton and carbon-13), ultra-violet absorption spectrum, melting point, and x-ray diffraction analysis. For the polychlorinated biphenyls, where the congeners possess closely similar properties, the latter technique was applied to four of the compounds to confirm their structure[29,30,60].

That confirmation of the identity of compounds of doubtful structure by an independent method is necessary, is underlined by the recent observation that the method given in the literature[31] for the synthesis of dibenz(a,i)acridine was found to yield another isomer, namely dibenz(a,j)acridine[32].

5. Homogeneity Testing

Each of the candidate reference materials was submitted to homogenisation in solution by a single batch recrystallisation, or by evaporation to dryness. Homogeneity checks were then made of the bulk material. If these proved satisfactory, the material was dispensed as measured units in glass vials or other appropriate containers and a further homogeneity check was made of the material distributed in each batch of vials to confirm that the material had remained homogeneous during the dispensing operation.

In the case of the bulk materials, each batch was mixed by mechanically shaking for 30 min prior to sampling, from which usually six samples (sample size 1.5-2.5 mg) were taken at random. For most of the PACs differential scanning calorimetry (DSC) was employed to establish the impurity distribution, but for thermally unstable PACs as well as for the nitro-PACs and PCBs analysis was made by gas chromatography or by high-performance liquid chromatography to measure impurity variations. Only if the batch was found to be homogeneous was quantitative dispensing of the compound carried out.

After dispensing the material, the between-bottle homogeneity of each batch of vials was investigated in one laboratory employing a method which had been shown in prior work to give reliable results. Six vials were taken at random from each series and typically 0.1 mg test portions were taken for the GC or HPLC measurements. Two sub-samples from each of the six vials were analysed. The purity of each test portion was obtained by quantifying the impurities. In general the standard deviation of the results obtained for the six different vials did not differ significantly from that of replicate measurements made on samples taken from a single vial.

6. Stability

The stability of samples of the solid compounds, stored at ambient room temperature, over the course of one year was studied by measuring their purities at regular intervals. In general the measured purity of the samples showed no significant change.

It was however separately observed that solutions of RMs were unstable if kept for some time, and photodecomposition in air could also occur. In the case of the nitro-PACs, samples of each compound were stored at -80 °C, +4 °C, 20 °C and 40 °C for periods of up to six months. Analysis at the start and after storage of one, three and six months were made of samples stored at +4 °C and at 40 °C.

All the nitro-PAC reference materials were found to be stable in the solid form at +4 °C and only 9-nitroanthracene and 3-nitrofluoranthene showed any significant changes when stored for six months at 40 °C[61].

7. Determination of Purity and Certification Procedure

7.1 General

The certified purity of each material, once it was dispensed in suitable vials and shown to be homogeneous as well as stable, was based on purity measurements made within the framework of a cooperative interlaboratory analytical exercise usually involving ten or more experienced laboratories of the European Community. The procedure which was normally adopted, was to divide the laboratories into two groups of five or six with at least two laboratories being common to each group. The set of purified candidate reference materials to be examined were also randomly divided into two sub-sets and each group of laboratories was asked to analyse a sub-set. For example where ten candidate compounds were available each group of laboratories was asked to analyse five of the compounds. Each laboratory was supplied with one or more dispensed samples of the candidate compound, selected at random, and the laboratory was requested to measure the purity of the compound by two, or more, independent analytical methods and to provide triplicate measurements by each method. This procedure was chosen to reduce the total burden of work on each laboratory whilst, at the same time, permitting the number of results available for technical and statistical evaluation to be of adequate size.

The independent methods of purity determination were chosen from the following:

1. (a) Gas-liquid chromatography (GLC): capillary column
   (b) Gas-liquid chromatography (GLC): packed column
2. (a) High-performance liquid chromatography (HPLC): normal phase (adsorp.)
   (b) High-performance liquid chromatography (HPLC): reversed phase
3. (a) Mass spectrometry (MS): gas-chromatography/mass spectrometry
   (b) Mass spectrometry (MS): direct inlet mass spectrometry

Since these methods of purity determination are based on the detection, separation, and quantitative measurement of the individual impurities present, with subsequent summation of the separate impurity amounts and subtraction from unit mass, the efficiency of their separation as well as the detector response characteristics of the applied system are of considerable importance.

7.2 Gas-Liquid Chromatography

This technique was utilised extensively for the certification measurements since it was available in all the participating laboratories. The advantages provided by GLC methods[33] are the high resolution achievable for impurities, and the similar response factors observed with the flame ionisation detector for most carbon compounds when the number of heteroatoms present is low.

In a typical GLC analysis of a PAH, a solution of it in a suitable organic solvent (e.g. toluene, dichloromethane, hexane) was chromatographed at temperatures between 180 and 305 °C. Column length varied between 1 and 10 m for packed columns (diameter 2-4 mm), and 20 to 50 m (diameter 0.2-0.5 mm) for capillary columns. Chromatography was conducted under isothermal conditions, or when more appropriate, under a temperature programme regime.

On general considerations there are three main sources of error which may affect the precision and accuracy of GLC measurements. These can be categorised as instrumental, chemical and data processing factors.

Instrumental factors relate mainly to the injection technique, the column and the detector. The injection technique must be such that transfer of the contents of the injected solution to the column is achieved without discrimination or losses.

The column selected for the particular analysis should be chosen to provide the highest efficiency compatible with the greatest selectivity for the impurities being quantified. Preliminary studies with known mixtures of the impurity components may be required to achieve optimum selectivity. The detector response factor of an impurity is frequently taken to be unity relative to the main component. However this may not always be the case and to reduce uncertainties of this kind it is desirable, (but often not possible on practical grounds), to measure the relative response factors of pure samples of the impurity compounds in prior studies. The linearity of response of the detector also needs to be confirmed for the range of impurity concentrations to be measured.

Chemical factors which may lead to erroneous measurements are the possible decomposition of the main or impurity components through photodecomposition in solution, or by thermal decomposition in the vaporiser or on the column, or through chemisorption on the column.

In GLC measurements the automated recording of data and computerised data processing need to be applied with care. Erroneous peak area integration values may be generated if there is inadequate recognition of baseline changes in the course of the chromatogram. Likewise poor matching of the detector response to the analog-digital input may give rise to an erroneous non-linear response. Preliminary calibration runs with pure samples of the impurities or suitable RMs can identify and provide a correction for these types of errors.

7.3 High-Performance Liquid Chromatography

HPLC methods[34,35] are, in effect, complimentary to those of GLC since they more readily permit the determination of organic impurities with high boiling points, or which undergo thermal decomposition at elevated temperatures. A further advantage is that impurities may be easily separated and submitted to further analysis. However, as in GLC, the factors that cause imprecise or inaccurate measurements are the retention of the impurity component on the column, or insufficient separation or resolution of the impurity components. A major source of error in HPLC is the fact that no universal detector is available

and the most widely used detectors (UV absorbance and UV fluorescence detection systems) require the determination of the response factor for each individual compound under measurement, and this in turn necessitates the availability of a macro sample (>1 mg) of each impurity compound. Only in a few instances could the impurity be obtained in such quantity to permit the determination of the detector response factor and therefore the response ratio of the impurity and the main component were taken to be unity. For HPLC analysis both normal (adsorption) phase, as well as reverse-phase packings, were applied.

In a typical HPLC analysis the column length was either 25 or 30 cm with internal diameter in the range of 4-4.6 mm. As in GLC, the concentration of the candidate reference material was around 1% by mass, and the injected sample size was between 0.2 and 20 μl. In general the UV absorbance was measured at a wavelength of 254 nm, or in some cases at a suitable absorption peak maximum of the impurity. Variable wavelength diode array detectors have been used in more recent work. In suitable cases fluorescence detection was also utilised.

7.4 Mass Spectrometry

Use of a mass spectrometer as the detector for the components eluted from a GLC column provides a very sensitive system, which permits quantification of the individual peaks of the chromatogram[36,37]. Modern mass spectrometry has the advantage of advanced computer techniques in the acquisition and processing of mass spectral data obtained from the combination of GLC and mass spectrometry. The mass spectrometer can be programmed to record a full scan or to provide selected-ion monitoring. Total ion current (TIC) chromatograms can be plotted, or the measured mass spectrum submitted to a library search for identification. Since high-resolution selected-ion monitoring mass spectrometry permits improved detection limits as well as higher selectivity, very often a more specific analysis can be achieved for a given impurity.

Additionally, in suitable cases, the purity of the candidate reference materials was also examined by direct inlet mode mass spectrometry. In this way impurities which are strongly retained on GLC and HPLC columns and might not be

observed by those techniques, could often be detected. However impurities giving parent ions with a similar m/z value as that of the main component (i.e. where isomeric impurities were present) would be masked, and were not readily detectable. At low ionisation potentials (20 eV or less) and with thermal programming from room temperature, a simple mass spectrum could be derived in which the parent ions were dominant[38]. The relative molecular ion masses gave some indication of the nature of the impurity and where the impurity was of a similar chemical structure quantitative analysis was possible.

Where a coupled gas-liquid chromatograph and mass spectrometer are utilised for impurity measurements, then the potential errors are similar to those indicated above for the GLC technique. Additionally errors may arise due to thermal decomposition of the impurity component under measurement. (By using glass or glass-lined interfaces, this can be minimised.) Over-fragmentation of the main compound may also give rise to errors, particularly when electron-impact ionisation is used. Mass spectrometer memory effects may also result in erroneous measurements, but can be obviated by solvent blank runs between sample runs. The relative response factors for the PCBs, under a selected-ion monitoring regime, have been shown to be essentially uniform[39].

7.5 Assessment of Organic and Inorganic Impurities

Those organic impurities which were detected by more than one laboratory by mass spectrometric methods, and for which the relative molecular mass had been established, were reported for each of the certified reference materials. Inorganic impurities that might be present were determined on a gravimetric basis by medium temperature ashing of the material, and gravimetric measurement of the ash content as well as measurement of some volatile elements present.

In the case of the nitro-PACs, whereas P, Cl, S and SiO_2 were determined when detectable, the determination of trace amounts of inorganically bound nitrogen was impractical in the presence of organic nitrogen. However if present, in line with other PACs of the series, the amount was considered likely to be close to the detection limit.

8. Evaluation of Data

In the early series the certified purity levels were calculated from the average of the individual results and the uncertainties were based on the standard deviations of individual measurements. In line with current Measurements and Testing practice[40], in the later series the certified purity values were derived from the means of the results of the individual methods in each laboratory and the uncertainty is based on the 95% confidence limits of the mean purity. In addition to the certified purity with the associated uncertainties that are stated on the certificate provided with each reference material, the upper limit of the organic and inorganic impurities present is also given.

In the first place, before calculation of the certifiable values, the distribution of the accepted mean results were tested for normality. Inspection of the mean values suggested, especially for some apparently high-purity compounds, that a skewed distribution would be more consistent with a log-normal distribution (i.e. one in which the logarithms of the mass fractions of the impurities are distributed normally).

Therefore the set means, expressed as a mass fraction of the impurities, and in a separate calculation, their logarithms, were tested for conformity to a log-normal distribution using the Kolmogorov-Lilliefors test. When this was done it was generally found that the results for the higher purity values could be fitted more satisfactorily to a log-normal distribution, which was not unexpected in view of the high purity of these compounds. The candidate reference compounds of lower purity usually conformed more satisfactorily to a normal distribution.

Where a normal distribution for the impurities was indicated, the confidence limits of the grand mean were calculated in accordance with normal practice for small sample statistics by using the relationship:

$$\overline{X} \pm \frac{t.s}{\sqrt{p}}$$

in which $\overline{X}$ is the grand arithmetic mean purity, given by

$$\overline{X} = 1 - \frac{1}{p} \sum_{i=1}^{p} \overline{Y}_i$$

where

$\bar{Y}_i$ = mean value of the impurity content found by one laboratory using one technique and p is the number of accepted set mean results.

t = Student's t factor (at 5% significance level) taken from tables for p-1 degrees of freedom.

s = calculated standard deviation of $\bar{X}$

Where a log-normal distribution for the set means was indicated, corresponding formulas employing the logarithms of the set means were used and in these cases asymmetric confidence limits resulted.

Bar graphs representing the purities with their associated uncertainties for the various groups of reference materials are presented in Section II.

As a final test of the overall homogeneity of the results for a whole series of compounds, the nonparametric Friedman test was applied to the rankings of the laboratories' means ($\bar{X}_l$) and also to the method means ($\bar{X}_m$). This showed that all laboratories were equally likely to report a high or a low result for any material analysed (i.e. no laboratory consistently produced high or low results). A comparison of the methods of analysis showed that packed column GC, GC/MS and direct inlet MS were more likely to give higher results for the purity value than were HPLC methods and capillary column GC.

The total estimated purity of the reference material was calculated by a linear combination of the inorganic and organic impurity results. This treatment yields the highest purity that can be certified on technical grounds. However, because detection limit values rather than true measured values are frequently utilised to establish the inorganic impurity content, it is likely that for a number of the certified reference materials the estimate of the total purity is somewhat pessimistic.

9. Applications

9.1 General Considerations

A reference material (RM) has been defined[41] by the International Organisation for Standardisation (ISO) as "a material or substance, one or more properties of

which are sufficiently well established for the calibration of an apparatus or for the verification of a measurement method. A reference material makes possible the transfer of the value of a measured quantity between one place and another". A certified reference material (CRM) is a reference material accompanied by a certificate stating the property values concerned, issued by an organisation, public or private, which is generally accepted as technically competent[42].

The PAC, nitro-PAC and PCB reference materials issued by the BCR fall into the CRM category since each unit is accompanied by a certificate which states the purity of the substance and the uncertainty attached to that value. These certified reference materials of pure compounds are of environmental and occupational significance, and are intended primarily for the calibration of an analytical system or for the verification of an analytical measurement technique. Both these applications are of basic importance when it is a question of measuring trace amounts of an organic pollutant in the environment or workplace, particularly if the permitted level of the pollutant is subject to regulatory control.

Where the reference materials are of high purity and where physicochemical data have been independently recorded for the same or similar high-purity samples the reference material can be indirectly used for the transfer of the physicochemical data. Spectral and other physicochemical data associated with the high purity PAC reference materials issued by the M & TP are published in a series of Atlases, the first three of which are available[43,44,45].

These RMs may also be used for a variety of biological tests and as reference substances in bioassays. In both cases a high purity is essential to minimise ambiguous results.

For some applications it is more convenient to use the RM in the form of a solution or matrix material containing a certified amount of the PAC or PCB reference material. A limited number of such RMs are issued by the M & TP (see sub-section 9.4).

Figure 7 (on the following page) gives in a condensed form the main areas of application of these types of RMs for environmental monitoring.

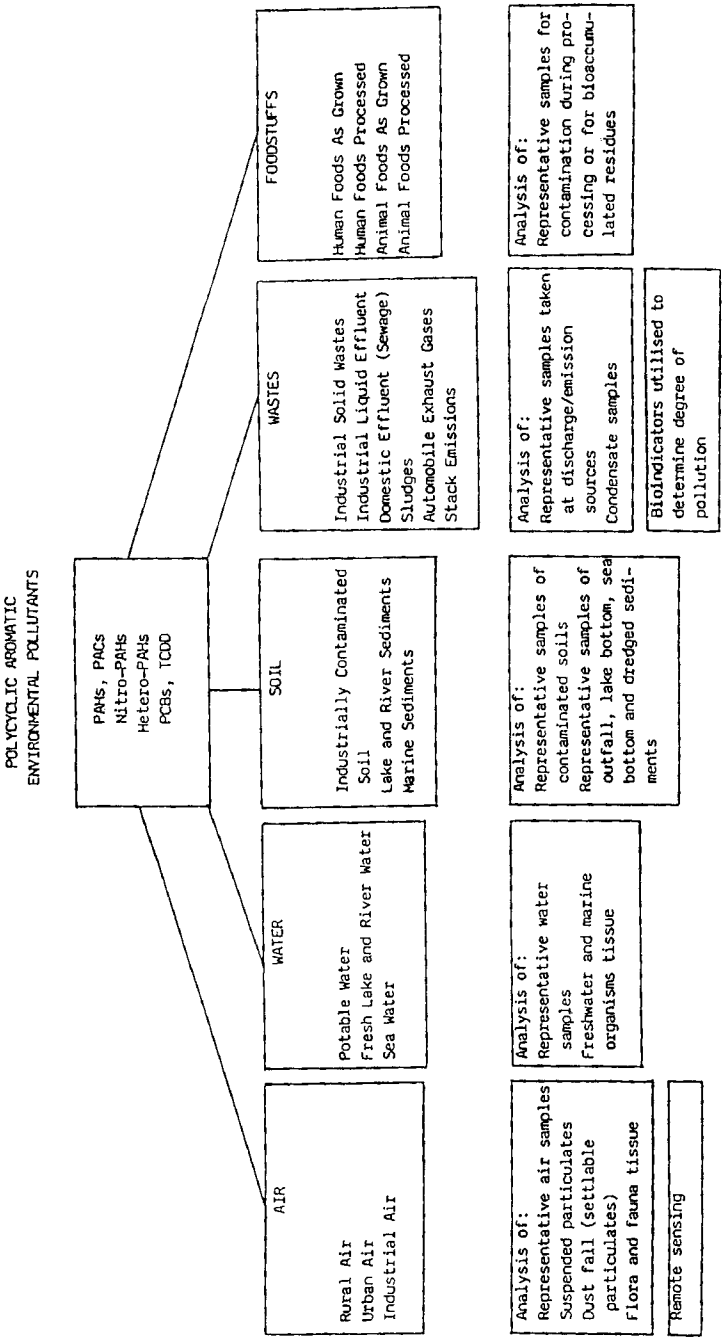


FIGURE 7
Methods Used for Monitoring Environmental Samples

9.2 Environmental and Occupational Monitoring

The widespread contamination of the environment by PAHs, nitro-PAHs, heterocyclic-PACs, PCBs and similar compounds has been well established. The use of RMs in the measurement of such pollutants has been described[19,28]. Sources of the contamination have been identified and recorded in a large number of publications. Major sources are the by-products from the combustion of fossil fuels such as coal, lignite, petrol and other petroleum oils.

The measurement of the concentration of a pollutant in an environmental sample, previously obtained by a suitable sampling procedure, generally requires the application of four fairly distinct analytical stages:

(i) Clean-up of the sample and extraction of the pollutant of interest, often obtained in admixture with similar co-pollutants.

(ii) Concentration of the pollutant mixture and separation of the pollutant of interest.

(iii) Calibration of the detection method and system.

(iv) Quantitative measurement of the individual pollutant.

Sometimes these analytical stages are applied in sequence but very frequently the stages are concurrent. Thus in gas-liquid chromatogaphy, separation of the pollutant and its quantitative measurement are combined in a single stage.

In order to achieve the optimum precision and accuracy of the final measurement of the pollutant concentration, a number of ideal requirements should be sought for each stage of the analytical technique applied. These are as follows:

ANALYTICAL STAGE	TECHNIQUE REQUIREMENTS
Clean-up and Extraction	Absence of losses High extraction efficiency (>80%) Good repeatability Good reproducibility
Separation and Concentration	Selectivity Good separation factor High enrichment factor Certainty of identity

Calibration	High-purity stable calibrants Linearity in range of interest Good response factor
Blank measurements	Ability to check detection limits Background level measurement Contamination level measurement
Final Determination	High specificity High sensitivity

Reference materials can be used in many analytical procedures to achieve these requirements, or at least make it possible to obtain reliable correction factors.

In the first stage of the analysis of an environmental sample, involving initial clean-up and extraction of the pollutants, a suitable RM may be added to the sample as an internal standard, which can be used to monitor any loss of material in this analytical stage, as well as to establish the extraction/clean-up efficiency and the repeatability of the procedure. For preference the RM should be chemically similar to the pollutant of interest and the assumption is usually made that the RM and the pollutant are extracted equivalently at the concentration levels examined. Recovery values based on an added standard will permit correction for losses during clean-up and at least partial correction for incomplete recovery.

In the second analytical stage an internal standard, either of the pollutant itself or of a closely similar compound, can be utilised to establish the separation and enrichment factors. Measurement of the relative retention time of a pollutant on a chromatographic column, with respect to an added standard, can also assist in confirming the identity of the pollutant. In addition in those cases where the pollutant can be isolated in sufficient quantity, one or more of its physical properties, such as the UV absorption spectrum, NMR spectrum or Shpol'skii spectrum can be compared with those of a pure RM of the pollutant, which if they have been independently measured, provides a confirmatory and an absolute method of identifying the pollutant.

Pure RMs of the pollutant or of suitable homologues may be used in the calibration stage to determine the response factors for the detection system used,

and to establish that the determination occurs within the region of linearity of measurement (i.e. the mass flow or concentration range for which the measured signal has a linear relationship with the concentration). Prepared solutions, or synthetic mixtures of pure pollutant RMs, can be used to determine the lower detection limit for the measurement procedure adopted, and, with the aid of blanks, can be used to establish the background levels of the pollutants, as well as possible adventitious contamination during the analytical stage.

A chromatographic technique is most commonly utilised as the end measurement method by which the pollutant component of interest is separated and quantified by the previously calibrated detection system. The main techniques most frequently applied are gas-liquid chromatogaphy (GLC) with a flame ionisation detector, high-performance liquid chromatography (HPLC) with UV or fluorescence detection, high-performance thin-layer chromatography (HPTLC) with densitometric measurement, and gas-liquid chromatography with mass-spectrometric measurement (GLC-MS).

9.3 Microbiological and Toxicological Assays

The availability of high-purity reference compounds, particularly those compounds which are present in environmental and workplace samples, offer a considerable advantage when testing their biological and toxicological properties. The discrepancies found when comparing the results of studies reported in the literature stem not only from differences in experimental conditions, but are also dependent on the purity and composition of the test substance, since impurities can significantly affect the final result of the assay.

Thus, in the Ames test, for example, the presence of an impurity that has a much higher activity than the main component can produce a result which is in fact an overestimation of the mutagenic activity. On the other hand, the impurities present may be toxic towards the bacterial strain being utilised, and thereby mask to a considerable degree the mutagenic activity of the main compound under test. Since the PAC and PCB reference materials are well characterised for purity and the detected impurities are indicated, use of the materials should reduce interferences and permit use of a standard substance in comparison assays. A

number of the certified PACs, especially the heterocyclic compounds have been used in tests of their mutagenic activity on various bacterial strains[46].

The PAH high-purity reference materials can also be used for the study of potential cocarcinogenic effects. Since PAH-derived carcinogenicity depends on arylmonooxygenase activity, which converts PAH compounds into the ultimate carcinogenic *trans*-dihydrodiolepoxides, stimulation or induction of this enzyme system plays a vital role in carcinogenesis[47]. In this context, certain of the high-purity PAH reference materials have been used to determine the induction potentials on the cytochrome P-448 monooxygenase system. Thus, induction factors from 4 to 6.5 were found for benzo(a)pyrene and benzo(b)-, benzo(k)- and benzo(j)fluoranthenes with the substrate benz(a)anthracene[48]. In similar experiments chrysene, benz(a)anthracene and anthanthrene were identified as potent inducers in the P-448 system[49].

It is known that to some degree the toxicity of a PCB is related to its molecular structure. Thus the specificity of toxicity and the inducibility of mixed-function oxidase systems, (i.e. cytochrome P-448 or P-450), in relation to the chlorine substitution pattern of PCBs has been shown in toxicological studies of PCB congeners[50]. The significance of the structure-activity relationship for PCBs based on structures derived from crystallographic diffraction analyses has been discussed by McKinney and coworkers[51].

9.4 Solutions and Matrix Materials

Analysts often prefer to utilise reference materials in the form of prepared solutions or as a suitably spiked matrix material.

Where the pure reference materials, prepared within the M & T programme, are known to be sufficiently stable under such conditions, suitable solutions or matrix RMs have been prepared or are pending.

Thus a solution in isooctane of nine PCB congeners at certified concentrations (around 10 μg/ml) is available (CRM 365), as well as fish oils containing certified contents of PCBs (CRM 349 and CRM 350). Also available are 12 separate "dioxins" (polychlorinated dibenzo-p-dioxins and dibenzofurans) in isooctane solution (CRMs 432-443)[52].

10. Handling, Disposal and Safety Aspects of Environmental Reference Materials

Many of the environmental RMs utilised for calibration purposes have been reported to possess carcinogenic or other biological activity[4,6,53,54,55,56] and therefore these compounds should only be handled by persons properly qualified and trained in the handling and use of potentially hazardous and toxic chemicals.

It should be the responsibility of the laboratory management and personnel to ensure that good laboratory practice is followed in handling these compounds so that contamination of the laboratory does not occur, and the health of the laboratory staff is protected. Thus skin contact should be avoided when handling the compounds, by wearing protective gloves. Likewise precautions should be taken against the inhalation or ingestion of solid particles of the materials arising from aerosol formation or vaporisation. This is best done by carefully opening the reference sample container in a restricted area and preferably in a protective hood or glove box set aside for this purpose.

Where a solution is required to be prepared from the solid reference material, sufficient sample should be taken to ensure accuracy of weighing, the sample being transferred to a graduated vessel by careful washing with the chosen solvent. Solutions of reference materials intended for calibration purposes should be freshly prepared and should not be exposed to light for extended periods.

Calibration solutions should be discarded after use, preferably into special waste containers to avoid contamination of ground or waste waters by PAC or PCB materials. Alternatively the recommendations of the International Agency for Research on Cancer for the destruction of PAC materials in laboratory wastes should be followed[57,58]. The destruction of small quantities of PACs by chemical oxidation methods has been described[57] and the disposal of polychlorinated biphenyls has been discussed[59]. Users should ensure that the disposal of waste PACs conforms to national regulations.

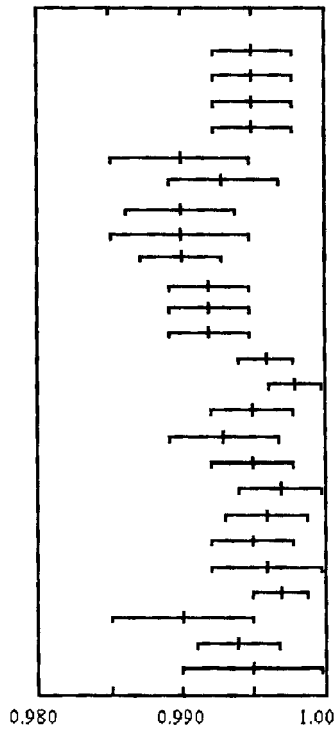
All environmental organic reference materials should be stored in darkness under cool conditions to prevent any photosensitised reactions.

II PURITY DATA

1. Purity Data of the Reference Materials

The purity measurement data of the following groups of organic reference materials have been published elsewhere as indicated. Here† are given summarising bar charts showing the purity mean values and uncertainties expressed as mass fractions (g/g). The identifying CRM Number is given alongside the name of each compound.

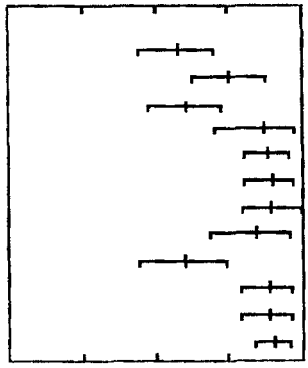
1.1 Polycyclic Aromatic Compounds, Group 1



46 Benzo(b)chrysene
47 Benzo(b)fluoranthene
48 Benzo(k)fluoranthene
49 Benzo(j)fluoranthene
50 Benzo(e)pyrene
51 Benzo(a)pyrene
52 Benzo(ghi)perylene
53 Indeno(1,2,3-cd)pyrene
77 1-Methylchrysene
78 2-Methylchrysene
79 3-Methylchrysene
80 4-Methylchrysene
81R 5-Methylchrysene
82 6-Methylchrysene
91 Anthanthrene
93 1-Methylbenz(a)anthracene
94 Dibenz(a,c)anthracene
95 Dibenz(a,j)anthracene
96 Dibenzo(a,l)pyrene
97 Benzo(a)fluoranthene
133 Dibenzo(a,e)pyrene
134 Benzo(c)phenanthrene
138 Dibenz(a,h)anthracene
139 Benzo(ghi)fluoranthene
140 Benzo(c)chrysene

†Polycyclic aromatic compounds, group 1[53]
†Polycyclic aromatic compounds, group 2[54]
†Polycyclic hetroaromatic compounds[54]
†Polychlorinated biphenyls[62]
†Nitro-polycyclic aromatic compounds[63]
Oxygenated-polycyclic aromatic compounds [63]

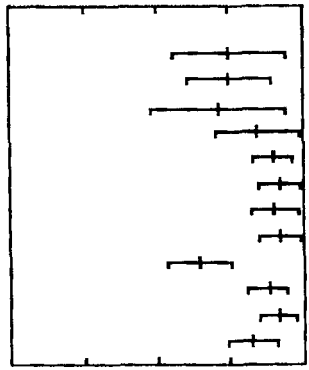
1.2 Polycyclic Aromatic Compounds, Group 2



159 Dibenzo(a,h)pyrene
160 Fluoranthene
161 Cyclopenta(cd)pyrene
168 Picene
177 Pyrene
265 Dibenzo(a,e)fluoranthene
267 Indeno(1,2,3-cd)fluoranthene
268 Dibenzo(a,i)pyrene
269 Chrysene
270 Triphenylene
271 Benz(a)anthracene
272 Coronene

0.980 0.990 1.000 g/g

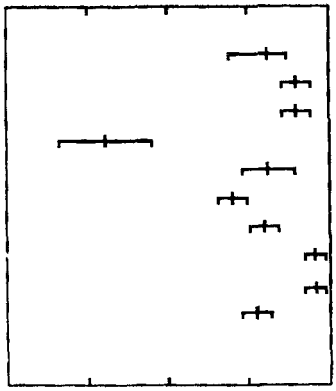
1.3 Polycyclic Heteroaromatic Compounds



92 10-Azabenzo(a)pyrene
135 Benzo(b)naphtho(2,1-d)thiophene
136 Benzo(b)naphtho(2,3-d)thiophene
137 Benzo(b)naphtho(1,2-d)thiophene
152 Dibenz(a,1)acridine
153 Dibenz(a,h)acridine
154 Dibenz(a,j)acridine
155 Dibenz(a,c)acridine
156 Dibenz(c,h)acridine
157 Benz(a)acridine
158 Benz(c)acridine
266 7H-Dibenzo(c,g)carbazole

0.980 0.990 1.000 g/g

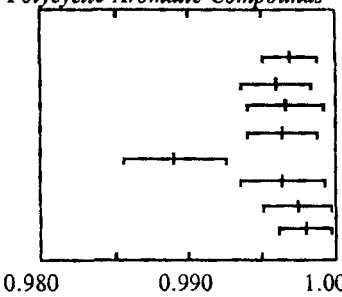
1.4 Polychlorinated Biphenyls



289 2,4′-Dichlorobiphenyl
290 2,3,3′-Trichlorobiphenyl
291 2,4,4′-Trichlorobiphenyl
292 3,3′,4-Trichlorobiphenyl
293 2,2′,5,5′-Tetrachlorobiphenyl
294 2,2′,4,5,5′-Pentachlorobiphenyl
295 2,3′,4,4′,5-Pentachlorobiphenyl
296 2,2′,3′4,4′,5′-Hexachlorobiphenyl
297 2,2′,4,4′,5,5′-Hexachlorobiphenyl
298 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl

0.980 0.990 1.000 g/g

1.5 Nitro-Polycyclic Aromatic Compounds



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