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Preparation and certification of solutions of perdeuterated polycyclic aromatic compounds intended for use as surrogate internal standards

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Abstract. Two standard solutions of deuterated polycyclic aromatic compounds (PACs) have been prepared for use as surrogate internal standards. Solution DPAC-1 contains 21 deuterated PACs, and is intended for use with mass spectrometric (MS) detection. Most of the difficulties in certifying concentrations in DPAC-1 arose from the fact that none of the individual compounds was 100% deuterated, so that effects of mass spectrometric fragmentation are convoluted with those of isotopic distributions. The best methods are discussed for using such internal standards so as to minimize these problems, together with those arising from kinetic isotope effects. Solution DPAC-2 contains 6 deuterated PACs, and is primarily intended for use with reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection (FLD, dual programmed wavelength mode), in which the signals for analyte and internal standard are separated chromatographically rather than via the detector. Full details of the preparation of these solutions are described. In addition, examples of their use in the analysis of a certified coal-tar extract (NIST SRM 1597) are described briefly. In one example a novel HPLC-MS technique was employed, and in the other the HPLC-FLD technique was used.

Introduction

Polycyclic aromatic compounds (PACs) form an important class of pollutants due to their widespread distribution together with their mutagenic and carcinogenic properties [1, 2]. Reliable quantitative analysis of these compounds, present at trace levels in complex matrices, still presents a considerable challenge to the analytical chemist. Improvements in procedures for extraction, selective concentration ("clean-up") and quantitation, continue to be made. Assurance of the quality of analyses of PACs in complex samples has been assisted by the development of certified reference materials from several natural matrices. The activities of the US National Institute of Standards and Technology (NIST) in this area have been summarized in a recent article [3]. In addition, the Marine Analytical Chemistry Standards Program (MACSP) of the National Research Council of Canada has produced a suite of marine sediment reference materials [4, 5] certified for the concentrations of the 16 polycyclic aromatic hydrocarbons (PAHs) which have been designated as priority pollutants by the US Environmental Protection Agency.

In addition to such natural matrix materials, however, it is essential that the analyst have available samples of the target analytes of a high (and known) degree of purity, for purposes of instrument calibration. The Community Bureau of Reference of the Commission of the European Communities has played a key role in making available more than 60 PACs of certified purity, in powder form [6]. However, many analysts prefer to use certified calibration solutions, such as those provided by NIST [3], for several reasons in addition to that of convenience. Thus, careful weighing procedures using standard analytical balances will provide masses with a precision of $\pm$ 0.1 mg or so, implying that a total mass of at least 10–20 mg must be weighed out each time if the associated uncertainty is to be $\leq 1\%$. This can represent a large investment, particularly for cases (including that of the PACs) where a large number of analytes are targetted simultaneously. In addition some PACs are proven carcinogens, and the health risks associated with weighing them as fine powders are not negligible.

To our knowledge, no certified standard solutions of isotopically labelled PACs are available for use as surrogate internal standards. The objective of the present work was to produce two such solutions of perdeuterated PACs, one (DPAC-1) for use in analyses employing gas or liquid chromatography with mass spectrometry as the quantitation technique, and the other (DPAC-2) primarily for use in analyses for which liquid chromatography with programmed wavelength fluorescence [7, 8] is suitable. (The various methods for using surrogate internal standards in quantitative trace organic analysis, together

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with competing techniques such as the method of standard additions, have been reviewed [9, 10] and are summarized below). In addition to all the problems involved in production of certified calibration solutions of unlabelled analytes, the related questions of isotopic purity and of distribution of isotopomers must be addressed, particularly if the solutions are to be used in conjunction with mass spectrometric detection. If the particular isotopic substitution is deuterium for hydrogen, it is necessary to consider also the possibility that kinetic isotope effects could result in significantly different degrees of ion fragmentation, within the ion source. For example kinetic isotope effects, in fragmentations of molecular radical cations of naphthalene and of naphthalene-d_8, have been extensively characterized [11]. This problem is anticipated to be much less serious in cases where heavy atom isotopic labelling, e.g. ^{13}C for ^{12}C, is used, but considerations of cost made this approach impractical in the present case.

Design of the solutions

The criteria used in selection of the deuterated PACs to be included in the two solutions, their approximate concentrations, and the solvent, were as follows:
(1) the compounds must be available at reasonable purities, both chemical and isotopic; (2) perdeuterated (rather than partially deuterated) compounds should be chosen, in order to minimize the possibility that the mass spectra of unlabelled analyte and labelled internal standard might overlap and thus avoiding the difficulties associated with deconvoluting the two contributions to the measured signals at the m/z values monitored [12–16]; the most important fragmentation reactions of perdeutero-PAHs, in this respect, are expulsion of D, D_2, CD_3 and C_2D_2 (2, 4, 18 and 28 Da, respectively), of which only the CD_3 loss is liable to cause such problems in the present case; however, the maximum number of hydrogen atoms in the analytes considered here is 14, so that only the $^{13}C_4$ isotopomer of the $(M - CD_3)^+$ fragment ion would interfere with the $M^{+\cdot}$ ion from the unlabelled analyte; in addition some degree of chromatographic separation of the perdeuterated and unlabelled compounds is observed [17]; (3) as wide a range of PACs as possible should be included in DPAC-1, compatible with the requirement that all components of the mixture should be resolvable by each of gas chromatography (GC), high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC), using a mass spectrometer for detection; this range should include as many different ring numbers as possible; (4) for DPAC-2, which is to be used primarily for LC with fluorescence detection, a reduced set of PACs that separate from sample components should be included; (5) some heterocyclic PACs should be included, in addition to PAHs; (6) concentrations should be sufficiently high, so that 1 mL of standard solution, added to a 10 g sample, would result in levels of internal standard in the range of 0.5 to 10 μg/g, typical of PAC concentrations in more highly contaminated samples such as the urban dust reference material SRM 1649 from NIST; smaller amounts of DPAC-1 would be used for

Table 1. Recommended concentrations of deuterated aromatic compounds in DPAC-1, representing totals for all isotopomers for each case

Compound	Molec. Formula[a]	RRT[b]	Purity[c]	c(μg/g)[d]	c(μg/mL)[e]
Naphthalene-d_8	$C_{10}D_8$ (136.11)	0.267	99.97	116	100
1-Methylnaphthalene-d_{10}	$C_{11}D_{10}$ (152.14)	0.368	94.1	23	20
Biphenyl-d_{10}	$C_{12}D_{10}$ (164.14)	0.427	99.6	(10)[f]	(8)[f]
Acenaphthylene-d_8	$C_{12}D_8$ (160.11)	0.491	99.0	23	20
Acenaphthene-d_{10}	$C_{12}D_{10}$ (164.14)	0.516	99.91	12	10
Dibenzofuran-d_8	$C_{12}D_8O$ (176.11)	0.548	99.8	12	10
Fluorene-d_{10}	$C_{13}D_{10}$ (176.14)	0.602	98.7	23	20
Dibenzo[b, d]thiophene-d_8	$C_{12}D_8S$ (192.08)	0.741	99.6	12	10
Phenanthrene-d_{10}	$C_{14}D_{10}$ (188.14)	0.762	99.2	92	79
Anthracene-d_{10}	$C_{14}D_{10}$ (188.14)	0.773	99.87	13	11
9-Methylanthracene-d_{12}	$C_{15}D_{12}$ (204.17)	0.889	95.3	12	10
Fluoranthene-d_{10}	$C_{16}D_{10}$ (212.14)	0.965	99.7	116	100
Pyrene-d_{10}	$C_{16}D_{10}$ (212.14)	1.000	99.8	93	80
Benz[a]anthracene-d_{12}	$C_{18}D_{12}$ (240.17)	1.204	99.98	58	50
Chrysene-d_{12}	$C_{18}D_{12}$ (240.17)	1.214	99.96	58	50
Benzo [b]fluoranthene-d_{12}	$C_{20}D_{12}$ (264.17)	1.377	99.90	35	30
Benzo[a]pyrene-d_{12}	$C_{20}D_{12}$ (264.17)	1.423	99.2	35	30
Dibenz[a, h]anthracene-d_{14}	$C_{22}D_{14}$ (292.20)	1.578	98.8	12	10
Benzo[ghi]perylene-d_{12}	$C_{22}D_{12}$ (288.17)	1.604	99.6	23	20
Coronene-d_{12}	$C_{24}D_{12}$ (312.17)	1.793	96.7	8	7
Dibenz[a, i]pyrene-d_{14}	$C_{24}D_{14}$ (316.20)	1.810	92.0	7	6

[a] Numerical values in parentheses are relative molecular masses ("molecular weights") for the monoisotopic species (^{12}C, ^2D, ^{16}O and ^{32}S only)
[b] Typical relative retention times for a 30m DB-5 GC column; RRT for pyrene defined as 1.000
[c] Percent chemical purity, determined by GC/FID and GC/MS
[d] Determined gravimetrically, with corrections for chemical impurities
[e] Gravimetric values multiplied by the density of the DPAC-1 solution at room temperature (0.862 g/mL)
[f] Values quoted are those established by GC/FID methods; the gravimetric value was 20% higher, but it is believed that losses resulted from evaporation prior to preparation of the solution

Table 2. Recommended concentrations of deuterated aromatic compounds in DPAC-2, representing totals for all isotopomers for each case

Compound	Molec. Formula[a]	RT[b]	Purity[c]	c(μg/g)[d]	c(μg/mL)[e]
Naphthalene-d_8	$C_{10}D_8$ (136.11)	15.75	99.97	(42)[f]	(36)[f]
Phenanthrene-d_{10}	$C_{14}D_{10}$ (188.14)	22.40	99.2	91.4	78.8
Anthracene-d_{10}	$C_{14}D_{10}$ (188.14)	24.30	–	(0.7)[g]	(0.6)[g]
Fluoranthene-d_{10}	$C_{16}D_{10}$ (212.14)	26.50	99.7	117	101
Benz[a]anthracene-d_{12}	$C_{18}D_{12}$ (240.17)	33.46	99.98	58.0	50.0
Perylene-d_{12}	$C_{20}D_{12}$ (264.17)	40.05	99.87	11.6	10.0
Dibenz[a, h]anthracene-d_{14}	$C_{22}D_{14}$ (292.20)	46.93	98.8	12.3	10.6

[a] Numerical values in parentheses are relative molecular masses ("molecular weights") for the monoisotopic species (^{12}C, ^{2}D) only
[b] Typical HPLC retention times (minutes) for the HPLC conditions used to obtain Fig. 1
[c] Percent chemical purity, determined by GC/FID
[d] Determined gravimetrically, with corrections for chemical impurities
[e] Gravimetric values multiplied by the density of the DPAC-2 solution at room temperature (0.862 g/mL)
[f] Determinations of concentration using HPLC/FLD and NIST SRM1647 and SRM1597 as internal standards in separate experiments, both failed to give concenterations of naphthalene-d_8 in DPAC-2 in agreement with that (59 μg/g) calculated from the gravimetric and chemical purity data. The value given is that obtained from GC/FID analysis of DPAC-2 using an independently weighed internal standard
[g] The value for anthracene-d_{10} corresponds to the impurity level in the phenanthrene-d_{10} sample

samples contaminated at lower levels; (7) relative concentrations of the components of the two solutions should be chosen to roughly match those of PACs found in many environmental samples, so as to maximize accuracy and precision in determining ratios of chromatographic peak areas; (8) the solvent must not be too volatile, and must be miscible with isooctane and with acetonitrile for GC and HPLC work, respectively.

A brief account of these broad design principles, and a progress report on their realization, was published previously [18]. Toluene was found to be a suitable solvent in the light of criterion (7). Tables 1 and 2 list the compounds chosen for the solutions DPAC-1 and DPAC-2 in accordance with criteria (2–6), together with their concentrations (totals of all isotopomers) and additional analytical information.

Preparation and analysis

The 22 deuterated PACs used in the preparation of DPAC-1 and DPAC-2 were obtained from Merck Sharpe and Dohme Isotopes, Montréal, Canada. The toluene solvent was Mallinckrodt "Nanograde", and was redistilled before use.

Prior to preparation of the solutions, the 22 constituent compounds and the toluene solvent were individually examined for chemical purity by high-performance liquid chromatography with detection by ultraviolet-visible spectroscopy (HPLC/UV, diode array detector), gas chromatography with a flame ionization detector (GC/FID), and gas chromatography with mass spectrometric detection (GC/MS). The chemical purity values listed in Tables 1 and 2 are those derived from the GC/FID data by assuming that the impurities had response factors equal to those of the respective main constituents. Since most of the chemical purities thus determined were in the range 99.2%–99.97%, any systematic errors associated with the assumption of equal response factors may be assumed to be unimportant. The most significant impurities in DPAC-1 are those described in Table 3, and were identified by GC/MS experiments. The contributions of those compounds present as impurities in other constituents have been included in the recommended concentrations listed in Tables 1 and 2.

The isotopic purity of each constituent compound was determined by GC/MS using electron impact (EI) ionization with as low as possible a value of the nominal ionizing electron energy. The data summarized in Table 4 were obtained using a VG Analytical ZAB-E double focussing mass spectrometer set for maximum transmission (resolving power about 600, 10% valley definition) in EI mode, with 4 eV nominal electron energy. In this way ionization was effected only by the high-energy tail of the electron energy distribution, and the fragmentations of the molecular radical cations $M^{+\cdot}$ were minimized. This feature is important for the deconvolution of the effect of fragmentation of the $M^{+\cdot}$ ions, and also of the natural $^{13}C:^{12}C$ ratio, from the desired distribution of $M^{+\cdot}$ ions containing zero, one, two, etc., ^{1}H atoms. This deconvolution procedure is described in detail in the documentation for the two DPAC solutions [19]. The distributions of isotopomers for each compound, listed in Table 4, are fixed properties. However, the intensities observed at the m/z values characteristic of the corresponding $M^{+\cdot}$ ions, or of the MH^+ ions if chemical ionization (CI) is used, are also functions of the characteristics of the particular ionization source reflecting differing degrees of fragmentation (EI) or of hydrogen-deuterium exchange with the CI reagent gas. Problems associated with such variations can be avoided by using the appropriate analytical method, as summarized below.

Each individual compound was weighed using a three-balance technique, in which each weighing was performed three times using three different analytical balances (Mettler AE-240, Mettler AE-260 and Sartorius MC1). Thus the mass of each compound used in each of the two solutions was determined as the mean (with a standard deviation) of three independently measured values. Each compound was washed quantitatively from its weighing

Table 3. Major chemical impurities in DPAC-1 solution

DPAC-1 Component	Impurity	μg/g	RRT[b]
1-Methylnaphthalene-d_{12}	naphthalene-d_8	0.51[a]	0.267
	dimethylnaphthalene(s)-d_{12}	0.33	0.420
	unknown (m/z 168, 150 ,136, 122)	0.19	0.429
	methylnaphthalene-d_{12} isomer	0.12	0.341
	unknown (m/z 160, 142, 128, 114)	0.10	0.335
Fluorene-d_{10}	unknown (m/z 180, 174, 162, 160, 146)	0.40	0.612
Phenanthrene-d_{10}	anthracene-d_{10}	0.64[a]	0.773
9-Methylanthracene-d_{12}	anthracene-d_{10}	0.35[a]	0.773
	dimethylanthracenes-d_{14}	0.18	0.932–0.963
Dibenz[a, i]pyrene-d_{14}	dideuterodibenzo[a, i]pyrene-d_{16}	0.48	1.685
Coronene-d_{12}	benzo[a]pyrene-d_{12} or isomer	0.23[a]	1.423
	benzo[ghi]perylene-d_{12} or isomer	0.05[a]	1.604

[a] These quantities have been included in the total concentrations given in Table 1
[b] Typical relative retention times for a 30m DB-5 column, RRT for pyrene-d_{10} defined as 1.000; same scale as used in Table 1

Table 4. ^1H Content of deuterated PACs from low-eV GC/EIMS data

Compound	Formula[a]	p_D(4eV)[b]	$F_H = n_H/n_D$	($^1H_0$: $^1H_1$: $^1H_2$: $^1H_3$)[c]
Naphthalene	$C_{10}X_8$	~ 0	0.00625	0.951: 0.048: 0.001
1-Methylnaphthalene	$C_{11}X_{10}$	0.050	0.00434	0.958: 0.041: 0.001
Biphenyl	$C_{12}X_{10}$	0.025	0.00588	0.943: 0.055: 0.001
Acenaphthylene	$C_{12}X_8$	0.011	0.00978	0.925: 0.072: 0.003
Acenaphthene	$C_{12}X_{10}$	0.115	0.00764	0.927: 0.071: 0.002
Dibenzofuran	$C_{12}X_8O$	~ 0	0.01120	0.915: 0.082: 0.003
Fluorene	$C_{13}X_{10}$	0.061	0.01711	0.844: 0.144: 0.011
Dibenzo[b,d]thiophene	$C_{12}X_8S$	~ 0	0.00883	0.932: 0.066: 0.002
Phenanthrene	$C_{14}X_{10}$	0.044	0.04693	0.633: 0.297: 0.063: 0.008
Anthracene	$C_{14}X_{10}$	~ 0	0.01034	0.902: 0.093: 0.004
9-Methylanthracene	$C_{15}X_{12}$	0.045	0.00319	0.963: 0.037: 0.006
Fluoranthene	$C_{16}X_{10}$	0.0003	0.00952	0.909: 0.087 0.004
Pyrene	$C_{16}X_{10}$	0.002	0.00994	0.906: 0.090: 0.004
Benz[a]anthracene	$C_{18}X_{12}$	~ 0	0.01271	0.859: 0.131: 0.009: 0.001
Chrysene	$C_{18}X_{12}$	0.002	0.01971	0.791: 0.187: 0.020: 0.002
Benzo[b]fluoranthene	$C_{20}X_{12}$	0.001	0.00603	0.930: 0.068: 0.002
Benzo[a]pyrene	$C_{20}X_{12}$	~ 0	0.00994	0.888: 0.106: 0.006
Perylene	$C_{20}X_{12}$	0.017	0.00923	0.896: 0.099: 0.005
Dibenz[a,h]anthracene	$C_{22}X_{14}$	~ 0	0.01350	0.829: 0.157: 0.014: 001
Benzo[ghi]perylene	$C_{22}X_{14}$	~ 0	0.00317	0.963: 0.037: 0.001
Coronene	$C_{24}X_{12}$	0.018	0.01953	0.793: 0.186: 0.020: 0.001
Dibenz[a,i]pyrene	$C_{24}X_{14}$	0.001	0.00383	0.948: 0.051: 0.001

[a] X = ^1H and/or ^2D
[b] For each compound C_iX_j, $[p_D \times (k/j)]$ is the probability that the molecular radical cation isotopomer $(C_iD_kH_{j-k})^{+\cdot}$ will expel a D atom under the ion source conditions of the particular experiment; this assumes that p_D is independent of k for each compound C_iX_j
[c] Molar ratios of isotopomers, calculated from the binomial distribution $(1 + F_H)^j$ (renormalized so that the sum of all isotopomers is unity) for each compound C_iX_j, where F_H is the ratio of total ^1H atoms to ^2D atoms in the sample of that compound used in DPAC-1. The $^{13}C/^{12}C$ ratio corresponds to the natural isotopic distribution in all cases

vial into the main solution, and the final total weight of the solution determined.

Before dispensing the solution into ampoules and sealing, an extensive series of checks was conducted to ensure that our filling and sealing procedure did not introduce contaminants into the sealed ampoules. It is known [20] that it is possible to form substantial quantities of PACs by pyrolysis of toluene (and other organic solvents) while flame-sealing ampoules. The present procedure involved dispensing approximately 1.1 mL of solution at ambient temperature directly into the foot of a 5 mL amber ampoule previously filled with argon, using a specially designed dispenser to avoid any deposition of solution on to the neck of the ampoule. The filled ampoules were then cooled in dry-ice in isopropanol just before flame-sealing under nitrogen, to minimize contact of the vapour with the seal point. The dispensers were also checked as a source of potential contamination, and one model was found to have some plastic components from which toluene leached a significant level of contaminants over a period of hours. This filling/sealing procedure was checked by filling each of 10 ampoules with 1.1 mL of toluene, sealing the pre-cooled ampoules, and analyzing the contents for PACs by HPLC-UV and by GC/MS. The ampoules themselves were subjected to a washing and drying procedure just prior to filling, using distilled

in-glass hexane followed by acetone, followed by overnight heating at 130 C. Provided that all of these precautions were taken, no detectable contamination due to the filling and sealing procedure was observed. The filled ampoules of each of the DPAC-1 and DPAC-2 solutions were labelled with sequential numbers to keep track of the order of filling.

Inter-ampoule homogeneity was checked for both solutions by randomly selecting 12 ampoules of each, covering the entire order of filling, and analyzing the contents of each vial in triplicate by GC/FID using non-deuterated 1-methylfluorene and 1-methylpyrene (plus perylene in the case of DPAC-1) as internal standards. An example of the results obtained for DPAC-1 is shown in Fig. 1. The inter-ampoule variation was thus estimated to be 0.04% (relative standard deviation (RSD) of the mean values for the 12 chosen ampoules), almost identical to the RSD for multiple GC/FID analyses of a single ampoule using on-column injection. The precision obtained using a splitless injector was worse by a factor of about 3, as expected for GC analyses of analytes and internal standards with widely varying volatilities [21, 22]. Some HPLC/UV and GC/MS analyses were also conducted on these selected ampoules, to check for contamination during the filling-sealing procedure. No contamination was detected.

The concentrations (Tables 1 and 2) derived from the three-balance gravimetric measurements, plus the chemical purity data, were checked by analyzing additional ampoules. In one series of experiments weighed quantities of the DPAC-1 and of the NIST SRM1491 solution (a standard solution of non-deuterated PAHs) were mixed together, and the resulting mixture analyzed by GC/MS with selected ion monitoring (SIM) of $M^{+\cdot}$ ions. However, the data obtained were not reliable, e.g. significantly different ratios were obtained using different mass spectrometers. It is believed that this reflected the influence of kinetic isotope effects [11] upon relative response factors for each PAC and its deuterated analogue.

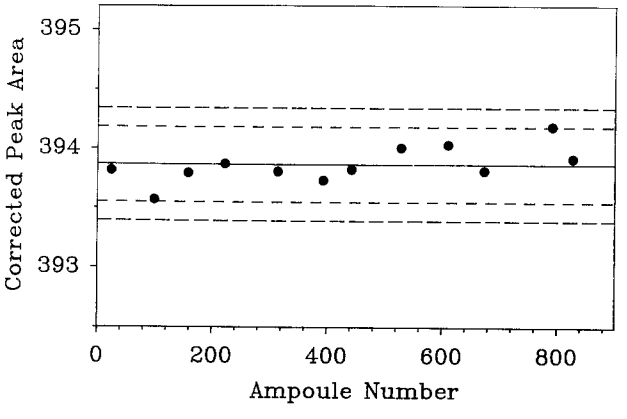

Fig. 1. GC/FID peak areas (on-column injection, mean values for triplicate injections) for fluoranthene-d_{10} relative to those of 1-methylfluorene as internal standard, as a function of ampoule number (filling order). The full line represents the mean value of the entire data set (36 determinations), i.e. all ampoules were treated as representing the same sample. Similarly the inner and outer dashed lines represent $\pm$ 2s and $\pm$ 3s, respectively, where s = 0.16 is the standard deviation of the entire dataset (corresponds to RSD = 0.04%)

Accordingly, checks on the recommended concentrations of Tables 1 and 2 were conducted by GC/FID using 1-methylfluorene and 1-methylpyrene as internal standards for DPAC-1, and by LC/FLD using the NIST SRM1647 (non-deuterated PAHs in acetonitrile) as an internal standard for DPAC-2. Relative response factors (RRFs) for the FID were first calculated on the assumption that, at least for the hydrocarbons, the molar RRF is proportional to the number of carbon atoms per molecule [23]; corrections to these first-order RRF values for the FID were obtained by direct measurement using proteo-PACs (including those in the NIST SRM1491). Unfortunately the experimental precision with which these RRF values could be determined was appreciably less (greater uncertainty) than that associated with the gravimetric determinations. In addition, their application to the GC/FID data for perdeutero-PACs involves the additional assumption that the FID is insensitive to H/D substitution. For these reasons these GC/FID determinations were treated as checks, valid to within a few per cent, on the concentrations derived from the weighings and purity determinations of the constituents of DPAC-1. In almost all cases these experiments showed that no gross discrepancies existed. However, these GC/FID results for DPAC-1 did consistently indicate that the concentration thus calculated for biphenyl-d_{10} was some 20% lower than that expected from the triple-balance weighing procedure, well outside combined uncertainties. It is suspected that this volatile PAH was partially lost between the time of weighing and the time at which the solution was prepared. For this reason the estimated concentration of biphenyl-d_{10} in DPAC-1, given in Table 1, is that derived from the GC/FID measurements rather than the gravimetric value. A similar discrepancy was found for the concentration of naphthalene-d_8 in DPAC-2, based upon the LC/FLD data. Accordingly, this discrepancy was further checked using GC/FID analysis of DPAC-2 with an independently weighed internal standard. These experiments confirmed that the concentration of naphthalene-d_8 in DPAC-2 is significantly lower than the gravimetric value, probably reflecting uncontrolled evaporation of this volatile constituent between the weighing procedure and its dissolution, and this is reflected in the recommended concentration given in Table 2.

Methods of use of surrogate internal standards

This topic has been reviewed recently [9, 10], so only a brief discussion is included here. Two quantities are usually measured in the analysis of trace constituents of a complex matrix, viz. the quantity Q_a' of the target analyte in the extract of a known mass W_s of sample, and an estimate of the fractional recovery F_a' of the analyte from the sample into the extract solution to be analyzed. The desired quantity Q_a, the amount (mass or number of moles) of analyte in the original mass (W_s) of sample, is

given by:

$$Q_a = Q'_a/F'_a \quad (1)$$

In the brief summary included in the Appendix, it is assumed that all calibration curves are linear and include the origin to within experimental uncertainty; cases in which these conditions are not fulfilled are discussed elsewhere [9, 10]. The condition of a zero intercept for the calibration curve implies that constant losses of analyte (as opposed to proportional losses accounted for by the parameter F'_a) during the extraction, clean-up and analysis steps, are all negligible. It is also important to bear in mind that experimental estimates of F'_a, based on recoveries of standards added to the raw sample, are upper limits reflecting possible occlusion of native analytes in the sample [24]. For this reason, values of Q_a obtained via Eq. (1) are lower bounds to the "true" values.

The simplest analytical method exploiting surrogate internal standards (method (i) of the Appendix) involves direct comparison of peak areas for analyte and internal standard, observed in the chromatogram of the spiked sample extract. The most important advantages of methods (ii) and (iii) (Appendix), in which the surrogate internal standard is used in conjunction with an external standard, are: (a) the concentrations of the internal standards need not be accurately known (though those of the external standard must be known), and (b) the relative instrumental response factors, for analytes and internal standards, need not be known at all. Point (b) is particularly important for deuterated internal standards used with mass spectrometric detection, for reasons discussed above. Therefore it is strongly recommended that either of methods (ii) and (iii) be used whenever possible. Whatever method is used, the most reliable values are always obtained when the ratio of chromatographic peak areas for analyte and standard is as close as possible to unity. It is for this reason that the profile of relative concentrations in DPAC-1 and DPAC-2 (Tables 1 and 2) was chosen to match that found in a range of environmental samples. In order to optimize this advantage, it is important to choose an appropriate quantity of DPAC-1 or DPAC-2 to add to the sample (and to the external standard if either of methods (ii) and (iii) is to be used). If the concentration of a convenient analyte (pyrene is a good choice) can be estimated first, the appropriate spiking level can be determined for subsequent accurate determinations. Alternatively, it may be possible to estimate a total PAC concentration using UV-visible spectroscopy, and hence the appropriate spike level.

Examples of application of the DPAC solutions

A separate publication will describe a full comparison of methods (i)–(iii) for exploiting the DPAC-1 solution as a surrogate internal standard, together with the method of standard additions, in certification of a marine sediment reference material. In the present work the application of this standard solution to development of a new quantitative instrumental technique for PACs will be described briefly. While capillary column GC/MS is the favored quantitative technique for PACs, it is limited with respect to the molecular weights of the PACs thus analyzable and also to the separation selectivity for isomers. Reverse-phase HPLC, although much inferior in terms of chromatographic efficiency, can provide advantages complementary to those of GC particularly with respect to the upper limit for molecular weights and to separation selectivity.

The main drawback in applying HPLC to quantitative trace analysis of PACs in complex mixtures has been the unavailability of a reliable LC/MS interface. A moving belt interface with EI ionization can provide qualitative analysis of PACs up to 580 Da [25], though no quantitation was attempted. The new technique described below involves reverse-phase HPLC separation of the PACs combined with on-line mass spectrometric detection incorporating atmospheric pressure chemical ionization (APCI). The LC/MS interface used was a heated pneumatic nebulizer. Additional details of this interface can be found elsewhere [26], but it is relevant to note here the features of the APCI mass spectra of PACs. These spectra are characterized by both $M^{+\cdot}$ and MH^+ ions due to charge transfer and gas phase protonation, respectively. The MH^+ ion dominates the spectra of higher molecular weight PACs (> 200 dalton) due to their larger proton affinities. No ion fragmentation is observed, so the only potential mass spectrometric complication is that of H/D exchange within the APCI plasma. However, this did not appear to be a significant factor under our experimental conditions.

The LC/APCI-MS system generally shows excellent performance in the external calibration mode. For example, one experiment with a serial dilution of the NIST SRM1647 PAH calibration solution showed a dynamic range of almost 4 orders of magnitude with a highly linear response ($r^2 = 0.99998$) and an intercept through zero within experimental error. Such results are in large part due to the excellent reproducibility of injection volumes when using modern LC injectors. The precision of quantitation of samples with external calibration was typically better than 3% RSD. With this kind of performance the value of internal standardization may not be immediately obvious. One of the problems we have observed, however, is the gradual evaporation of volatile solvent from standards and samples, leading to erratic and erroneous results with external calibration (relative errors up to 50% have been observed). This problem can be greatly reduced through the use of volumetric internal standards. Of course, as discussed above, the use of perdeutero-PACs as surrogate internal standards also provides the advantage of correcting for losses in sample work-up. The experiment described below was conducted to evaluate only the ability of this LC/APCI-MS technique to quantify PACs in a complex mixture using internal standardization, and did not involve any estimates of fractional recoveries from raw environmental samples.

A calibration curve was generated to determine linearity of response and intercept. This was accomplished by mixing SRM1647 (NIST's PAH calibration solution in acetonitrile) with DPAC-1 in various proportions

(SRM1647/DPAC-1 volume ratios of 0, and approximately 0.9, 4.6, 9.3 and 14.0), determined accurately by weighing. The m/z values for both M^+ and MH^+ ions, for both proteo-and deutero-PACs, were measured in an LC/MS selected ion monitoring experiment. Each solution was analyzed in triplicate and the H/D peak area ratios for the M^+ and MH^+ ions of each compound were averaged. Figure 2 shows the resulting calibration curve for one representative analyte, benzo[a]pyrene. Excellent linearity ($r^2 = 0.9997$), over a range of H/D ratios from 1:3 to 3:1, is observed, and the curve includes the origin to within experimental error (y-intercept = 0.014 $\pm$ 0.017). We have observed that such curves are linear even if solutions are allowed to partially evaporate. In fact, the data in Fig. 2 were generated from solutions that had reduced in volume by as much as 10% due to uncontrolled evaporation. However, the slope of the least-squares linear fit to these data was 0.932 $\pm$ 0.009, significantly different from unity, indicating that the response factors for deuterated and non-deuterated benzo[a] pyrene were not the same in these LC/APCI-MS experiments. No ion fragmentation was observed under these soft ionization conditions, so no uncertainties arose from kinetic isotope effects in this case. It was hoped that accounting for both the $M^{+\cdot}$ and MH^+ ions would correct for any differences in gas phase basicities of the proteo- and deutero-PACs, but this effect may in fact account for the discrepancy. Note that the calculation of the molar ratio of perproteo- to perdeutero-PAC took into account the isotopomer distributions listed for the latter in Table 4.

These data were useful in establishing that H/D peak area ratios less than or greater than 1:1 can generate accurate results though, of course, the best precision would be achieved with a 1:1 peak area ratio. Since the perdeutero-PACs in DPAC-1 are in approximately the same relative proportion to each other as the PACs in most environmental samples, it is possible to arrange that most of the analytes will have a closely eluting internal standard in a roughly 1:1 proportion. Unfortunately, we still have one problem with calibration. Because SRM1647 and other NIST calibration solutions have all their PACs at about the same concentrations, a set of calibration solutions such as those used to generate Fig. 2 should be made to ensure that for each PAC there is a calibration point at the optimum 1:1 ratio.

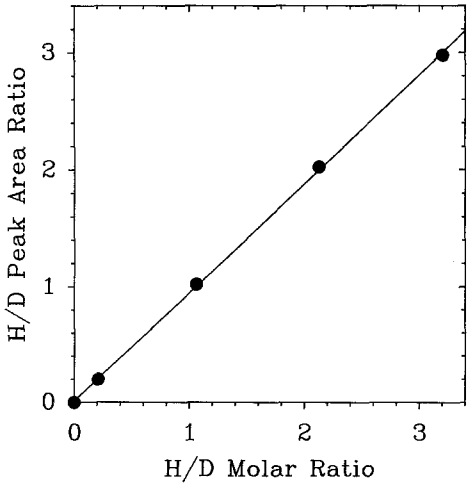

Fig. 2. Calibration curve for the analysis of benzo[a]pyrene (BaP) by LC/APCI-MS using perdeutero-BaP as an internal standard. Solutions were prepared by mixing varying proportions of SRM1647 and DPAC-1. Each point represents the mean of triplicate analyses of each solution; the ratios of proteo- to deutero-BaP peak areas, for M^+ and MH^+ ions, were averaged

Quantitation of the NIST SRM1597 coal tar extract was then performed using LC/APCI-MS and internal standardization with the DPAC-1 solution. Calibration was performed with the same SRM1647/DPAC-1 solutions as were used to obtain Fig. 2. The results obtained are summarized in Table 5. Except for fluorene, fluoranthene and benzo[*a*]pyrene, the deviation of the calculated concentrations from the values certified by NIST ("relative errors" in Table 5) were less than 7% (the average of all the absolute relative errors was 5.0%). The partial coelution of another isomer of molecular weight 202, viz. acephenanthrylene, with fluoranthene, accounted for the high value obtained (Table 5) for the concentration of the latter compound. Similarly, a mass 166 isomer coeluting with fluorene in the coal tar solution gave rise to the high calculated concentration for this compound. At this time, an explanation for the low (-11%) measured concentration of benzo[a]pyrene cannot be given. The average precision of 6.3% for this data set is not as good as we have achieved at other times using the LC/APCI-MS system with external calibration. This may be due in part to the fact that, as noted previously [27], use of a volumetric internal standard can sometimes decrease the reproducibility of LC analyses through simple propagation of error from dealing with double the number of measured quantities. It is gratifying, however, that use of DPAC-1 as an internal standard provided concentrations for the constituent PACs in SRM1597 in satisfactory agreement with the certified values.

The coal tar extract SRM1597 was also analyzed using LC-FLD with DPAC-2 as internal standard. Figure 3 shows a chromatogram obtained from the analysis of DPAC-2 mixed with SRM1597 using LC-FLD with dual programmed wavelengths. Good separation of deutero-PACs from the proteo-PACs, as well as other sample components, was achieved, except for naphthalene-d_8. Calibration was accomplished with mixtures of SRM1647 and DPAC-2. Again, as for LC/MS, good linearities for the H/D peak area ratios were observed. Table 6 presents a summary of the results of the quantitative analyses. The average precision of measurement for each PAC was a very acceptable 2% RSD. Except for chrysene, there was excellent agreement between the determined concentrations and those reported by NIST (the average of all the absolute relative errors was 3.4%). The only really poor result ($+21\%$ error) for chrysene appears to be due partly to the weak signal and partly to the near coelution of other components that also fluoresce. The result for naphthalene was generated by using phenanthrene-d_{10} as the internal standard. It was not possible to

Table 5. LC/APCI-MS quantification results for the NIST SRM1597 (coal-tar extract) using internal standardization with the DPAC-1 solution of perdeuterated PACs. Mixtures of NIST SRM1647 and DPAC-1 were used for calibration

Compound	Certfd. concn. (μg/g)	Present results (μg/g)	Std. Dev. (n = 3)	% RSD (n = 3)	Rel. Error (%)
Naphthalene	1160	1149	33	2.9	− 1.0
Acenaphthylene	250[c]	247	9	3.6	− 1.1
Acenaphthene	–	9.2	0.09	1.0	N.A.
Fluorene	140[c]	161	7.4	4.6	+ 15
Phenanthrene	462	461	15	3.2	− 0.2
Anthracene	101	107	5.7	5.4	+ 6.1
Fluoranthene	322	351	40	11	+ 9.0
Pyrene	235	241	12	5.0	+ 2.6
Benz[a]anthracene	98.6	105	4.8	4.6	+ 6.8
Chrysene	71.7	70.4	5.3	7.5	− 1.8
Benzo[b]fluoranthene	61[c]	63	5.0	7.9	+ 2.6
Benzo[k]fluoranthene	39[c]	41[a]	3.3	8.0	+ 5.7
Benzo[a]pyrene	95.8	84.8	1.0	1.1	− 11
Benzo[ghi]perylene	53.7	57.0	1.9	3.3	+ 6.2
Dibenz[a, h]anthracene	6.8[d]	7.4	0.5	6.6	+ 8.8
Indeno[1,2,3-cd]pyrene	60.2	60[b]	15	25	− 0.4

[a] Perdeutero-benzo[b]fluoranthene used as internal standard
[b] Perdeutero-benzo[ghi]perylene used as internal standard
[c] Not certified by NIST; value quoted is that obtained by NIST using one of HPLC or GC
[d] Reported but not certified by NIST in Ref. [28]

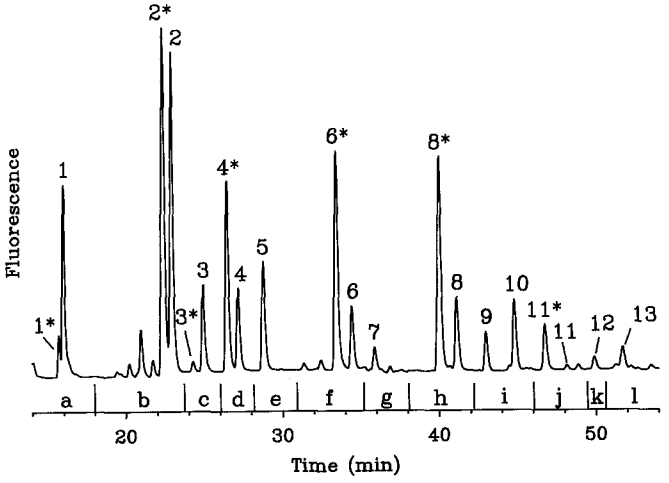

Fig. 3. HPLC/FLD chromatogram of a mixture of DPAC-2 plus NIST SRM1597. The compounds are numbered as follows (asterisk indicates deuterated compound): *1* naphthalene; *2* phenanthrene; *3* anthracene; *4* fluoranthene; *5* pyrene; *6* benz[a]anthracene; *7* chrysene; *8* perylene; *9* benzo[k]fluoranthene; *10* benzo[a]pyrene; *11* dibenz[a, h] anthracene; *12* benzo[ghi]perylene; *13* indeno[1,2,3-cd]pyrene. Excitation (Ex) and emission (Em) wavelength pairs (nm), and detector gain settings (G), for each retention time window, were as follows: Window *a*: Ex = 280, Em = 340, G = x2. Window *b*: Ex = 249, Em = 380, G = x1. Window *c*: Ex = 250, Em = 442, G = x1. Window *d*: Ex = 285, Em = 450, G = x2. Window *e*: Ex = 333, Em = 390, G = x1. Window *f*: Ex = 285, Em = 385, G = x1. Window *g*: Ex = 285, Em = 385, G = x4. Window *h*: Ex = 406, Em = 440, G = x8. Window *i*: Ex = 296, Em = 405, G = x1. Window *j*: Ex = 298, Em = 394, G = x2. Window *k*: Ex = 380, Em = 419, G = x2. Window *l*: Ex = 300, Em = 500, G = x16

use naphthalene-d_8 as the internal standard due to its incomplete resolution from naphthalene. In retrospect it was probably a mistake to include naphthalene-d_8 in DPAC-2, but its presence should be useful for occasions where this solution is used in LC-MS or GC-MS analyses.

Table 6. LC-FLD quantification results for NIST SRM1597 (coal tar extract) using internal standardization with the DPAC-2 solution of perdeuterated PACs. A mixture of SRM1647 and DPAC-2 was used for calibration

Compound	Certfd. conc'n (μg/g)	Present result (μg/g)	Std. dev. (*n* = 4)	% RSD	Relative error (%)
Naphthalene	1160	1188	7	0.6	+ 2.4
Phenanthrene	462	452	8	1.7	− 2.2
Anthracene	101	109	2	1.8	+ 7.9
Fluoranthene	322	327	3	1.0	+ 1.5
Pyrene	235	252	6	2.5	+ 7.2
Benz[a]anthracene	98.6	99.9	0.6	0.6	+ 1.3
Chrysene	71.7	86.5	0.4	0.5	+ 21
Perylene	26.1	27.0	0.4	1.6	+ 1.1
Benzo[k]fluoranthene[a]	38.8	38.7	1.5	3.9	− 0.3
Benzo[a]pyrene	95.8	92.1	1.8	2.0	− 3.9
Dibenz[a, h]anthracene[b]	na	10.9	0.6	4.6	na
Benzo[ghi]perylene	53.7	50.5	1.8	3.6	− 6.0

[a] Not certified; NIST's LC result given
[b] Not measured by NIST

Again, it is gratifying that use of DPAC-2 as an internal standard provided concentration values for SRM1597 in satisfactory agreement with the certified values.

Conclusions

Preparation of instrument calibration solutions of deuterated PACs, for use as surrogate internal standards, entailed appreciable difficulties in addition to those involved for non-deuterated analogues. Most of these difficulties arose from the fact that none of the individual standards was 100% deuterated. For a sample of a compound which contains m H/D atoms per molecule, and for which x% of the total H/D content is hydrogen,

approximately xm% of the molecules contain one H-atom (Table 4). This is extremely important for quantification using mass spectrometric detection, and is further complicated by the possibility of kinetic isotope effects in the subsequent ion fragmentation reactions within an EI ion source. The effect of the xm% of the isotopic contaminant will be unnoticeable for LC/FLD determinations provided that x is not too large.

The DPAC-1 and DPAC-2 solutions, whose preparation has been described here, provide satisfactory results when used to quantify PAHs in a certified coal-tar extract (NIST SRM1597). Examples of their use as surrogate internal standards, in certification of PAHs in a marine sediment reference material, will be published shortly.

Appendix: Summary of methods in organic trace analysis exploiting surrogate internal standards

The following is a summary of useful results whose derivation is described elsewhere [9, 10]. The most important assumption is that the appropriate calibration curves are linear and include the origin to within experimental error.

(i) *Use of a surrogate internal standard without an external standard.* In this approach, prior to extraction the weighed sample (W_s) is spiked with a known quantity Q_i of the surrogate internal standard, usually as a measured volume V_{IS} of a solution of concentration C_{IS}. After extraction and clean-up, the sample is analyzed by e.g. GC/MS, and the ratio of the GC peak areas A'_a and A'_i, for analyte and internal standard respectively, is measured from the same experiment (thus removing the injection volume from consideration). Then the desired concentration of analyte in the original sample is given [9, 10] by:

$$Q_a/W_s = (A'_a/A'_i)\cdot(F'_i/F'_a)\cdot C_{IS}\cdot V_{IS}/W_s \quad (2)$$

Clearly, the value thus derived for Q_a depends directly upon those of C_{IS} and V_{IS}, and is thus subject to any errors in C_{IS} due to solvent evaporation or other causes. In addition, use of Eq. (2) requires the following explicit assumptions:
- the instrumental response factors for the native analyte and internal standard are equal (see discussion above in *Preparation and analysis* section);
- the fractional recovery efficiencies F'_a and F'_i are taken to be equal, an assumption most likely to be valid if these parameters are close to unity.

A value of F'_i, the fractional recovery into the sample extract of the internal standard spiked into the sample prior to extraction, can be obtained via comparison of the peak area A'_i with that observed (A'''_i) for a separate injection of a (suitably diluted) solution of the internal standard. If D'''_{IS} is the dilution factor for this diluted version of the original internal standard solution (so that $C'''_{IS} = C_{IS}\cdot D'''_{IS}$), then:

$$F'_i = (A'_i/A'''_i)\cdot(v'''/v')\cdot(V'/V_{IS})\cdot D'''_{IS} \quad (3)$$

where V' is the total volume of sample extract prior to any aliquots being removed for analysis, and v''' and v' are the injection volumes (highly uncertain in the case of GC though not for HPLC). In general F'_i is an upper bound to F'_a, the desired quantity, since the native analyte is subject to occlusion by the matrix in a fashion not applicable to freshly spiked internal standard [9, 10]. The disadvantages of this method of use of a surrogate internal standard are apparent. Its advantages are its speed and ease of use.

(ii) *Use of a surrogate internal standard in conjunction with an external standard.* In this method both the weighed sample and an accurate external standard solution of the target analyte (i.e. *not* isotopically labelled) are spiked with identical quantities of the solution of the surrogate internal standard. This quantity (usually measured as $C_{IS}\cdot V_{IS}$) does not enter the calculations for Q_a or F'_i, so that an accurate value for C_{IS} is not required. The working relationships [9, 10] are Eqs. (4) and (5):

$$Q_a/W_s = (A'_a/A'_i)\cdot(A''_i/A''_a)\cdot(F'_i/F'_a)\cdot(Q''_a/W_s) \quad (4)$$

where Q''_a is the (weighed) quantity of standard analyte in the external standard solution at the moment of spiking with the internal standard, and A''_a and A''_i are the chromatographic peak areas for analyte and internal standard, respectively, measured in the separate experiment on the spiked external standard solution.

$$F'_i = (A'_i/A''_i)\cdot(v''/v')\cdot(V'/V'') \quad (5)$$

where V'' is the total volume of the external standard solution immediately after spiking with the internal standard solution, and v' and v'' are the injection volumes of the sample extract solution and of the spiked external standard solution, respectively. The advantage of method (ii) over method (i) thus lies mainly in the reliability of the values deduced for Q_a, which are now (Eq. (4)) independent of the value C_{IS}, the concentration of the internal standard solution (e.g. DPAC-1). In method (ii) the role of C_{IS} is played by Q''_a, the weighed quantity of analyte in the external standard solution (e.g. the NIST SRM 1491). The disadvantages of method (ii) are:
- the values of F'_a and F'_i must still be assumed (Eq. (4)) to be equal;
- it is important to ensure that the quantities of internal standard, used to spike the sample and the external standard solution, are equal even if they need not be accurately known; in practice such reproducibility of V_{IS} can be achieved to within excellent precision through use of modern electronic pipettes;
- the estimate of F'_i still depends explicitly upon injection volumes (Eq. (5));

The advantages of method (ii) over method (i) are:
- the relative response factor, for target analyte relative to surrogate internal standard, does not enter the calculations (since all quantification is now done relative to the external standard); this removes all uncertainties arising from possible kinetic isotope effects on mass spectrometric fragmentations or H/D exchange if Cl mass spectrometry is employed, provided that the spiked sample extract and the spiked external standard are analyzed using the same experimental conditions (preferably on the same day);
- the concentration of the internal standard (C_{IS}) need not be accurately known.

(iii) Use of a surrogate internal standard in conjunction with both a volumetric internal standard and an external standard. This method is similar to method (ii). However, in addition to spiking both the raw sample (prior to extraction) and the external standard solution with equal quantities of the surrogate internal standard (e.g. DPAC-1), both the sample extract solution and the external standard solution are also spiked with equal quantities of a suitable volumetric standard [9, 10]. This volumetric internal standard need not be an isotope-labelled analyte, although it could be, but must be chromatographically distinguishable from all constituents of the sample extract and of the surrogate internal standard. This additional spiking makes no difference to the estimation of Q_a, and Eq. (4) still applies. However, use of the volumetric internal standard removes the dependence of F'_i on the injection volumes (see Eq. (5)). If subscript i denotes the surrogate internal standard as before, and subscript j the additional volumetric internal standard, the relevant expression for F'_i is now Eq. (6):

$$F'_i = (A'_i/A'_j)\cdot(A''_j/A''_i) \quad (6)$$

where single primes refer to the (doubly-spiked) sample extract solution, and double primes to the (doubly spiked) external standard solution. All of the same advantages and disadvantages listed for method (ii) also apply to method (iii), with the exception that F'_i is now independent of any injection volumes.

Note, however, that the intrinsic problem of possible differences between F'_i and F'_a applies to all three methods. Usually $F'_i \leq F'_a$, so that values obtained for Q_a by assuming $F'_i = F'_a$ are generally lower limits to the true values [9, 10]. An example of this effect for PAHs in environmental samples has been described [24]. Extraction rates by supercritical fluid extraction were much larger for the deuterated PAHs added to the samples than for the native PAHs, this effect being most pronounced for volatile PAHs (e.g. naphthalene) which must be tightly bound in order to remain associated with the matrix. This exemplary work [24]

used method (iii), with deuterated PAHs as surrogate internal standards, 1-chloroanthracene as volumetric internal standard, and NIST SRM 1647a (16 PAHs in acetonitrile) as the external standard.

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