NRC Publications Archive
Archives des publications du CNRC

Quantitative trace analysis by combined chromatography and mass spectrometry using external and internal standards
Boyd, R. K.

For the publisher's version, please access the DOI link below./ Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

NRC Publications Record / Notice d'Archives des publications de CNRC:
https://nrc-publications.canada.ca/eng/view/object/?id=28dc857e-1762-4057-a359-d0d011b1ccb5
https://publications-cnrc.canada.ca/fra/voir/objet/?id=28dc857e-1762-4057-a359-d0d011b1ccb5

Questions? Contact the NRC Publications Archive team at
PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

National Research Council Canada   Conseil national de recherches Canada

Canada

M·uin c. 2

b 200122-24X An

Quantitative Trace Analysis by Combined Chromatography

and Mass Spectrometry Using External and Internal Standards.*

IMB Technical Report #63.

R.K. Boyd

Marine Analytical Chemistry Standards Program,

Institute for Marine Biosciences,

National Research Council,

1411 Oxford Street, HALIFAX, Nova Scotia, CANADA B3H 3Z1.

b 200122-24X An

CONTENTS.

I.   Introduction.

Quantitative analysis of analytes present at trace levels in complex mixtures is one of the most demanding experimental tasks facing the modern scientist. Concerns about environmental pollution by trace amounts of highly toxic contaminants, for example, have placed ever-increasing demands upon the analytical chemist to increase the accuracy and precision of such measurements, and to decrease detection limits, for an ever-widening range of analytes.

This document represents an attempt to assemble and systematise methods which have been developed to facilitate quantitative analyses of this kind through use of external and internal standards. Most chemists have a general appreciation of these terms; their definitions as used in this document are given in Section II. However, in the course of several years of operation of the Marine Analytical Chemistry Standards Program (MACSP) of the National Research Council of Canada, it has become clear to us that many of our clients for the instrument calibration solutions and isotope-labelled internal standards, which the MACSP supplies, are unaware of some of the better methods of exploiting such materials.

This document contains no original work. It is in effect a review of methods invented by others (see *e.g.* Refs. 1-3 for earlier reviews). In addition, it is largely a theoretical treatment, but the mathematics required involve only low-level algebraic manipulations. It is hoped that this report can fill a gap in bringing together and systematising the various methods, while drawing attention to their advantages and drawbacks. **The treatment focusses on chromatography combined with mass spectrometry, but many of the considerations apply also to procedures using other chromatographic detectors.** Further, in order to keep the length within reasonable bounds, the question of errors and statistical treatment of data will not be treated systematically; excellent user-oriented reviews, of applications of statistical methods to analytical chemistry, are available[4-6].

The experimental equipment used for modern quantitative analysis is highly sophisticated, usually incorporating a high resolution chromatographic stage (most often capillary column gas chromatography (GC) or high performance liquid chromatography (HPLC)), coupled to on-line detectors of a wide range of complexity of which mass spectrometry (MS) is the most flexible and informative. Faced with this sometimes bewildering array of technological marvels, it is easy to forget that **all quantitative analyses ultimately depend upon the availability of pure analyte standards, weighed out to the highest possible accuracy and precision on an analytical balance (see Section VI).** The sophisticated GC/MS or HPLC/MS apparatus simply provides a means of comparing an electrical signal, generated by passage of the analyte from the sample through the apparatus, to the corresponding signal generated by a known (*via* weighing) amount of the analyte standard. This

3

document describes methods which have been devised to make this comparison process as free as possible from experimental errors.

Such experimental errors are conveniently classified into **random** and **systematic** errors. The smaller the random errors, the greater is the experimental **precision**. In contrast, the experimental **accuracy** is a measure of how successfully the systematic errors have been reduced, *i.e.* how closely the experimental values approach the "true" or "accepted" value for the parameter being measured. The random errors (experimental precision) are usually estimated experimentally *via* the **repeatability**, *i.e.* the spread in values obtained on multiple consecutive repeats of the same analysis, performed consecutively. The repeatability is often distinguished from the **reproducibility** of the analytical procedure which refers to comparison of the results of an experimental estimate of precision (repeatability), obtained on one occasion, with those of a similar estimate obtained some time later or perhaps in a different laboratory. Thus defined, the experimental reproducibility clearly involves both random errors and uncontrolled systematic errors. Further, systematic errors are conveniently sub-divided into **bias** errors (constant offset of the experimental from the "true" value) and **proportional** errors (systematic differences between experimental and "true" values, whose magnitude is proportional to that of the "true" value).

The theory of random errors is highly developed[4-6]. Systematic errors are more difficult to deal with due, at least in part, to the difficulty in determining "true" values. Detection and estimation of systematic errors are of crucial importance in the pharmaceutical industry[3], where errors in the concentration of the active ingredient in a formulation can be literally a matter of life and death. It is thus not surprising that analytical chemists employed by the pharmaceutical industry have been greatly concerned with methods for estimating bias and proportional errors in quantitative analysis; this concern is exemplified by the work of Cardone[7-12] which represents a significant extension of earlier ideas of Youden[13-15].

These approaches to the elucidation of systematic errors (both constant and proportional), exemplified by the work of Cardone[7-12], are of great generality. The corresponding treatment offered here is less general in most respects, reflecting the somewhat different contexts involved in environmental *vs.* pharmaceutical analysis. For example, the fractional recovery F_a' of the target analyte, from the raw sample into the sample extract solution, is invariably a parameter of major concern in environmental analysis. Since pharmaceutical formulations are generally less complex and less variable, and more homogeneous (by design), it is often possible to design analytical procedures for which F_a' is close to 100%. **Methods for estimating F_a', however, will be a major focus of the present work.**

In the mathematical treatment developed in this document, it has been explicitly **assumed that fractional recovery is the only important source of proportional systematic error.** This assumption clearly implies a loss of generality. However, by

focussing on the F_a' parameter it will be possible to show that the treatment developed by Cardone[7-12] is not completely general, either. Thus, the relative lack of concern with this parameter in the pharmaceutical context is exemplified by the almost complete lack of attention paid[7-12] to surrogate internal standards, widely used in other contexts to provide estimates of analyte recoveries F_a'. Further, one of the more important conclusions of Cardone[7-12] is that the slope of a plot of analytical response vs. quantity of analyte should be identical for the Youden plot[13-15] (variations in analyte quantity achieved by varying sample size) and for the Method of Standard Additions (see Section IIIB(iii) below). This conclusion is valid only if the recovery efficiency for the analyte originally present in the raw sample is identical to that for additional analyte added as the pure standard to the sample. This condition can not be assumed to be valid for many environmental samples, in which low values for F_a' reflect occlusion of the analyte in the sample matrix. (A problem which is, almost by definition, unimportant for pharmaceutical formulations for which the active ingredient must be fully available). These qualitative discussions are more fully discussed, and expressed in mathematical form below. Sections III and IV provide brief qualitative discussions of the various methods, and present the corresponding working relationships. More detailed algebraic derivations are presented in the Appendices, which have been numbered to correspond with the appropriate parts of Sections III and IV.

Finally, it is appropriate to discuss briefly the **measurement and dispensing of volumes of liquid solutions.** The use of standard volumetric flasks, for the accurate "making-up-to-volume" of solutions of the order of 1mL or greater, is well established. Dispensing liquid volumes on the μL scale is a more demanding procedure, e.g. when spiking a solution of standard (or internal standard) into a raw sample or extract solution, or when injecting an aliquot of the extract solution into a chromatograph. Modern automatic pipettes are capable of delivering μL - sized volumes with high accuracy and precision, and are essential for the best practice in spiking, accurate dilutions, etc. Injections of extract solutions into a high-performance liquid chromatograph (HPLC) can be accomplished with similarly high accuracy and precision using modern loop-injector technology. However, injection of samples in liquid solution into a stream of high-temperature carrier gas in a gas chromatograph (GC) will always present problems in attempting to achieve high accuracy and precision: this generalization holds also for "cold on - column" injection techniques. Examples of documented problems arising from this problem, for various designs of GC injectors, have been published[16-18]. Use of volumetric internal standards solves this injection problem in GC, as well as more general problems arising from inadvertent evaporation of volatile solvents from sample extract solutions and standard solutions. These principles will be elucidated below, and are signalled by the absence of volume parameters in the expressions derived for the desired quantity of analyte.

II. Definitions of Symbols and Nomenclature.

An **external standard** is either a solution of the target analyte at known concentration, or a known quantity of the analyte. An **internal standard** is a substance added in known quantities to the sample to be analysed, and which is therefore measured in the same chromatographic run as is the analyte itself. An internal standard should be a stable compound not present in the original sample; it must either be chromatographically resolvable from the analyte and other substances in the sample extract, or provide a unique signal without interference (*e.g.* a characteristic ion at a unique m/z value for mass spectrometric detection). For the best accuracy and precision an internal standard should also preferably have a retention time similar to that of the analyte, and be present at a similar concentration. A **volumetric internal standard** is an internal standard which is added to the sample extract; it need not be chemically related to the analyte of interest, but should fulfil the other requirements described above for internal standards. A volumetric internal standard is used in conjunction with an external standard solution, in order to circumvent uncertainties in volumes of solutions and/or in the injection volumes in GC (injection volumes are generally much more reproducible in HPLC). A **surrogate internal standard** is an internal standard added to the sample itself, prior to any extraction, clean-up, *etc.*, in order to account for analyte losses during these steps; such an internal standard must therefore possess physico-chemical characteristics which are identical (or as nearly so as possible) to those of the analyte. When mass spectrometric detection is used, the surrogate internal standard is often an isotope-labelled version of the analyte, and its use amounts to a special case of Isotope Dilution Analysis. See Section IV for a more complete discussion of internal standards.

For all methods described in this document, the simplest procedure applies if the **calibration curve**, *i.e.* a plot of the appropriate instrumental signal *vs.* amount of analyte injected (or some simple multiple thereof), can be shown to be a linear function with a zero intercept. In the simple example of a method employing an external standard (Method IIIA (i)), such a demonstration would involve injections of varying quantities q_a'' of analyte *via* accurate dilutions of a stock external standard solution, and recording the corresponding chromatographic peak areas A_a''. If a plot of A_a'' *vs.* q_a'' is a straight line through the origin, then the response is uniquely defined as the slope of this line. **The best experimental practice always involves establishing the full calibration curve by direct experiment;** however, if pressure of time makes this impractical, the calibration may be estimated *via* a **single-point calibration** procedure in which just a single value of q_a'' is used; it is preferable to show previously that the necessary conditions (linear calibration with zero intercept) are satisfied. Note that the "single-point calibration" nomenclature does not imply that a single calibration chromatography run is all that is employed; a statistically meaningful result should be determined by multiple injections of the same calibration solution.

LIST OF SYMBOLS USED.

Symbols marked with a **prime** ' refer to the <u>sample extract solution</u>, possibly spiked with surrogate internal standard. **Un-primed symbols** refer to the <u>original sample</u>, prior to extraction, clean-up, *etc.* **Double-primed symbols"** refer to a standard calibration solution (<u>external standard</u>) of unlabelled analyte, possibly spiked with an internal standard of one kind or another. **Triple-primed symbols** ''' refer to a quantity of <u>pure solvent</u> spiked with surrogate internal standard.

Subscripts are used extensively. Their meanings are as follows:

a	=	the target analyte;
e	=	analyte spiked into a raw sample in the form of an external standard solution, as in the Method of Standard Additions;
i and/or j	=	internal standard (surrogate and/or volumetric);
s	=	analyte spiked into a blank (control) sample;
x	=	unknown substance **X**, which interferes with the chromatographic response of the target analyte;
y	=	pertaining to the Youden Sample Response Curve;
msa	=	pertaining to the Method of Standard Additions.

The **major symbols** used in this document are listed below in alphabetical order:

A_z = chromatographic peak area (or height) for injection of a quantity q_z of substance Z; the number of primes indicates the type of solution injected;

b = y-intercept of an experimental response curve, usually obtained by linear least-squares regression;

C_a = concentration of analyte in the sample (= Q_a/W_s)

C_{is} = concentration of internal standard in the solution used to spike the sample, sample extract and/or the external standard solution;

C_a'' = concentration of analyte in the external standard solution;

F_z' = fractional recovery of substance Z from sample into extract solution, partially defines $Q_z' = F_z'.Q_z - L_z'$;

f_z = fractional transmission of quantity q_z of substance Z from chromatographic injector to the detector, partially defines detector response *via* $A_z = R_z.(f_z.q_z - I_z)$;

7

k = slope of an experimental response curve, usually obtained by linear least-squares regression;

L_z' = fixed loss of substance Z occurring during extraction and clean-up, partially defines $Q_z' = F_z'.Q_z - L_z'$;

I_z = fixed loss of substance Z after injection into chromatograph but before exiting the detector, partially defines detector response *via*: $A_z = R_z . (f_z . q_z - I_z)$;

Q_z = total quantity of substance Z in a sample, sample extract, calibration solution, *etc.*;

q_z = quantity of substance Z in solution, injected (in injection volume v) into the chromatograph;

R_z = detector response for substance Z, per unit quantity of Z reaching the detector, partially defines detector response *via* $A_z = R_z.(f_z.q_z - I_z)$;

S_a = quantity of pure analyte spiked into raw sample (or control sample) in the form of external standard solution;

S_a' = total quantity of analyte in extract solution derived from a spike (S_a) into the sample;

V = total volume of a solution (either sample extract or a calibration standard) prior to removal of the first injection volume v, contains the corresponding total quantity Q_z of substance Z;

v = chromatographic injection volume of solution;

V_{IS} = volume of solution of internal standard (concentration C_{IS}) used to spike the sample or sample extract and/or the external standard solution;

V_a = volume of solution of target analyte (concentration C_a'') used to spike the raw sample (Method of Standard Additions) or a blank (control) sample;

W_a = mass of sample represented in the sample extract solution.

All of the quantities Q and q may be assumed to be masses, though amounts of matter (moles) may sometimes be used. In cases where <u>both</u> a surrogate and a volumetric internal standard are employed, these are denoted by subscripts **i** and **j**, respectively.

III   Analytical Methods Using External Standards with No Internal Standard in Quantitative Chromatography.

In this Section the methods of use of external standards in quantitative chromatography, without the intervention of an internal standard of some kind, are discussed.

In order to introduce the present approach using the simplest possible example, in Section IIIA it is assumed that the appropriate calibration curve is linear with a zero intercept (and thus that a single-point calibration procedure (see Section II) is valid). Section IIIB deals with circumstances in which these conditions are not fulfilled.

IIIA   Linear Response Curve with Zero Intercept.

In this case, the necessary conditions for a single-point calibration procedure to be valid are assumed to be satisfied. However it is always preferable, where possible, to use a full calibration curve procedure.

IIIA(i) Standard Calibration Curve Method.

This method involves establishing the **instrumental response curve**, using standard solutions of the analyte covering an appropriate range of concentrations C_a'' and corresponding weighed quantities of analyte $Q_a'' = (C_a''.V'')$. Then the assumption that the resulting calibration curve is linear with zero intercept (Figure 1) leads to the working relationship given by eq [5] (see Appendix for details).

$$C_a = Q_a/W_s = A_a'.(1/R_a.f_a).(V'/v').(1/F_a')\,(1/W_s) \qquad [5]$$

In the special case where only a single-point calibration procedure is used, the appropriate form is eq.[6]:

$$C_a = Q_a/W_s = (Q_a'/F_a')/W_s = (V'/v')(v''/V'')(A_a'/A_a'')(Q_a''/F_a')\,(1/W_s) \qquad [6]$$

$$= (V'/v')(A_a'/A_a'')(v''.C_a'')/(F_a'.W_s)$$

where $C_a'' = (Q_a''/V'')$.
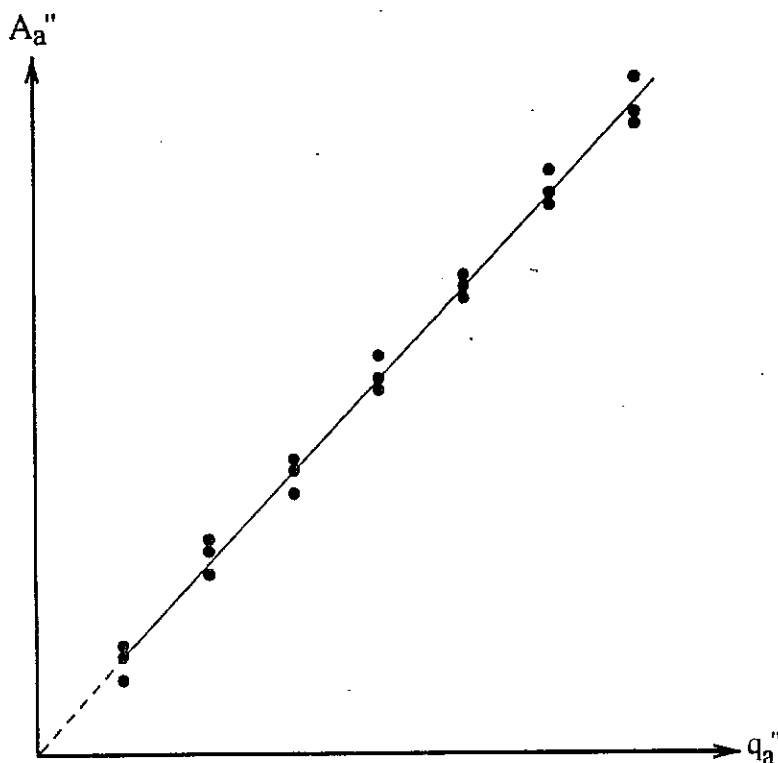
Figure 1.   Calibration curve obtained for an external standard, in which the intercept is not significantly different from zero. In III A (i) and (ii) it is assumed that such calibration data are fitted to a straight line forced through the origin, with a slope interpreted as $(R_a'' \cdot f_a'')$ or as k_s', in III A (i) and III A (ii), respectively. The injected quantities of analyte, q_a'' and q_s' are defined operationally as $[\,(v''/V'') \cdot Q_a''\,]$ and $[\,(v_s'/V_s') \cdot S_a\,]$, respectively.

All of the disadvantages of the Standard Calibration Curve method are evident in eqs.[5] and [6]. Thus, volumes and concentrations appear explicitly; **systematic uncertainties** in V', V'' and C_a'' due to solvent evaporation, and **random errors** in the injection volumes v' and v'' (not well controlled in GC, but much better in HPLC using injection loop methodology), directly affect the final result. The peak area A_a' and its calibration (either $(R_a \cdot f_a)$ in eq.[5] or (Q_a''/A_a'') in eq.[6]) must be measured in separate chromatographic runs, introducing the possibility of systematic error due to instrumental drift. **The fractional recovery F_a' is not measurable from such experiments alone**, and the requirement for an assumed value of unity introduces a **proportional error** into the values of Q_a thus calculated **(yielding values for Q_a which are lower limits to the true value).** Note also that uncertainties in the injection volumes imply that the ordinate (independent variable) in Figure 1 is NOT free of experimental uncertainties, which is a necessary assumption[4-6], in the simple linear regression routines commonly used for such purposes.

IIIA(ii) Calibration by Spiking Control (Blank) Samples.

In this context a control sample is taken to mean a sample which is identical in every way to the sample to be analysed, except that it contains an undetectable quantity of the analyte. The degree to which such a sample can be said to exist will vary strongly with the situation, but it is relatively easy to achieve in a pharmaceutical formulation context, for example.

This method involves many of the same assumptions as the described in Section III A (i). Now, however, the calibration curve actually used is obtained by spiking different quantities S_a of standard into different aliquots of the control sample, which are then taken through the complete analytical procedure. The most reliable method of determining the quantities S_a is by direct weighing of the pure standard. However, in practice the spiking is often done by dispensing volumes V_a of concentration C_a'' ($S_a = V_a.C_a''$); this method is more convenient, but carries the risk of introducing both systematic errors (*e.g.* from evaporation of solvent and inaccuracy of the volumentric equipment) and random errors (*via* imprecision in the volumes dispensed).

By varying S_a over an appropriate range, and determining the corresponding chromatographic peak areas A_s' for injections of aliquots of the extracts, a calibration curve can be constructed. Under the general assumptions of Section III, this calibration curve is assumed to be a straight line passing through the origin (Figure 1). Then, as discussed in the Appendix, **Eq [9] is the working empirical relationship for analyses using a calibration obtained by extracting blank samples spiked with known amounts of analyte.**

$$C_a = Q_a/W_s = (1/k_s') . (V'/v') . (A_a'/W_s) \qquad [9]$$

where k_s' is the slope of the calibration curve (see Appendix).

In summary, the method of Calibration by Spiking of Control Samples is subject to all of the **systematic and random errors** described in Section IIIA(i) for the Standard Calibration Curve Method, with the important exception that the **proportional errors associated with uncertainties in the fractional recovery are now less serious.** By combining the two methods it is possible (see Appendix) to measure values of the fractional spike recovery F_s', a valuable parameter for purposes of Quality Control. The present method is clearly more time-consuming than IIIA(i), which may be a significant consideration in some circumstances. **Further, the present method depends on the availability of sufficient quantities of a suitable blank sample.**

IIIA(iii)   Method of Standard Additions.

This method is similar to that described in Section IIIA(ii), but now the spikes of pure standard are added to different aliquots of the actual sample to be analyzed rather than to a control (blank) sample. Known variable amounts S_a of the external standard, preferably determined by direct weighing (see Section IIIA(ii)) are spiked into separate aliquots of the raw sample prior to extraction, clean-up, *etc.* The method thus provides its own calibration (no additional calibration experiments are required). In a qualitative way, it is easy to understand that extrapolation to zero quantity of added (spiked) analyte standard will yield the desired quantity of native analyte. The response curve, obtained in an ideal analysis by the Method of Standard Additions (see Appendix for details of the assumptions involved), is of the form illustrated in Figure 2.
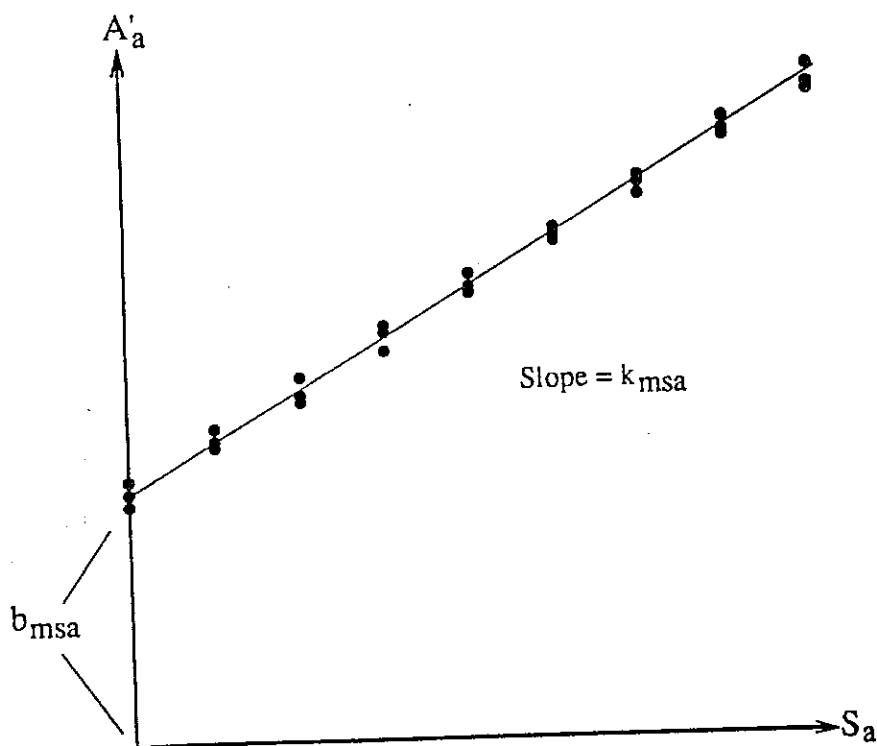

Figure 2.   Ideal response curve for an analysis by the Method of Standard Additions using a fixed mass of sample (W_s), fixed extract volume (V') and fixed injection volume (v'). The slope and intercept are the quantities b_{msa} and k_{msa} in eq [19].

The desired quantity C_a is given (see Appendix for details of assumptions) by **eq.[19], the working relationship for Method IIIA(iii):**

$$C_a = Q_a/W_s = (b_{msa}/k_{msa}) \cdot (F_e'/F_a') \qquad [19]$$

where k_{msa} and b_{msa} are determined empirically as the slope and intercept of the plot of A_a' *vs.* S_a (Figure 2). Note an advantage here of the present method over that described in Section IIIA(ii); in the present case the recovered spike and analyte from the sample are contained in the <u>same</u> sample extract solution, so that the volumes v' and V' are the same for both and thus do not appear (i.e. cancel) in calculation of Q_a from eq [19]. In the method described in Section IIIA(ii), on the other hand, the standard spike and the unknown analyte are contained in different solutions, accounting for the appearance of the additional volume variables v_s' and V_s' in eqs.[7] - [13]. The irreproducibility of the injected volumes v' and v_s', especially for GC analyses, can be a major contributor to the random error in the final analytical result.

Since F_a' (fractional recovery of native analyte into the extract solution) is not measurable, practical application of eq.[19] requires the further assumption that $F_a' = F_e'$ (fractional spike recovery), leading to a **proportional error** in Q_a yielding a lower limit to the true value, since in general $F_e' > F_a'$ (see discussion of F_s' in Section IIIA(ii)). However, this proportional error is likely to be considerably smaller than that in Method IIIA(i) where it is necessary to set F_a' = unity.

In practice, observation of a linear plot of A_a' *vs.* S_a is a necessary but not sufficient condition for the validity of all the assumptions involved; this point is discussed in Section IIIB. However, note that it is possible to derive (see Appendix) a value for Fe':

$$F_e' = k_{msa} \cdot (V'/v')/(R_a \cdot f_a) \qquad [20]$$

where F_e' is usually an upper bound to F_a' (no occlusion effects in the extraction of the standard spike). However, any such information on recovery efficiencies is invaluable as an indicator for Quality Control of the overall analytical procedure. Evaluation of eq [20] requires measurement of the instrument response factor ($R_a \cdot f_a$) in separate experiments using standard solutions of analyte, as in Section III A (i).

Although the present document does not pretend to deal with the proper statistical approach to experimental data with associated random errors (see the excellent reviews by Miller and Miller[4,5]), a cautionary word is appropriate at this point. The commonly employed unweighted least-squares linear regression fit to experimental values of y as a function of x (with the explicit assumption of zero uncertainty in x), gives the following expression for slope k and intercept b:

$$k = \sum \left[(x_i - X)(y_i - Y) \right] / \sum (x_i - X)^2 \qquad [21]$$

$$b = Y - kX \qquad [22]$$

where X and Y are the arithmetical means of the experimental values x_i and y_i, respectively. The point here is that k and b, thus estimated, are negatively correlated *via* eq [22]. A positive deviation, of the experimental estimate (eq [21]) for k from the "true" value, inevitably leads to a corresponding negative deviation for b, and thus an even more important error in the ratio b/k. Thus although the Method of Standard Additions does indeed possess the advantage of requiring that only the ratio (F_e'/F_a') be assumed to be unity (rather than F_a' itself), it also has a potential disadvantage common to all methods which evaluate a desired quantity as the ratio of a least-squares intercept to the slope. This latter disadvantage can be substantially avoided by using less restrictive least-squares fitting algorithms. **However, in the present context it is important to realise that eq [19], as commonly evaluated (eqs [21] and [22]), is subject to errors of a purely mathematical origin.**

Under the assumptions and restrictions of Section IIIA, the Method of Standard Additions is susceptible to simplification to a form analogous to a single-point calibration. Such a simplification would involve only two analyses, *e.g.* of unspiked sample and of one spiked aliquot (see Appendix). Such a single-point calibration version of the present Method of Standard Additions is the only option if limited amounts of sample are available. Indeed, the requirement for large amounts of sample is the main operational disadvantage of the Method of Standard Additions. **A minimal sample size is determined not only by requirements of adequate signal/noise ratios in the measurement of the signals A_a', but also by the requirement that the sample analyzed be statistically significant, free of significant random variations in the concentration of analyte due to intrinsic inhomogeneity; the latter consideration is often important in environmental analysis.**

It is of interest that the present account of the Method of Standard Additions refers to experiments in which varying amounts of standard S_a are spiked into a fixed quantity of sample. It has been shown[19] that the converse method, *viz.* a fixed amount of standard spiked into varying quantities of sample, has no advantages. However, the analysis of varying quantities of sample <u>can</u> provide information concerning bias errors (constant systematic errors), and this approach is discussed in Section IIIB(iii).

IIIB. Calibration Curves with a Useful Linear Range but a Non-Zero Intercept.

It is not uncommon, in analytical practice, to observe a calibration curve which is linear to within the experimental precision, but which has a statistically significant non-zero intercept. (Statistical tests, to determine whether the uncertainty limits on the intercept do or do not encompass the origin, have been given by Cardone[12] and by Miller[5]). Such behaviour, if observed for solutions of pure standards, signals a fundamental problem in the analytical method. There are two broad classes of such problems, corresponding to positive and negative values for the y-intercept b (eq [22], see Figure 3).
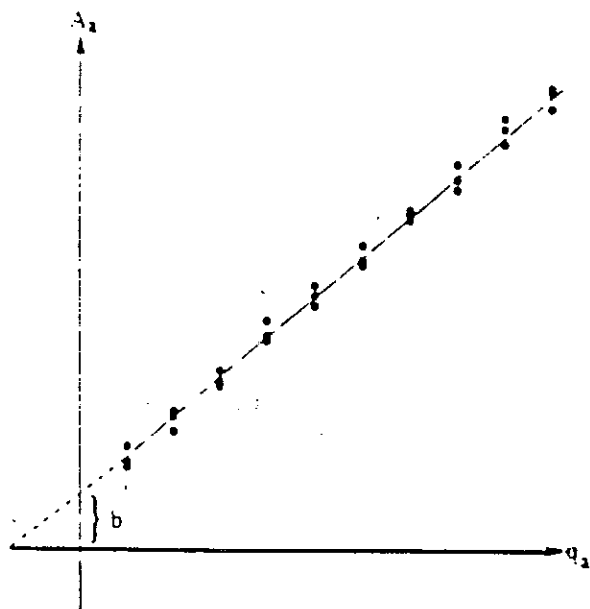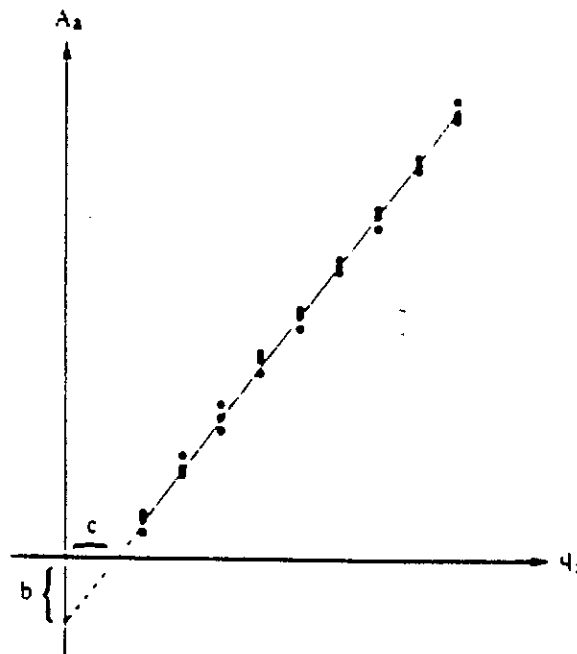
Figure 3a

Figure 3b

Figure 3.   Generalised response curves with intercepts significantly different from zero.

A positive value for b corresponds to a non-zero analytical signal for a solution known to contain none of the analyte. This effect usually involves chemical interferences and rarely occurs for highly selective analytical techniques, *e.g.* those incorporating high-resolution chromatography with mass spectrometric detection. A well-known example of such a problem, however, is provided by analyses for tetrachlorodibenzo-p-dioxins ("dioxin") at ultra-trace levels (*e.g.* parts-per-trillion) in samples containing much larger quantities (*e.g.* parts-per-million) of other chlorinated aromatic pollutants. Examples are known where even capillary-column GC with high-resolution mass spectrometric detection (up to 1.8×10^4, 10% valley definition) could not resolve the desired "dioxin" signals from interferences co-eluting from a 60m capillary column! If it were possible to acquire a calibration curve as in Section IIIA(ii), by spiking "dioxin" standard into a (probably mythical) control sample which contained all of the interfering species but none of the "dioxin" analyte, the curve would indeed show a positive y-intercept. It would be highly unusual, however, if the instrumental calibration curve, obtained using pure standard (Section IIIA(i)) were to show a positive value of b for a technique of such high specificity. One would suspect presence of an impurity in the solvent in such a case. (An impurity in the standard sample of the analyte would show up as a proportional error in the external calibration curve, rather than as a constant bias). Where possible, it is good practice to attempt to remove or minimise problems resulting in positive y-intercepts, by first identifying their causes. Where this is not possible, the algebraic treatments described below may be used.

The second class of problems corresponds to negative values for the y-intercepts of the appropriate calibration curves. The physical meaning of such negative values is best approached *via* the implied positive values for the x-intercepts c (Figure 3):

$$c = -b/k \qquad [23]$$

where the least-squares linear regression expressions for b and k are given as eqs [21] and [22]. Such a circumstance implies a threshold value for q_a'', below which no signal is observed. Such observations, particularly for calibration experiments using solutions of the pure standard (Section IIIA(i)), are usually interpreted in terms of irreversible losses of analyte on "active sites" on the column[20,21] or on the injector or other components of the chromatography train[22], or in the mass spectrometer ion source[23,24]. A major problem with such effects is that they tend to be irreproducible, and thus not susceptible to accurate calibration. Thus, it is preferable to investigate the source of such effects and if possible to eliminate them, to the point where the calibration curve passes through the origin to within the experimental precision. Otherwise, the algebraic approaches described below must be adopted.

Such non-zero intercepts in calibration curves are examples of **bias errors**, as defined in Section I above. The detection and characterization of bias errors, often in conjunction with simultaneous proportional systematic errors (*e.g.* those associated with values of $F_a' < 1$), is a major thrust of the approach promoted by Youden[13-15] and

more recently by Cardone[7-12]. Many of the principles described in Section IIIB are adapted directly from their work.

IIIB(i) Standard Calibration Curve Method.

Observation of a non-zero intercept b", in the calibration curve obtained using pure analyte standards, leads to a modified form of eq [5], as the working relation:

$$Q_a/W_s = (A_a' - b'') (V'/v') / [(F_a' . k'') . W_s] \qquad [26]$$

where the empirical slope parameter k" may be interpreted as $(R_a.f_a)$.

The derivation of eq [26] (see Appendix) is an algebraic triviality, but its implications for the propagation of experimental error are not trivial[5]. This conversion also carries chemical implications whose validity is by no means guaranteed in any particular case. **The values obtained for the experimental slope k", and particularly for b", in the calibration experiments will not necessarily apply to the sample extracts.** For example if b" is found to be positive, often interpreted in terms of co-eluting interferences, the amount of such interfering substances in the sample extract could well be very different from that in the calibration solution. If such interfering substances were derived from the solvent different effective values for b" would pertain, depending upon the total volumes of solvent employed in extracting and dissolving the sample as opposed to dissolving the standard. On the other hand if b" were negative, corresponding to a positive x-intercept often interpreted in terms of a constant loss of analyte on active adsorption sites in the chromatographic train, this amount of lost analyte could well vary depending upon the quantity of co-extractives from the sample which could compete for these active sites.

The point of the foregoing discussion is to emphasise that non-zero values for b" usually signal potential uncertainties which are best avoided, if possible, by diagnosing the cause and taking appropriate remedial actions. Such actions might involve a more selective analytical method if b" > 0, e.g. different chromatography and/or increased mass spectrometer resolution, or (for b" < 0) changing columns and/or silylation of appropriate portions of the chromatographic train, etc. However, if none of these remedies improve the situation, assumption of the applicability of the calibration parameters k" and b", to analysis of sample extracts, gives the working relation eq [26].

IIIB(ii) Calibration Curve with a Non-Zero Intercept Using Spiked Control Samples.

As in IIIA(ii), the quantities of analyte whose values are known, and for which analytical responses are determined, are the quantities S_a of standard spiked into a (fixed) quantity of blank sample. Here, however, the calibration curve is <u>not</u> presumed to pass through the origin (Figure 3).

The non-zero intercept b_s' is subject to a discussion very similar to that for b'' in IIIB(i). Thus, a value $b_s' > 0$ usually implies that a co-eluting interference is contributing to the analytical response A_s', but in this case the interfering compound(s) could arise from the control sample as well as from the solvent. Similarly, if $b_s' < 0$ the corresponding positive value for the x-intercept could now reflect loss of a fixed quantity of analyte on "active sites" of some kind during the extraction and/or clean-up procedures, as well as in the chromatographic train as discussed in Section IIIB(i).

Comments made in IIIB(i), concerning the advisability of diagnosing and removing the causes of non-zero intercepts if at all possible, also apply here. However, if this can not be done, the Appendix shows that the working relationship is eq [28].

$$C_a = Q_a/W_s = (A_a' - b_s')\ (V'/v')\ (1/k_s')\ (1/W_s) \qquad [28]$$

However, in the present case it is possible to demonstrate (see Appendix) how, for simple specific examples, the calibration parameters k_s' and b_s', determined experimentally as the slope and intercept for the spiked controls, will be in error when applied to determination of Q_a/W_s for the real sample *via* eq [28]. **Since in general $F_a' \leq F_s'$, use of the calibration value k_s' for the slope parameter can result in a proportional error, while a bias error could result if $L_a' \neq L_s'$. This simple example reinforces the recommendation that, if possible, the cause of a calibration curve not passing through the origin should be sought and rectified.**

IIIB(iii)   Method of Standard Additions and the Youden Sample Response Curve.

This Section is that which owes the largest direct debt to the work of Cardone[7-12] and of Youden[13-15]. In its most developed form, this approach[7-15] is a powerful protocol of great generality for the detection of both bias and proportional systematic errors in an analytical procedure. In order to explain the principles involved in a reasonable space, **a particular model for the analytical procedure will be employed:**

(a) the chromatography/detection system used is sufficiently selective that no interferences intervene (as discussed in IIIB(ii), for example); the present discussion could readily be extended to include this effect, if desired;

(b) the detector response is directly proportional to the quantity of analyte reaching it, thus:

$$A_a' = R_a (f_a . q_a' - l_a) \qquad [38]$$

where l_a represents the (assumed fixed) quantity of analyte lost to "active sites" located between the injector and detector, and f_a is the fraction of injected analyte which reaches the detector if l_a is zero;

(c) extraction of analyte from the sample, and the associated selective concentration procedures ("clean-up"), are subject to losses of both the proportional and bias type; it is assumed that the proportional loss may vary according to whether the analyte is that originally present that is spiked into the sample, but the fixed loss (possibly on "active sites") is assumed to be the same:

$$Q_a' = (F_a' . Q_a) + (F_e' . S_a) - L_a' \qquad [39]$$

While the model specified in (a) - (c) is not completely general, it does cover the majority of circumstances which can lead to significant error in quantitative trace analysis.

Youden[13-15] realised that a general approach to determining whether or not bias errors were present was to determine a response curve in which the only variation in quantity of analyte Q_a was due to controlled variations in sample size W_s:

$$Q_a' = F_a' . (Q_a/W_s) . W_s - L_a' \qquad [40]$$

Eq [40] is a special case of eq [39] with $S_a = 0$ (no spiking) and written so that W_s becomes the independent variable. The implicit assumption that $C_a = (Q_a/W_s)$ is constant amounts to ensuring that the sample is effectively homogeneous over the range of values for W_s to be used. Then, under the assumptions implicit in eqs [38] and [40], it is straightforward to derive the corresponding functional form of the Youden Sample Response Curve:

$$A_a' = R_a . (f_a . q_a' - l_a) = R_a . f_a . q_a' - R_a . l_a$$

$$= [R_a . (v'/V') . F_a' . f_a . (Q_a/W_s)] . W_s - R_a . f_a . (v'/V') [L_a' + l_a (V'/v') / f_a]$$

19

$$= \quad k_y \cdot W_s + b_y \qquad\qquad\qquad [41]$$

where k_y and b_y are the slope and intercept, respectively, of the Youden Sample
Response plot. Note that, under assumptions (a) - (c), only negative or zero values
for b_y are predicted (see Figure 4). If b_y turns out to be positive, the most probable
cause is that the bias errors are dominated by co-eluting interferences as discussed in
IIIB(ii). A statistically significant non-zero value for b_y indicates the presence of bias
errors in the analytical procedure, although this alone can not determine whether
these errors arose during the extraction and clean-up steps or in the chromatographic
train. Under the present assumptions (a) - (c), this question could be approached by
determining the dependence of b_y upon (V'/V'):

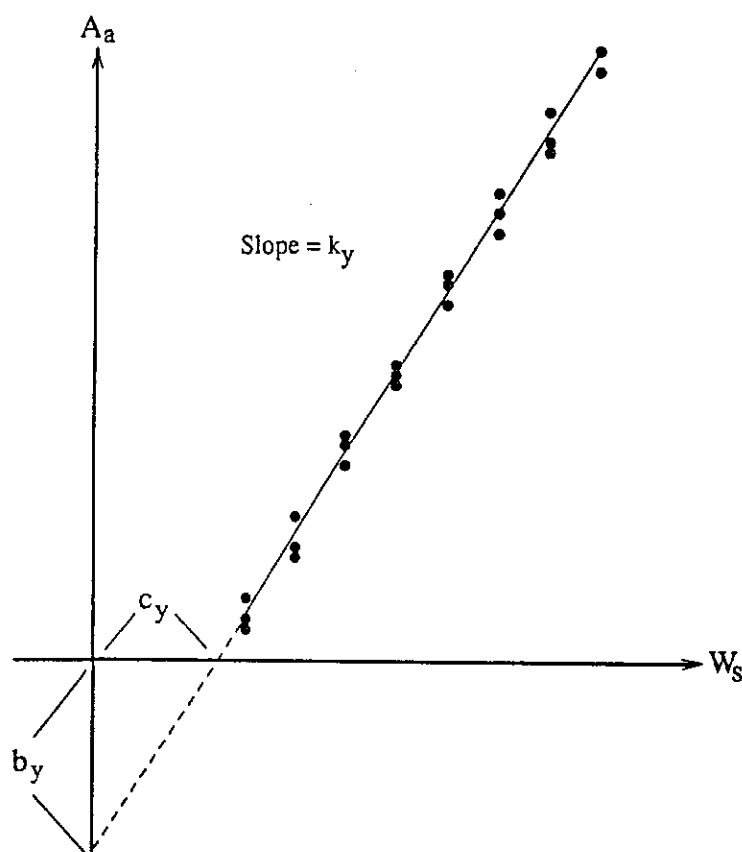Figure 4.   Illustration of typical Youden plot in which the independent variable is the
quantity of (unspiked) sample analyzed, keeping the volumes (V',V')
constant. The apparent threshold value for W_s, $c_y = -b_y/k_y$, reflects a bias
systematic error due to analyte loss on "active sites" in either or both of
the extraction/clean-up step and the final quantitative analytical
procedure.

$$b_y = -R_a \cdot [L_a' \cdot f_a \cdot (v/V') + I_a] \qquad [42]$$

A value for (Q_a/W_s) can be obtained from the Youden slope k_y (eq [41]) if it is assumed that the proportional error factors F_a' and f_a are both unity, and if R_a can be measured. This is not generally feasible, and **the Youden method is usually applied as a diagnostic approach to the detection of bias errors** (but see eq [46] below).

The Method of Standard Additions, described in IIIA(iii) for the simple idealised case, can be adapted to the more realistic model defined by assumptions (a) - (c). In such a procedure, eq [39] applies with the restriction that Q_a is fixed (*via* a fixed value for W_s), and S_a is the independent variable. Then, as detailed in the Appendix, the most reliable value of (Q_a/W_s) from the Method of Standard Additions is probably eq [46]:

$$C_a = (Q_a/W_s) = [(b_{msa} - b_y)/k_{msa}] \cdot (F_e'/F_a')/W_s \qquad [46]$$

As before the ratio of analyte recovery efficiencies, for spiked and original analyte, is difficult to determine experimentally and is always a major source of uncertainty. The use of the Youden intercept b_y as the appropriate correction for b_{msa}, rather than *e.g.* the intercept b_b' of the Standard Calibration Curve (eq [27]), is in accord with the more general conclusions of Cardone[7-13].

IV.   Methods Involving Internal Standards.

Internal standards are used in quantitative chromatography for two reasons: to remove or reduce the dependence of the final analytical result on the values of volumes of solutions, and to provide some measure of the fractional recovery of analyte from the sample into the extract. A compound used for the first purpose is often referred to as a **volumetric internal standard,** and is usually added to the extract solution itself. A **surrogate internal standard,** on the other hand, is added in known quantities to the sample at as early a stage as possible. The fractional recovery of the surrogate internal standard can provide some information on the efficiency of recovery of the native analyte.

The principles upon which these two objectives can be achieved, and their limitations, are discussed below. However, it will be convenient here to summarise the **desirable properties for an internal standard:**

21

(a)   It must be completely resolved in the chromatogram from all other known and unknown substances in the sample extract. (Note: for mass spectrometric detection, the retention time of the internal standard need not be well-separated from those of other components, provided that it is characterized by at least one unique m/z value within the appropriate retention time window and that the total sample flux into the ionization source is low enough that suppression effects are negligible).

(b)   The internal standard should elute as closely as possible to the target analyte, in order to minimise effects of instrumental drift.

(c)   The internal standard must be chemically stable under the conditions of the analytical procedure.

(d)   Best accuracy and precision are obtainable if the peak height (area) for the internal standard is as close as possible to that for the target analyte.

(e)   The internal standard must be wholly absent from the sample.

(f)   In the case of a *volumetric internal standard* lack of volatility is an important consideration, when volatile solvents are used.

(g)   A *surrogate internal standard* must be as chemically similar to the analyte as possible. Where possible, this is arranged to be a stable-isotope-labelled version of the analyte, implying that mass spectrometric detection must generally be used. In view of requirement (e), the degree of isotopic substitution should be sufficiently large that the probability of observing such a molecule in the natural material (natural isotopic abundances) is negligible.

IVA.   Volumetric Internal Standards in Conjunction with External Standards.

The simple case of a Standard Calibration Curve will be worked through in some detail (Section IVA (i)), in order to establish principles of more general applicability and also the inherent limitations of the approach. The general intent of a volumetric internal standard is to avoid the uncertainties associated with volumes such as v' and V' (see Section III above). The most common of such uncertainties are:

(a)   low precision in chromatographic injection volumes v', particularly for gas chromatography;

(b)   potential for uncontrolled systematic errors in total solution volumes V', when volatile solvents must be used.

These volume parameters are replaced by quantities Q_i of internal standard, which are thus ultimately defined by weighing procedures. However, in practice these quantities are most often dispensed as volumes of a solution of the internal standard. This apparent contradiction is resolved by noting that, as explained below, all that is required of the volume-dispensing in this context is that it be reproducible, in the sense that the same volume (of the same solution of internal standard) be added to the sample extract solution to be analyzed, as to the stock solution of external standard. Modern digital dispensers can achieve this with a high degree of precision. The absolute values, of the dispensed volume and of the concentration of volumetric internal standard, are not required. In order that the concentration of internal standard solution be the same for all experiments, it is important that all such additions be made at the same time to all solutions to be analyzed (particularly if the solvent is volatile). Similar remarks apply also to certain applications of surrogate internal standards, to be discussed below in Section IVB.

IVA(i). Standard Calibration Curve.

As for any procedure incorporating a calibration curve, the important characteristics of the latter must be determined, particularly the question of whether or not it encompasses the origin, and also the extent of the linear dynamic range. In the present context, a suitable calibration procedure could involve the following steps:

(a) using accurate weighings and appropriate dilutions (best not serial dilutions, for which errors accumulate), prepare a series of standard solutions of the analyte; however, before filling to the calibration mark on each standard volumetric flask, add a fixed volume V_{IS} of the solution of internal standard, of concentration C_{IS} (see general comments under Section IV A). If a volatile solvent is used, it is important to perform all of these additions at the same time, so that each solution contains the same quantity of volumetric internal standard: $(Q_i'' = V_{IS} \cdot C_{IS})$.

(b) using weighings and dilutions, prepare a series of standard solutions of the internal standard alone.

(c) conduct the quantitative chromatography experiments on all solutions prepared in (a) and (b), including as many replicate injections of each solution as are feasible.

The present treatment assumes the validity of a linear response curve with a (possibly) non-zero intercept; this assumption must apply to both the analyte and to the volumetric internal standard. **Application of such a calibration to quantitative analysis of sample extracts requires that these extracts be spiked with the**

23

identical quantity of volumetric internal standard as was used in the calibration experiments, *i.e.* $Q_i' = Q_i''$. Note that all of these spiking procedures (calibration solutions plus sample extracts) should be done at the same time, to minimise drift in the values of V_{IS} and/or of C_{IS}. Then the following equation analogous to eq [49] applies to analyses of the sample extracts (see Appendix):

$$Q_a' = [(A_a' - b_a') / (A_i' - b_i')] / [(R_a \cdot f_a)/(R_i \cdot f_i)/Q_i'']$$

$$C_a = Q_a/W_s = Q_a'/(F_a' \cdot W_s) \tag{50}$$

where $[(R_a \cdot f_a)/(R_i \cdot f_i)/Q_i'']$ is determined experimentally as the slope of the plot corresponding to eq [49] (see Appendix), for calibration experiments conducted using the external standard solutions spiked with volumetric internal standard, prepared as in (a) above. In addition, evaluation of eq [50] requires the additional assumption that the intercepts b_a' and b_i', pertinent to the sample extract solutions, are given by the experimental values b_a'' and b_i''' from the calibration experiments (see Appendix).

It is not possible to generalise about the validity or otherwise of this assumption, and this emphasises yet again the importance of investigating the causes of any non-zero intercepts with a view to remedial action to reduce them to zero. If this can in fact be done, the long calibration procedure (a) - (c) can subsequently be shortened to a **single-point calibration procedure,** involving just a single calibration solution containing an accurately known quantity Q_a'' of analyte standard, and a reproducible (but not necessarily accurately known) quantity Q_i'' of volumetric internal standard.

The way in which a volumetric internal standard corrects for systematic errors associated with uncontrolled evaporation of volatile solvent (effectively uncertainties in V') can now be appreciated. This procedure uses $Q_i' = Q_i''$ as the normalising parameter, rather than solution volumes. It is thus extremely important that the volumetric internal standard itself be involatile, and that it be added in precisely equal quantities to the analyte (external) standard solution(s) and to the sample extract(s).

The effect of a volumetric internal standard on the overall analytical precision is less clearcut, however. If the random error is dominated by that in the injection volume v', as is commonly the case for GC analyses, it might be expected that use of a volumetric internal standard would improve the precision. However, modern sample loop injectors for HPLC can achieve a precision of injection of 0.05%; it seems inherently unlikely that use of a volumetric internal standard could improve on this, and indeed the question arises as to whether it might impair the overall precision. This question has been considered in detail by Haefelfinger[26], in an interesting analysis of the propagation of error in such experiments; this statistical analysis was illustrated by real-life examples, including at least one in which the random error associated with the manipulations of the volumetric internal standard were large enough to significantly decrease the overall precision, relative to that achievable using only the simple

24

external standard procedure (Section IIIA(i)). Clearly, an informed assessment is required in each individual case[26]. Despite such uncertainties concerning the effect of a volumetric internal standard on reproducibility if controlled by injection volumes, use of an internal standard can always provide a correction for instrumental drift.

IVA(ii).　　Calibration Using Spiked Control Samples.

In this Section, we discuss modifications to the method described in IIIB(ii) appropriate to incorporation of a volumetric internal standard. **It is assumed in this treatment that analyte losses, associated with "active sites" in the chromatography train, have been reduced to negligible values,** *i.e.* l_a is effectively zero. However, the corresponding assumption is <u>not</u> made for the extraction/clean-up procedure (L_a' not necessarily zero).

In this context, a fixed quantity Q_i'' of volumetric internal standard would be used to spike all such final <u>extracts</u> from the blank samples, in a fashion analogous to that described in IVA(i) for the analyte standard solutions.

The least-squares-fit parameters $k_{s,i}''$ and $b_{s,i}''$, determined from the calibration experiments using the extracts of spiked control samples (see Appendix), are now <u>assumed</u> to be directly applicable to analyses of real sample extracts, also spiked with volumetric internal standard:

$$A_a'/A_i' \;=\; k_{s,i}'' \cdot Q_a + b_{s,i}''$$

$$C_a = Q_a/W_s = [\,(A_a'/A_i') - b_{s,i}''\,] / (k_{s,i}'' \cdot W_s) \tag{56}$$

In order for eq [56] to be valid within the assumptions made to this point, the interpretation in terms of the model (see Appendix) shows that all of the following conditions must also be satisfied:

$$Q_i' \;=\; Q_i''\,; \quad F_s'' \;=\; F_a'\,; \quad L_s'' \;=\; L_a' \tag{57}$$

The first of these conditions may be satisfied experimentally by careful attention to control of both C_{is} and V_{is}, as discussed in IVA(i). The recovery efficiency F_a'', for analyte standard spiked into a blank sample, is likely to be an upper limit for F_a' but the discrepancy can be minimised if the recovery efficiencies are developed to the extent that both approach 100%. The extraction/clean-up losses L_s'' and L_a'' are not necessarily constants (see discussion in Section IVB(i)), and should preferably be reduced to zero if possible. The advantage of the spiked blank calibration procedure, over that employing standard solutions of pure analyte (IVA(i)), is that the two

i

assumptions (eq [57]) concerning equality of parameters describing recovery efficiency are less stringent than those required in IVA(i), *viz.* $F_a' = 1$ and $L_a' = 0$.

A different comparison involves the precision achievable using the method described here, and the analogous method (IIIB(ii)) which does not use a volumetric internal standard. This same question was discussed in IVA(i), and need not be repeated here except to emphasise that an informed decision must be made in each case[26].

IVA(iii)   Method of Standard Additions with a Volumetric Internal Standard.

In this section we consider the modifications to Section IIIB(iii) brought about by incorporation of a volumetric internal standard into the sample extracts, as discussed above. The intrinsic advantage of the Method of Standard Additions is that it supplies its own internal calibration procedure.

Again, in order to keep the algebra tractable, **it will be assumed here that the quantitative chromatography is well-behaved in the sense that I_a is zero to within experimental uncertainty.** An analogous assumption must be made for the internal standard. However, the possibility of a bias error, arising during the extraction and clean-up steps, is not excluded. Then, eq [42] of Section IIIB(iii) still applies to the Youden Sample Response experiments, but with $I_a = 0$, with a corresponding expression for the analytical response for the volumetric internal standard (see Appendix).

The volume ratio (v'/V') again cancels exactly despite random variations in v', and is replaced by the quantity of internal standard Q_i', to give the theoretical expression for the Youden Sample Response curve:

$$A_a'/A_i' = [(R_a \cdot f_a /R_i \cdot f_i) \cdot (F_a' /Q_i') (Q_a/W_s)] \cdot W_s - (R_a \cdot f_a/R_i \cdot f_i) (L_a'/Q_i') \qquad [59]$$

$$= k_{y,i} \cdot W_s + b_{y,i}$$

As discussed in IIIB(iii), the main practical purpose of this procedure is to determine whether or not a bias error exists, *via* a value for $b_{y,i}$ which is (or is not) statistically different from zero. If random errors in v' dominate those for the overall procedure, use of the volumetric internal standard will generally improve the precision[26], and the degree of confidence in evaluating $b_{y,i}$ will correspondingly increase.

With regard to the analyses corresponding to the Method of Standard Additions itself, however, the extract solutions have been spiked with the same fixed quantity Q_i' of volumetric internal standard. We obtain a relationship independent of the volume ratio (v'/V') (see Appendix for details):

26

$$A_a'/A_i' = [(R_a \cdot f_a/R_i \cdot f_i)(F_e'/Q_i')] \cdot S_a + (R_a \cdot f_a/R_i \cdot f_i)(F_a' \cdot Q_a - L_a')/Q_i' \qquad [60]$$

$$= k_{msa,i} \cdot S_a + b_{msa,i}$$

where:

$$b_{msa,i} = (R_a \cdot f_a/R_i \cdot f_i) \cdot (F_a'/Q_i') \cdot Q_a + b_{y,i} \qquad [61]$$

$$k_{msa,i} = k_{y,i} \cdot (F_e'/F_a') / (Q_a/W_s) \qquad [62]$$

As in Section IIIB(iii), the most reliable value of Q_a/W_s, obtainable from the combined Youden Sample Response and Method of Standard Additions, is probably given by combining eqs [61] and [62]:

$$C_a = Q_a/W_s = [(b_{msa,i} - b_{y,i})/k_{msa,i}] \cdot (F_e'/F_a') / W_s \qquad [63]$$

with the necessary assumption that $F_e' = F_a'$. If the Youden Sample Response intercept is not determined, it is necessary to assume in addition that $b_{y,i} = 0$, (*i.e.* $L_a' = 0$, see Appendix).

The same comments, concerning the likelihood that use of the volumetric internal standard will improve or impair the overall precision[26], apply here also. Note, however, that if volatile solvents are used the incorporation of a non-volatile volumetric standard will always provide insurance against bias errors introduced by uncontrolled solvent evaporation from the final sample extract. Even if a volumetric internal standard is included for this latter reason, there is of course no subsequent requirement to evaluate the data using eqs [59] - [63]. The internal standard can be ignored, if it is judged to be wise to do so[26], and the methods of Section IIIB(iii) used instead.

IVB. Methods Exploiting Surrogate Internal Standards.

The ideal surrogate internal standard is an isotope-labelled version of the target analyte, with sufficient isotope labels in each molecule that the natural abundance of this species in the sample, is negligible (condition (g) in the introductory comments to Section IV). **The mass spectrometric responses A_a' and A_i', corresponding to analyte and internal standard in the sample extract, do not then interfere with one another, and this simplification is assumed to be valid in the entire discussion of this section.** (Incidentally, it is worth noting here that a high level of

27

deuteration usually results in a significant shift of retention time, relative to that of the non-deuterated analyte. This provides further separation of the two responses).

However, it is frequently difficult to acquire a surrogate internal standard which fulfils this condition. As a result, the analyte and internal standard will probably co-elute, and the (higher) m/z value monitored for the surrogate will contain interfering contributions from naturally occurring isotopic variants (mostly ^{13}C) of the native analyte. In such cases it is necessary to deconvolute the two signals from one another, using regression techniques. This deconvolution is complicated even further if appreciable ion fragmentation, particularly hydrogen losses, occur in the mass spectrometer. In such a case, one is faced also with interferences of the labelled internal standard on the m/z value monitored as characteristic of the unlabelled analyte. This problem has been extensively reviewed by De Leenheer et al.[27] .

At some point, uncertainties introduced during the deconvolution procedure will outweigh the advantages conferred by use of a surrogate internal standard, but it is not possible to generalise further. Each case must be considered[27] on its own merits. In some cases, it may even be preferable to use a closely related compound (e.g. a methyl homologue) as a surrogate internal standard, rather than a partially isotope-labelled version of the analyte, if clean separation of the mass spectrometric signals turns out to be more critical than increased assurance that the recovery efficiently F_i' provides a good estimate for F_a' (see below). Note, however, that if a chemically similar but non-identical compound is used as surrogate internal standard, the relationship $F_i' \geq F_a'$ is no longer necessarily valid. The idea that F_i' provides an upper limit to F_a' is based on the combined assumptions that the surrogate is chemically identical to the analyte, but that the original analyte may be more difficult to extract (occlusion effects) but the surrogate added externally. The latter effect may, in some cases, be minimised by allowing sufficient time for the internal standard and native analyte to re-distribute themselves in the sample, prior to extraction.

IVB(i).   Use of a Surrogate Internal Standard with No External Standard.

As mentioned above, the ideal surrogate internal standard is an isotope-labelled version of the target analyte, with sufficient isotope labels in each molecule that the natural abundance of this species, in the sample, is negligible. This is the case assumed here. When such an analytical aid is available, it provides a means to achieve an analytical result of a reasonable degree of reliability when a limited quantity of sample is available. Procedures such as the Method of Standard Additions require, in principle, multiple sample aliquots. All analyzed samples must be of a size sufficient that sample homogeneity is assured, and that the analyte quantities Q_a fall within the dynamic range of the analytical procedure.

28

The simplest procedure is to add a measured quantity Q_i of the surrogate internal standard to a measured quantity W_s of the sample. The spiked sample is than taken through the extraction and clean-up steps, and analyzed for both native and isotope-labelled analytes in the same chromatography experiment. In the present treatment it will be necessary to assume that no bias errors exist in the chromatographic quantitation of either pure analyte or pure internal standard, i.e. that I_a and I_i are both zero. If this is found experimentally to be not so, it is strongly recommended that the causes be identified and the situation remedied; otherwise the algebraic expressions become intractable.

The ratio of chromatographic response factors ($R_a . f_a/R_i . f_i$), a crucial quantity (see Appendix) can be measured using solutions containing known quantities of both analyte and internal standard. **Frequently, in the case that the surrogate internal standard is an isotopically labelled version of the analyte, it is <u>assumed</u> that these response factors are equal for mass spectrometric detection.** While this assumption is probably valid for most cases when applied to total ionisation yields, it can break down due to kinetic isotope effects if molecular ions are monitored and are subject to appreciable ion fragmentation. A dramatic example of this effect has been published[28]. It is good pratice to establish whether or not such an effect is operating by comparing mass spectra of the analyte and its isotope-labelled analogue, as a function of ion source temperature, ionizing energy, etc.

Deduction of values for Q_a also requires knowledge of, or assumptions regarding, the recovery parameters F_a', F_i', L_a', L_i'. Some experimental information on this question can be obtained if sufficient sample is available (see Appendix). The most usual procedure, however, is to assume that $F_i' = F_a'$ (in most cases F_a' will be less than F_i'), and that the constant loss parameters L_a' and L_i' are both zero. **Note that it is possible that non-zero values of L_a' and L_i' may interact with one another, due to competition for the "active sites". This potential complication is related to the so-called "carrier effect", whose importance has been the subject of considerable debate[20,29,30].**

Finally, it is worthwhile to add a few comments about the quantity Q_i of surrogate internal standard, which directly determines the value of Q_a. The best accuracy and precision are achieved when Q_i is determined by direct weighing of a sample of known purity (both chemical and isotopic). However, such internal standards are generally scarce and expensive, and it is common practice to dispense Q_i as a measured volume V_{IS} of a solution of concentration C_{IS}. In this case the calculated value for Q_a depends directly upon the product $C_{IS} . V_{IS}$ (no cancellation, as in the methods using volumetric internal standards described in Section IVA). Thus, errors in the dispensed volume V_{IS} and in the concentration C_{IS} are reflected directly in the value deduced for Q_a.

29

In summary, the simplest and most commonly used method employing a surrogate internal standard, uses eq [67]:

$$C_a = Q_a/W_s = (A_a'/A_i') \cdot C_{IS} \cdot V_{IS}/W_s \qquad [67]$$

Apart from the disadvantages associated with explicit dependence on C_{IS} (potential systematic errors due to solvent evaporation) and V_{IS}, eq [67] implies assumptions concerning equal chromatographic response factors (usually, but not invariably[28], valid), equal recovery efficiencies $(F_a' = F_i')$, and zero constant losses in both the extraction/clean-up sequence $(L_a' = 0 = L_i')$ and in the chromatographic train $(l_a = 0 = l_i)$.

IVB(ii).   *Use of a Surrogate Internal Standard in Conjunction with an External Standard.*

In this method, the surrogate internal standard is used to spike both the raw sample (as in IVB(i)) and an external standard solution(as was done for the volumetric internal standard in IVA(i)). As a result, the quantitation is done by measuring Q_a relative to Q_a'' (a weighed quantity, rather than to $Q_i = C_{IS} \cdot V_{IS}$ as in IVB(i)), while the surrogate internal standard plays a dual role of correcting (partially) for extraction efficiency and also that of a volumetric internal standard. This dual role will become apparent in the detailed treatment (see Appendix).

The problem of constant losses L_a' and L_i', occurring during extraction and clean-up, must be faced. As for the corresponding chromatographic losses l_a and l_i these will interact with one another in a manner related to the question of the carrier effect[20,29,30]. Thus, the only hope for an accurate and precise analysis is that these losses are reduced to zero by appropriate experimental precautions. Under these conditions the volume ratios cancel exactly to give the working relation eq [73], on condition that $Q_i'' = Q_i$:

$$C_a = Q_a/W_s = (A_a'/A_i') \, (A_i''/A_a'') \, (F_i'/F_a') \cdot (Q_a''/W_s) \qquad [73]$$

Note that the absolute values of Q_i and Q_i'' need not be known. The only condition required is that they be equal, a condition met experimentally by using a good-quality digital dispenser (good precision for V_{IS}), and by spiking the sample extract and the

external standard solution at the same time (same value for C_{IS}, not necessarily known accurately). This feature (lack of dependence on the absolute value of C_{IS}) is particularly important when a volatile solvent must be used. Since surrogate internal standards are usually scarce and expensive, they are usually not available in quantities sufficient that a quantity Q_i can be weighed out accurately and precisely each time (a minimum of several milligrams, for most analytical balances). The same restriction does not usually apply to the unlabelled standard, so sizable quantities Q_a'' can be weighed out, and external standard solutions made up fresh, each time. This is a considerable advantage of the present method over that described in IVB(i).

In addition, the procedures described here permit an estimate of the recovery efficiency F_i', of the surrogate internal standard, to be made (see Appendix):

$$F_i' = (A_i'/A_i'') \, (v''/v') \, (V'/V'') \quad\quad\quad [74]$$

provided $Q_i = Q_i''$. Such an estimate of F_i' is subject to combined errors in the volumes, and in any event provides only an upper limit to F_a'. However, practical application of eq [73] requires the assumption that $F_a' = F_i'$, and this is most likely to be valid when F_i' is close to unity. Thus, estimation of F_i' via eq [74] provides a check on the internal consistency of this procedure.

IVB(iii)   *Analysis Using a Surrogate Internal Standard in Conjunction with Both a Volumetric Internal Standard and an External Standard.*

This procedure is identical to Method IVB(ii) except that a volumetric internal standard is also used, as described below, in order to provide more reliable measurements of F_i' (fractional recovery of the surrogate spiked into the raw sample). Monitoring F_i' is essential for quality control of the overall analytical procedure, and is possible with very little additional effort over and above that required for Method IVB(ii). However, in cases where precision of injection volumes or detector drift are <u>not</u> major problems (compare discussion in IVA(i)), Method IVB(ii) is adequate (eq [74]) and may even be superior[26] to the approach described below.

It is necessary to use two different symbols to denote the two internal standards. Subscript i will denote the surrogate, as for Method IVB(ii), while a subscript j will denote the volumetric internal standard.

In practice, the surrogate internal standard is used to spike the raw sample and the external standard solution, exactly as in Method IVB(ii). However, prior to analysis by (usually) chromatography with mass spectrometry, both the sample extract and the external standard solution are <u>also</u> spiked with a volumetric internal standard. (A realistic example would involve analysis for a specific PCB congener, using a

31

surrogate internal standard which is an all-^{13}C isotopic variant of the congener, and using octachloronaphthalene as the volumetric internal standard which also serves as a retention time reference point).

The volumetric standard does not enter the analytical result Q_a itself; eq. [73] applies under the same restrictions, including an assumed validity of a single-point calibration, as in Method IVB(ii). Under these restrictions **eq [73] is valid as the working relationship for the present method,** but can be applied only if the fractional recovery F_a' is assumed equal to F_i'. Note that, in particular, eq [73] assumes that the surrogate spike quantities Q_i and Q_i'' are equal; this condition is readily fulfilled experimentally, as discussed above.

However, the chromatograms obtained for the sample extract and external standard also contain peaks corresponding to the volumetric internal standard, and this information permits reliable measurement of F_i'. If these chromatograms are regarded as analyses for the <u>surrogate</u> internal standard (subscript i) by Method IVA(i), using the external standard in conjunction with the volumetric internal standard (subscript j), the relationships derived for Method IVA(i), but with the subscript substitutions **a --> i** and **i --> j,** apply here.

By assuming that the relative response factors (R_{ij} . f_i/R_j . f_j) are equal for the two chromatographic runs (sample extract and external standard), the following result is readily obtained (see Appendix) if it was arranged by experiment that the volumetric internal standard quantities were equal, *i.e.* $Q_j' = Q_j''$, and if in addition the surrogate internal standard quantities were also equal, *i.e.* $Q_i'' = Q_i$:

$$Q_i'/Q_i \equiv F_i' = (A_i'/A_j') \cdot (A_j''/A_i'') \qquad [76]$$

In this way, reliable measurements of F_i' can be obtained from two peak area ratios, obtained from the same chromatograms as those used to quantitate the analyte itself (eq [73]). These values of F_i' are invaluable for quality control of the overall analytical procedure but are upper bounds to F_a' if the surrogate is an isotopically labelled version of the analyte.

In summary, Method IVB(iii) has no advantage over Method IVB(ii) as far as the desired analytical result (Q_a/W_s) is concerned (still uncertain due to lack of knowledge of the ratio of fractional recoveries), but provides more reliable measurements of F_i' with little additional effort, in cases where injection volumes v are the limiting factor in the overall precision (usually true of GC methods). In other cases (*e.g.* HPLC loop injection), injection volumes are dispensed with high precision and use of a volumetric internal standard could actually impair the overall precision[26].

IVB(iv)   *Use of a Surrogate Internal Standard which is NOT an Isotopic Variant of the Target Analyte.*

In some cases it may be necessary to use a surrogate internal standard which is not an isotopic variant of the analyte. The most common circumstance under which such a situation would arise corresponds to a case where an entire <u>class</u> of analytes is of interest, but where isotopic variants are available for only a few members of the class. For example, polychlorobiphenyls (PCBs) comprise a class of 209 congeners, of which only a few are available as all-^{13}C variants. Another example is afforded by the polycyclic aromatic hydrocarbons (PAHs), for which the number of isomers increases dramatically as the molecular weight (and thus the number of fused rings) increases; again, only a very few compounds are available as perdeuterated internal standards. (For the PAHs, only a small fraction of the unlabelled isomers are available as <u>external</u> standards!).

The discussion of Methods IVB(i)-(iii) was explicitly restricted to cases where the surrogate internal standard is indeed an isotopic variant of the analyte. This Section considers the additional implications of circumstances where this condition is not fulfilled. The first part of the discussion concerns circumstances where, although the isotopic variant is <u>not</u> available, the pure analyte <u>can</u> be used as an external standard. Finally, the implications of having neither external standard nor true surrogate internal standard available for the analyte of interest, but where one or both of these <u>are</u> available for a closely related analyte, will be described.

An example of the first circumstance is provided by PCB analysis where only one or two all-^{13}C versions of chemically pure congeners are available, but where all of the <u>unlabelled</u> congeners of interest as analytes <u>are</u> available. The discussion may be separated for convenience into the extraction and clean-up step, and the quantitative chromatographic analysis.

Extraction and Clean-up. The fractional recoveries F′ reflect the efficiency with which the analyte or internal standard can be extracted from the spiked sample, and successfully taken through subsequent fractionation procedures to the final cleaned-up sample extract. In all of the methods discussed thus far, estimates of the desired quantity $F_a{}'$ *via* measurements of $F_e{}'$ (Methods IIIA(iii) and IIIB(iii)) or of $F_i{}'$ can be taken to be <u>upper</u> limits to the true value of $F_a{}'$, corresponding to the likelihood of discrimination against the native analyte due to occlusion within the sample matrix. As discussed above, this effect leads to potential systematic errors corresponding to values of (Q_a/W_s) which are <u>lower</u> limits to the true value. The same physical and chemical effects are operative when using a surrogate which is an isotopic variant of a compound <u>related</u> to the analyte of interest. However, not even the <u>sign</u> of the systematic error can now be assigned with any confidence since *a priori* it is just as likely that $F_i{}' < F_a{}'$ as that $F_i{}' > F_a{}'$. While no fully satisfactory solution to this problem

seems possible, some information on the nature of such systematic errors in any given case can be obtained from additional experiments. Thus, in the present case where an external standard is presumed to be available for the analyte of interest, F_e' can be measured by the Method of Controlled Additions (see IIIA(iii) and IIIB(iii), and this <u>does</u> provide an <u>upper</u> limit to F_a', to be compared with F_i'. Comparisons among different extraction/clean-up procedures can also provide helpful information.

Quantitative Chromatographic Analysis. An example of both systematic and random uncertainties, arising from the chromatography *per se*, is provided by the case of a mixture of analytes covering a wide range of volatilities, such as the PCBs; significant quantitative errors can arise[16] when using a Grob-type splitless injector for capillary GC. These errors arise because the fraction of any given analyte successfully entering the column is governed not only by carrier gas flow rate, valve actuation purging time, and solvent properties, but also by non-reproducible effects such as partial aerosol formation and partial adsorption on the injector surface. These non-reproducible effects are more important for the less volatile components, as has been demonstrated[16] experimentally for the PCBs. The use of internal standards can alleviate this problem, but for quantitative PCB analyses a range of internal standards, covering the volatility range of the analytes, is required[17]. For example, an all-^{13}C tetrachlorobiphenyl used as a single internal standard for all the PCB congeners can adequately correct for such injector errors for all tetrachlorobiphenyl isomers, and reasonably well for tri-and pentachlorobiphenyl congeners, but the correction becomes progressively less applicable for congeners with increasing degrees of chlorination[17]. Cool on-column injection techniques were shown[16] to circumvent these problems of irreproducible discrimination against less volatile components, provided that proper attention is paid to the necessary precautions[18]. In the case of the intrinsic mass spectrometric sensitivities, it is not possible to predict with confidence even relative total ionization efficiencies although various schemes based upon additive atomic contributions have been proposed. Even less is known concerning the fragmentation yields. Thus, although EI mass spectrometric sensitivities for selected ion monitoring (SIM) are reasonably reproducible, relative values are not predictable even for analytes which are isotopic variants of one another[28]. In the present case, where it is presumed that the unlabelled versions of both the analyte and surrogate IS are available, the relative responses in SIM can be determined experimentally.

We now discuss briefly cases where neither external standard nor <u>true</u> surrogate is available. Unfortunately, this circumstance is all too common in environmental analysis. To continue with the example of PCB analysis, it is frequently necessary to estimate the amount of a particular congener from experiments in which none of the available external nor internal standards are chemically identical to the target analyte, though usually isomers of the target PCB congener are available.

Extraction and Clean-up. As above, measured values of F_i' can be systematically larger or smaller than F_a', and there is now no possibility of using the Method of Controlled Additions to measure F_e'. For the example of PCB analysis, using *e.g.* one specific all-^{13}C tetrachlorobiphenyl as surrogate, the measured value of F_i' will be, as usual, an upper limit to F_a' for the corresponding native congener and hopefully should not be too different for isomeric tetrachlorobiphenyls. Some knowledge about the dependence of F_e', and thus of F_i', on degree of chlorination, can be obtained by using the Method of Controlled Additions for representative PCBs other than the analyte of interest (which is presumed here to be not available as a pure standard). In this way, it is possible to obtain some semi-quantitative feel for the likely variations of F_i', and thus for the correction factors to be applied to the values obtained for Q_a/W_s, by assuming $F_a' = F_i'$. However, this is the best that can be done in these circumstances.

Quantitative Chromatographic Analysis. All of the comments made above apply in this case also, but now there is no possibility of accurately determining the relative responses for unlabelled analyte and internal standard. Again, the best that can be hoped for is that some semi-quantitative trends can be established. Any values of C_a estimated in this way must be treated with appropriate scepticism.

V. Comments on Non-Linear Calibration Curves.

Although at first sight a situation involving non-linear calibration curves may appear to be mathematically intractable, a simple decision renders it no more difficult algebraically than any other method. As an illustrative example we shall use the method involving a surrogate internal standard in conjuction with an external standard (Section IVB(ii)), since this method is reasonably complicated but provides results of a high degree of reliability.

If the calibration procedure, using external standard solutions spiked with constant amounts Q_i'' of surrogate internal standard, reveals a highly non-linear relationship between (A_a''/A_i'') and (Q_a''/Q_i''), possibly with a non-zero intercept, the most obvious course would be to describe the dependent variable in these calibration experiments, *viz.*, (A_a''/A_i''), as a function of the independent variable (Q_a''/Q_i''). Such a course *does* lead to algebraic difficulties, basically because in the analyses of the unknown samples the roles of the variables are reversed.

Accordingly the calibration data are best treated, by appropriate curve-fitting procedures, to give directly the inverse functional relationship in which the peak area is assumed to be the independent variable. While such an inversion of dependent and independent variables is indeed an algebraic triviality, it carries implications for the propagation of experimental errors as has been emphasised by Miller[5].

It is now necessary to make the crucial assumption that the _same_ functional form applies equally to the analyses of the extract solution from the spiked sample and to the spiked external standard solutions.

Another necessary simplification is to _assume_ that the offset losses L_a' and L_i' are zero to within experimental error, and that $F_a' = F_i'$ for a surrogate internal standard, **the working relationship for this method becomes eq.[80]** (see Appendix):

$$C_a = Q_a/W_s = [\Phi_{ai}''\{(A_a'/A_i')\}/\Phi_{ai}''\{(A_a''/A_i'')\}] \cdot (Q_a''/W_s) \qquad [80]$$

It can be seen that eq[73], the working relationship for Method IVB(ii), is just a special case of eq[80]. Although eq[80] may look forbidding, it merely requires determination of the functional form Φ_{ai}'' from appropriate calibration experiments (_e.g._ determining the values of the coefficients in polynomial fits to the data), followed by substitution of the experimental peak-area ratios determined for the extracts of the spiked samples, into these functions. Similar treatments can be developed for the other methods if the calibration curves turn out to be non-linear over the desired range. However, the question of propagation of errors, associated with the inversion of dependent and independent variables[5], will always require considerable attention.

VI. When is the Result of Weighing Equal to the Mass of the Object Weighed?

As emphasised in the Introduction, all quantitative chemical analyses refer ultimately to a mass, as measured on an analytical balance, of a standard sample of the analyte **with a known degree of purity**. Although the purpose of the present document is not to describe details of experimental procedures, this essential (if non-glamorous) fact is sufficiently important that some amplifying discussion is included here.

It is important first to define some terminology. The **mass** of an object is an intrinsic property of that object, in the same way as the **amount of substance** (proportional to the number of constituent atoms or molecules) is fixed; there are no known chemical or physical phenomena which can alter these properties without destroying part of the object. (We here take the term **mass** to mean the **rest-mass**, since relativistic effects are of no significance for analytical chemistry in the present context.) It is this aspect of these properties which has led to their adoption as fundamental quantities in the Système Internationale d'Unites (SI units), with the kilogram and the mole, respectively, as the units which permit SI to be a coherent system[7]. There is thus no possibility for debate concerning the meaning of "mass".

The same is not true, however, of the widely used terms "weight" and "to weigh". Most physics textbooks interpret the **weight** of an object to mean the **gravitational force** acting on that object, which is thus a function of both the intrinsic mass of the object and of its position in space. In common usage "weight" is most often used as a synonym for "mass". The verb **"to weigh"** is almost universally taken to mean an operation in which the mass of an object is measured *via* comparison of the local gravitational force upon it with that upon a standard object of certified mass. (True confusion often arises since such a standard mass is, in common usage, also referred to as a "weight"). The archetypal weighing experiment involves use of a two-pan balance in a comparison *via* the lever principle of the unknown object and the standard-mass artifact. Then, when the lever is at equilibrium, the moments of the two forces about the pivot must be equal:

$$m_u.g.L_u = m_s.g.L_s \tag{81}$$

If the arms of the lever are of equal lengths L, and since the value of the gravitational acceleration (g) can be assumed to not vary over the length of the balance arm, this reduces to:

$$m_u = m_s \tag{82}$$

However, eq.[81] is only an approximation to the full expression of balanced forces for the vast majority of cases where an object is "weighed" in this way. In almost every case, the "weighing" is done in ambient atmospheric air, so both the object being weighed and the standard mass are subject to buoyancy forces in accordance with the *Principle of Archimedes*. Since any object immersed in a fluid displaces its own volume of the fluid, and since this fluid volume would have been in equilibrium with its own "weight" (in the sense of the gravitational force upon that fluid volume), the buoyancy force on the displacing object must be equal and opposite to the "weight" of the displaced fluid. Therefore, eq.[81] must be amended thus:

$$(m_u - V_u.d_a).g.L_u = (m_s - V_s.d_a).g.L_s \tag{83}$$

where d_a is the density of the ambient atmosphere, and V_u and V_a are the volumes of the unknown and standard masses, respectively. On substituting:

$$V_u = m_u/d_u \quad \text{and} \quad V_s = m_s/d_s \tag{84}$$

eq.[83] becomes:

$$m_u = m_s.[(1 - d_a/d_s)/(1 - d_a/d_u)] = m_s.k_{asu} \tag{85}$$

where the buoyancy correction factor k_{asu} is a function of the nature of all of the ambient atmosphere and the standard and unknown masses. This correction factor has the value of unity only under two circumstances, *viz.* either d_a = zero (the analytical balance is operated in a vacuum, an option seldom used in practice in a busy analytical laboratory), or $d_s = d_u$ (standard and unknown have equal densities). The standard reference masses are, by international convention, made of stainless steel with d_s = 8 g cm^{-3}, while most chemical materials thus weighed out have densities of the order of 1 g cm^{-3}, so even the second option for reducing k_{asu} to unity is not available in practice.

It is necessary therefore to investigate the values of k_{asu} for typical conditions. The most important variable in determining d_a is altitude, with values ranging from about 1.2 mg cm^{-3} at sea level to about 1.0 mg cm^{-3} at an altitude that of Denver; the variations due to the weather (humidity) amount to only some ±3%. For example, if an object of density 1 g cm^{-3} is weighed at sea level, with standard masses of density 8 g cm^{-3}, the value of k_{asu} calculated from eq.[85] is 1.00105, corresponding to a systematic error of about 0.1% (too <u>low</u> a value of m_s, the parameter actually recorded, is required to balance the unknown mass.) If *e.g.* dried argon is used to flush the balance when used to weigh air-sensitive compounds, the value for d_a must be calculated *a priori*.

This discussion has focussed on the two-pan lever balance, as a readily visualised experimental arrangement. In fact the same considerations apply to modern single-pan electronic balances which must be calibrated against a standard mass; the fact that the standard is placed on the same pan, as is used subsequently for the unknown object, does not alter the fact that buoyancy corrections are required. The question as to whether or not these corrections are significant must be answered for each individual set of circumstances.

VIII   Conclusions

There are no easy answers in quantitative trace organic analysis. The "best" procedure must be determined for each particular case. For cases where the matrix is sufficiently complex that analyte recovery efficiency is an important question, surrogate internal standards are an essential tool if unavailability of large quantities of sample precludes use of the Method of Standard Additions. As emphasised in Section IVA, use of a volumetric internal standard can greatly improve experimental precision in some cases, but can also have a deleterious effect in others. In this, as in other aspects, it is the responsibility of the analyst to make an informed decision.

Another aspect in which professional judgement is an essential feature of a successful analysis involves the question of whether or not the signal, supposedly corresponding to the target analyte, is partly of entirely due to a co-eluting interference. This

question of qualitative analysis, as a necessary preamble to quantitation, if frequently overlooked. The probability of such co-elutions is surprisingly high, as has been demonstrated[31-33] both theoretically and experimentally. Demonstration of "peak purity" usually involves full-spectrum analysis (*e.g.* full-scan mass spectrum or diode-array detection of the complete UV-visible spectra) across the chromatographic peak(s) of interest. Such detailed qualitative analysis is also advisable when testing a control sample to ensure that it is free of the target analyte(s).

Statistical analysis of data, and experimental design and sampling strategies, are essential features of quantitative analysis which are not treated in the present document. A more mundane problem, which can lead to large uncertainties, involves determination of chromatographic peak areas (or heights). Particularly when the peaks of interest are superimposed upon a significant unresolved background, appreciable unceretainties can arise in determining the appropriate baselines. It is essentail that the rules programmed into the detector datasystem are fully understood. Perferably the datasystem should provide an interactive feature which permits user-defined "rubber-banding" of chromatographic baselines, for cases where the pre-programmed routines are not acceptable.

Finally, use of certified reference materials has not been mentioned here. Generally, such materials are used as quality control checks, but they can also be employed as the basis for calibration of an integrated analytical procedure. However, reference materials are generally expensive, representing a scarce resource, and while they do offer the means of probably the most satisfactory calibration procedure, it is not often that is is feasible to do so.

ACKNOWLEDGEMENTS.

REFERENCES.

1. Quantitative Mass Spectrometry with Stable Isotope Labeled Internal Standard
    as a Reference Technique.
    I. Björkhem.
    Stable Isotopes, (H.-L. Schmidt, H. Förstel and H. Heinzinger, Eds.), Elsevier,
    Amsterdam (1982), pp.593-604.

2. Automation and Validation of HPLC-Systems.
    F. Erni, W. Steuer and H. Bosshardt.
    Chromatographia **24**, 201-207 (1987).

3. Analysis of Drugs and Other Toxic Substances in Biological Samples for
    Pharmacokinetic Studies.
    G.W. Peng and W.L. Chiou.
    J. Chromatogr. **531**, 3-50 (1990).

4. Basic Statistical Methods for Analytical Chemistry. Part 1. Statistics of
    Repeated Measurements: a Review.
    J.C. Miller and J.N. Miller.
    Analyst **113**, 1351-1356 (1988).

5. Basic Statistical Methods for Analytical Chemistry. Part 2. Calibration and
    Regression Methods; a Review.
    J.N. Miller.
    Analyst **116**, 3-14 (1990).

6. Chemometrics in Analytical Chemistry; a Review.
    R.G. Brereton.
    Analyst **112**, 1635-1657 (1987).

7. Detection and Determination of Error in Analytical Methodology. Part I. In the
    Method Verification Program.
    M.J. Cardone.
    J. Assoc. Off. Anal. Chem. **66**, 1257-1282 (1983)

8. Detection and Determination of Error in Analytical Methodology. Part II.
    Correction for Corrigible Systematic Error in the Course of Real Sample
    Analysis.
    M.J. Cardone.
    J. Assoc. Off. Anal. Chem. **66**, 1283-1294 (1983).

9.   New Technique in Chemical Assay Calculations. 1. A Survey of Calculational
     Practices on a Model Problem.
     M.J. Cardone.
     Anal. Chem. **58**, 433-438 (1986).

10. New Technique in Chemical Assay Calculations. 2. Correct Solution of the
     Model Problem and Related Concepts.
     M.J. Cardone.
     Anal. Chem. **58**, 438-445 (1986).

11. Exchange of Comments on a New Technique in Chemical Assay Calculations.
     R. Ferrus and M.J. Cardone.
     Anal. Chem. **59**, 2816-2827 (1987).

12. Method Validation Revisited: A Chemometric Approach.
     M.J. Cardone, S.A. Willavize and M.E. Lacy.
     Pharmaceutical Research **7**, 154-160 (1990).

13. On the Question of Duplicate Analyses.
     W.J. Youden.
     Biometrics **3**, 61 (1947).

14. Technique for Testing the Accuracy of Analytical Data.
     W.J. Youden.
     Anal. Chem. **19**, 946-950 (1947).

15. The Sample, the Procedure and the Laboratory.
     W.J. Youden.
     Anal. Chem. **32**, 23A-37A (1960).

16. An Evaluation of Splitless and On-Column Injection Techniques for the
     Determination of Priority Micropollutants.
     F.I. Onuska, R.J. Kominar and K. Terry.
     J. Chromatogr. Sci. **21**, 512-518 (1983).

17. Mass Spectrometric Quantification of Polychlorinated Biphenyl Congeners Using
     Multiple Carbon-13 Internal Standards.
     F.L. Shore, J.D. Martin and L.R. Williams.
     Biomed. Env. Mass Spectrom. **13**, 15-19 (1986).

18. Factors Affecting the Accuracy and Precision of Cold On-Column Injections in
     Capillary Gas Chromatography.
     K. Grob Jr. and H.P. Neukom.
     J. Chromatogr. **189**, 109-117 (1980).

19. A Systematic Approach to Standard Addition Methods in Instrumental Analysis.
M. Bader.
J. Chem. Educ. **57**, 703-706 (1980).

20. A Polemic on the Uses and Functions of Deuterated Analogues in Quantitative Mass Spectrometry.
R. Self.
Biomed. Mass Spectrom. **6**, 315-316 (1979).

21. Comprehensive Standardized Quality Test for Glass Capillary Columns.
K. Grob, Jr., G. Grob and K. Grob.
J. Chromatogr. **156**, 1-20 (1978).

22. Quantitative Determination of Tiamenidine Hydrochloride in Human Serum using Gas Chromatography Mass Spectrometry.
H.-W. Fehlhaber, K. Metternich, D. Tripier and M. Uihlein.
Biomed. Mass Spectrom. **5**, 188-191 (1978).

23. Variations in Mass Spectral Fragmentation Produced by Active Sites in a Mass Spectrometer Source.
A. Wegmann.
Anal. Chem. **50**, 830-832 (1978).

24. Glass-Lined Field Ionization Sources.
J.H. McReynolds, N.W. Flynn, R.R. Sperry, D. Fraisse and M. Anbar.
Anal. Chem. **49**, 2121-2122 (1977).

25. Vapor Phase Silylation of Laboratory Glassware.
D.C. Fenimore, C.M. Davis, J.H. Whitford and C.A. Harrington.
Anal. Chem. **48**, 2289-2290 (1976).

26. Limits of the Internal Standard Technique in Chromatography.
P. Haefelfinger.
J. Chromatogr. **218**, 73-81 (1981).

27. Isotope-Dilution Mass Spectrometry in Clinical Chemistry.
A.P. De Leenheer, M.F. Lefevere, W.E. Lambert and E.S. Colinet.
Advan. Clinical Chem. **24**, 111-161 (1985).

28. Isotope Effect in Negative Ion Chemical Ionization Mass Spectrometry of Deuterium Labbled Lormetazepam.
S. Takahashi.
Biomed. Env. Mass Spectrom. **14**, 257-261 (1987).

29. Evidence for the Lack of a Carrier Effect in the Solvent Extraction and Determination of Octopamine by Gas Chromatography Mass Spectrometry.
B.J. Millard, P.A. Tippett, M.W. Couch and C.M. Williams.
Biomed. Mass Spectrom. **4**, 381-384 (1977).

30. A "Carrier Effect" Observed in Assays for Antidiarrhoeal Compounds.
N.J. Haskins, G.C. Ford, S.J.W. Grigson and K.A. Waddell.
Biomed. Mass Spectrom. **5**, 423-424 (1978).

31. Theoretical Limitations of Gas Chromatography/Mass Spectrometric Identification of Multicomponent Mixtures.
D. Rosenthal.
Anal. Chem. **54**, 63-66 (1982).

32. Statistical Theory of Component Overlap in Multicomponent Chromatograms.
J.M. Davis and J.C. Giddings.
Anal. Chem. **55**, 418-424 (1983).

33. Probability Distributions of the Number of Chromatographically Resolved Peaks and Resolvable Components in Mixtures.
M. Martin, D.P. Herman and G. Guiochon.
Anal. Chem. **58**, 2200-2207 (1986).

APPENDIX: DETAILED DERIVATIONS OF WORKING RELATIONSHIPS.

This Appendix presents more detailed algebraic derivations of relationships discussed in the main text. There is therefore considerable overlap between the two. It was decided that it is more important here to ensure clarity than to pursue conciseness, so that considerable duplication of material will be found. Sections and sub-sections within the Appendix are numbered in strict accordance with those in the main text.

III   Analytical Methods Using External Standards with No Internal Standard in Quantitative Chromatography.

IIIA   Linear Response Curve with Zero Intercept.

In this case, the necessary conditions for a single-point calibration procedure to be valid are satisfied. However it is always preferable, where possible, to use a full calibration curve procedure.

IIIA(i) Standard Calibration Curve Method.

This method involves establishing the **instrumental response curve,** using standard solutions of the analyte covering an appropriate range of concentrations C_a'' and corresponding weighed quantities of analyte $Q_a'' = (C_a''.V'')$. Then the assumption of a linear calibration curve with zero intercept corresponds in this case to a relationship (eq [1]) between the independent variable Q_a'' and the dependent variable A_a'';

$A_a'' = R_a''$. (quantity of analyte delivered to the chromatographic detector)

$$= R_a''.(f_a''.q_a'' - l_a'') = R_a''.f_a''.q_a'' \quad IF \ l_a'' = zero$$

$$= R_a''.f_a''.(v''/V'').Q_a'' \tag{1}$$

where l_a'' (the constant analyte loss in the chromatographic train) must be zero if the response curve is to pass through the origin. If this is found experimentally to <u>not</u> be the case, it is good practice to determine the reasons and take remedial action since non-zero values are often irreproducible (see introduction to Section IIIB, below). The instrumental response factor $(R_a''.f_a'')$, thus determined for the external standard

solution as the slope of a plot of A_a'' vs. Q_a'', can be written for the special case of a single-point calibration procedure (*i.e.* single value of Q_a''), as:

$$R_a''.f_a'' \equiv A_a''/q_a'' = (A_a''/Q_a'')(V''/v') \tag{2}$$

since $(q_a''/Q_a'') = (v''/V'')$.

Refs. 5 and 12 describe statistical tests for deciding whether or not the intercept, derived from a linear fit of A_a'' vs. Q_a'', is significantly different from zero. The corresponding relationship for chromatographic analysis of the extract solution from the sample, can be rearranged to give an expression for Q_a' as the dependent variable;

$$Q_a' = A_a'.(V'/v')/(R_a'.f_a') \tag{3}$$

where again the constant loss l_a' has been assumed to be zero. It is not possible to measure $(R_a'.f_a')$ directly (Q_a' is not known), so **it must be assumed that $R_a'.f_a' = R_a''.f_a'' = R_a.f_a$. This is a general assumption made throughout the present treatment.** Associated (proportional) systematic errors can arise from drift of the intrinsic detector sensitivity R_a or from variations in the fractional transmission factor f_a. The value of R_a will undoubtedly drift with time and exposure to sample, but the effects of such drift can be controlled by alternating injections of the external standard solution with those of the sample extract, permitting continuous monitoring of $(R_a''.f_a'')$ *via* eq[2]. More subtle uncertainties, associated with this assumption of constant instrumental response, can arise for example if the chromatographic peak used to measure A_a' is not as well defined or resolved as the corresponding peak used to measure A_a'' for the solution of the pure standard. Such uncertainties can be resolved only by repeating the analysis using a chromatographic method of significantly different separation selectivity, although use of full-scan mass spectrometry can also assist in such cases.

However, under this assumption of a constant or slowly varying response factor $(R_a.f_a)$ eq.[3] can be used to transform the experimentally observed chromatographic signal A_a' to Q_a', the quantity of analyte present in the sample extract. **Such an inversion of the calibration procedure (eq [1]) carries implications for the propagation of associated experimental uncertainties, as discussed by Miller[5].** Conversion of Q_a' to the desired quantity Q_a requires knowledge of the fractional recovery of analyte during the extraction and clean-up steps of the overall analytical procedure. The present case of an assumed ideal linear relationship corresponds to an <u>assumption</u> of the relationship given as eq.[4]:

$$Q_a' = F_a'.Q_a - L_a' = F_a'.Q_a \tag{4}$$

with the implication that the fractional recovery F_a' is a constant (note also the assumption of zero intercept L_a'). Then eqs.[3] and [4] may be combined to give eq.[5], the working relationship for Method IIIA(i):

$$C_a = Q_a/W_s = A_a'.(1/R_a.f_a).(V'/v').(1/F_a') (1/W_s) \qquad [5]$$

In the special case where only a single-point calibration procedure is used, eqs.[1] or [2], [3] and [4] are combined to give eq.[6]:

$$Q_a/W_s = (Q_a'/F_a')/W_s = (V'/v')(v''/V'')(A_a'/A_a'')(Q_a''/F_a') (1/W_s) \qquad [6]$$

$$= (V'/v')(A_a'/A_a'')(v''.C_a'')/(F_a'.W_s)$$

where $C_a'' = (Q_a''/V'')$.

All of the disadvantages of the Standard Calibration Curve method are evident in eqs.[5] and [6]. Thus, volumes and concentrations appear explicitly; **systematic uncertainties** in V', V'' and C_a'' due to solvent evaporation, and **random errors** in the injection volumes v' and v'' (not well controlled in GC, but much better in HPLC using injection loop methodology), directly affect the final result. The peak area A_a' and its calibration (either $(R_a.f_a)$ in eq.[5] or (Q_a''/A_a'') in eq.[6]) must be measured in separate chromatographic runs, introducing the possibility of systematic error due to instrumental drift. The fractional recovery F_a' is not measurable from such experiments alone, and the requirement for an assumed value of unity introduces a **proportional error** into the values of Q_a thus calculated **(yielding values for Q_a which are lower limits to the true value).**

IIIA(ii) Calibration by Spiking Control (Blank) Samples.

In this context a control sample is taken to mean a sample which is identical in every way to the sample to be analysed, except that it contains an undetectable quantity of the analyte. The degree to which such a sample can be said to exist will vary strongly with the situation, but it is relatively easy to achieve in a pharmaceutical formulation context, for example.

This method again assumes the validity of eq.[1] for the instrumental response. Now, however, the calibration curve actually used is obtained by spiking different quantities S_a of standard into different aliquots of the control sample, which are then taken through the complete analytical procedure. The most reliable method of determining

46

the quantities S_a is by direct weighing of the pure standard. However, in practice the spiking is often done by dispensing volumes V_a of concentration C_a'' ($S_a = V_a.C_a''$); this method is more convenient, but carries the risk of introducing both systematic errors (e.g. from evaporation of solvent and inaccuracies in the volumetric equipment) and random errors (via imprecision in the volumes dispensed).

By varying S_a over an appropriate range, and determining the corresponding chromatographic peak areas A_s' for injections of aliquots of the extracts, a calibration curve can be constructed. Under the general assumptions of Section III, this calibration curve is assumed to be a straight line passing through the origin:

$$A_s' = k_s'. (v_s'/V_s'). S_a \qquad\qquad [7]$$

where k_s' is the experimental slope of the calibration curve from the spiked (thus subscript $_s$) blank samples. If this calibration is then assumed to be applicable to the analysis of the real (unspiked) sample, the desired quantities are calculated as follows:

$$Q_a' = (1/k_s'). (V_s'/v_s'). A_a' \qquad\qquad [8]$$

In the absence of any additional information, it is now necessary to assume 100% recovery of the analyte from the sample, i.e. that $Q_a = Q_a'$, to give:

$$\boxed{C_a = Q_a/W_s = (1/k_s'). (V'/v'). (A_a'/W_s) \qquad\qquad [9]}$$

Eq [9] is the working empirical relationship for analyses using a calibration obtained by extracting blank samples spiked with known amounts of analyte.

Eqs [7], [8] and [9] are straightforward empirical relationships, with experimentally measured parameters. However, under the various assumptions of this section (Section IIIA), the wholly phenomenological eq [7] (with the experimentally determined parameter k_s') can be interpreted in terms of more fundamental parameters in an appropriate form of eq [1], viz:

$$A_s' = (R_a.f_a). (v_s'/V_s'). S_a' \qquad\qquad [10]$$

where $(R_a.f_a)$ is the (assumed constant) instrumental response factor for the analyte, and S_a' is that portion of the quantity of analyte, originally spiked into the blank sample, which was successfully transferred to the sample extract (of volume V_s'). Again under the approximations made in this Section, the constant loss L_s' in the extraction/clean-up procedures is assumed to be zero:

$$S_a' = F_s' . S_a \qquad \qquad [11]$$

so that eq [10] becomes:

$$A_s' = (R_a.f_a) .F_s' . (v_s'/V_s'). S_a \qquad \qquad [12]$$

Comparison of eqs [7] and [12] shows that the experimental slope k_s' may be interpreted, under the stated assumptions, as:

$$k_s' = (R_a.f_a) . F_s' \qquad \qquad [13]$$

so that the empirical eq [9] can be similarly interpreted to give:

$$Q_a/W_s = (1/R_a.f_a) . (F_a'/F_s') . (V'/v') . (A_a'/W_s) \qquad \qquad [14]$$

The practical consequences of the discussion of eqs [10 - [14] arise from the understanding that <u>combination</u> of the external standard calibration method (III A(i)) with the spiked blank sample calibration method (IIIA(ii)) provides much better information than either separately. Thus, IIIA(i) permits independent evaluation of $(R_a.f_a)$, which can be applied directly to eq [13] to provide a value for F_s', an upper bound to F_a' since the native analyte in the sample may be subject to "occlusion effects" which do not affect the analyte added as an external spike. Such an estimate of recovery efficiency is nonetheless an important quality control parameter in judging whether the extraction procedure is adequate.

The working relationship (eq [9],) for the calibration technique discussed in the present section, can be understood (eq [14]) in terms of the assumptions of the model used here. The advantage over the method described in IIIA(i) is that it is the <u>ratio</u> (F_a'/F_s') which is now the unknown quantity reflecting extraction efficiencies, and the usual default option of setting this ratio to unity is likely to be much more accurate than the corresponding assumption in IIIA(i), *viz.* that F_a' itself must be set to unity.

In summary, the method of Calibration by Spiking of Control Samples is subject to all ·of the **systematic and random errors** described in Section IIIA(i) for the Standard Calibration Curve Method, with the important exception that the **proportional errors associated with uncertainties in the fractional recovery are now less serious.** By combining the two methods it is possible to measure values of F_s', a valuable parameter for purposes of Quality Control. The present method is clearly more time-consuming than IIIA(i), which may be a significant consideration in some circumstances. Further, the present method depends on the availability of sufficient quantities of a suitable blank sample.

IIIA(iii) Method of Standard Additions.

This method is similar to that described in Section IIIA(ii), but now the spikes of pure standard are added to different aliquots of the actual sample to be analyzed rather than to a control (blank) sample. Known amounts S_a of the external standard, preferably determined by direct weighing (see Section IIIA(ii)) are spiked into separate aliquots of the raw sample prior to extraction, clean-up, *etc.* Then the total quantity of analyte, recovered from the spiked sample and present in the sample extract, is:

$$Q_a' = (Q_a.F_a' + S_a.F_e') - (L_a' + L_e') \qquad [15]$$

where F_e' is the fractional recovery of that portion of the analyte which was spiked into the sample as a standard solution (compare F_s' in Section IIIA(ii)). In general $F_e' \geq F_a'$ since the latter accounts for losses due to imperfect extraction from the sample matrix as well as for losses during clean-up. As in all of Section IIIA, it is assumed here that all F' parameters are constants, independent of sample size and analyte levels. The constant losses L_a' and L_e' are assumed to be zero in the present case, but non-zero values are discussed in Section IIIB(iii), below.

Also in common with all methods discussed in Section IIIA, it is assumed that the instrumental response curve is the ideal version given by eq.[1], with the chromatographic constant losses l_a' and l_e' also zero. Then Q_a' is also given by eq.[16]:

$$Q_a' = (V'/v').q_a' = (V'/v').A_a'/(R_a.f_a) \qquad [16]$$

Combining eqs.[15] and [16] gives eq.[17]:

$$A_a' = [(v'/V').(R_a.f_a).F_e']. S_a + [(v'/V').(R_a.f_a).F_a'.Q_a] \qquad [17]$$

If the value of Q_a is made constant by always extracting a fixed quantity W_s of sample (assumed homogeneous), and if the volume v' is maintained constant for all injections (to within the available precision, a shortcoming of all methods not involving an internal standard particularly if the chromatographic method is GC), eq.[17] predicts that a plot of A_a' vs. S_a should be linear, with slope k_{msa} and intercept b_{msa} given by:

$$k_{msa} = [(v'/V').(R_a.f_a).F_e'] \quad \underline{and} \quad b_{msa} = [(v'/V').(R_a.f_a).F_a'.Q_a] \qquad [18]$$

From eq.[18], the desired quantity C_a is given by **eq.[19], the working relationship for Method IIIA(iii):**

$$C_a = Q_a/W_s = (b_{msa}/k_{msa}).(F_e'/F_a')/W_s \qquad [19]$$

where k_{msa} and b_{msa} are determined empirically as the slope and intercept of the plot of A_a' *vs.* S_a (eqs [17] and [18]). Note an advantage here of the present method over that described in Section IIIA(ii); in the present case the recovered spike and analyte from the sample are contained in the <u>same</u> sample extract solution, so that the volumes v' and V' are the same for both and thus do not appear in calculation of Q_a from eq [19]. In the method described in Section IIIA(ii), on the other hand, the standard spike and the unknown analyte are contained in different solutions, accounting for the appearance of the additional volume variables v_s' and V_s' in eqs.[7] - [13]. The irreproducibility of the injected volumes v' and v_s', especially for GC analyses, can be a major contributor to the random error in the final analytical result.

Since F_a' is not measurable, practical application of eq.[19] requires the further assumption that $F_a' = F_e'$, leading to a **proportional error** in Q_a yielding a lower limit to the true value, since in general $F_e' > F_a'$ (see discussion of F_s' in Section IIIA(ii)). However, this proportional error is likely to be considerably smaller than that in Method IIIA(i) where it is necessary to set $F_a' = $ unity.

In practice, observation of a linear plot of A_a' *vs.* S_a is a necessary but not sufficient condition for the validity of all the assumptions underlying the explicit form, eq.[16]; this point is discussed in Section IIIB. However, note that eq.[18] gives:

$$F_e' = k_{msa} \cdot (V'/v')/(R_a \cdot f_a) \hspace{3cm} [20]$$

where F_e' is usually an upper bound to F_a' (no occlusion effects in the extraction of the standard spike). However, any such information on recovery efficiencies is invaluable as an indicator for Quality Control of the overall analytical procedure.

Under the assumptions and restrictions of Section IIIA, the Method of Standard Additions is susceptible to simplification to a form analogous to a single-point calibration. Such a simplification would involve only two analyses, *e.g.* of unspiked sample and of one spiked aliquot. Then two independent versions of eq.[17] will result, which can be solved for the unknowns Q_a and F_a' (<u>assumed</u> equal to F_e'). Such a single-point calibration version of the present Method of Standard Additions is the only option if limited amounts of sample are available. Indeed, the requirement for large amounts of sample is the main operational disadvantage of the Method of Standard Additions. **A minimal sample size is determined not only by requirements of adequate signal/noise ratios in the measurement of the signals A_a', but also by the requirement that the sample analyzed be statistically significant, free of significant random variations in the concentration of analyte due to intrinsic inhomogeneity; the latter consideration is often important in environmental analysis.**

It is of interest that the present account of the Method of Standard Additions refers to experiments in which varying amounts of standard S_a are spiked into a fixed quantity

of sample. It has been shown[19] that the converse method, *viz.* a fixed amount of standard spiked into varying quantities of sample, has no advantages. However, the analysis of varying quantities of sample <u>can</u> provide information concerning bias errors (constant systematic errors), and this approach is discussed in Section IIIB(iii).

IIIB. Calibration Curves with a Useful Linear Range but a Non-Zero Intercept.

It is not uncommon, in analytical practice, to observe a calibration curve which is linear to within the experimental precision, but which has a statistically significant non-zero intercept. (Statistical tests, to determine whether the uncertainty limits on the intercept do or do not encompass the origin, have been given by Cardone[12] and by Miller[5]). Such behaviour, if observed for solutions of pure standards, signals a fundamental problem in the analytical method. There are two broad classes of such problems, corresponding to positive and negative values for the y-intercept b (eq [22]).

A positive value for b corresponds to a non-zero analytical signal for a solution known to contain none of the analyte. This effect usually involves chemical interferences and rarely occurs for highly selective analytical techniques, *e.g.* those incorporating high-resolution chromatography with mass spectrometric detection. The second class of problems corresponds to negative values for the y-intercepts of the appropriate calibration curves. The physical meaning of such negative values is best approached *via* the implied positive values for the x-intercepts c:

$$c = -b/k \tag{23}$$

where the least-squares linear regression expressions for b and k are given as eqs [21] and [22]. Such a circumstance in the context of eq [1] implies a threshold value for q_a'', below which no signal is observed. Such observations, particularly for calibration experiments using solutions of the pure standard (Section IIIA(i)), are usually interpreted in terms of irreversible losses of analyte on "active sites" on the column[20,21] or on the injector or other components of the chromatography train[22], or in the mass spectrometer ion source[23,24]. A major problem with such effects is that they tend to be irreproducible, and thus not susceptible to accurate calibration. Thus, it is preferable to investigate the source of such effects and if possible to eliminate them, to the point where the calibration curve passes through the origin to within the experimental precision. Otherwise, the algebraic approaches described below must be adopted.

Such non-zero intercepts in calibration curves are examples of **bias errors**, as defined in Section I above. The detection and characterization of bias errors, often in conjunction with simultaneous proportional systematic errors (*e.g.* those associated with values of $F_a' < 1$), is a major thrust of the approach promoted by Youden[13-15] and

51

more recently by Cardone[7-12]. Many of the principles described in Section IIIB are adapted directly from their work.

IIIB(i) Standard Calibration Curve Method.

Observation of a non-zero intercept, in the calibration curve obtained using pure analyte standards, is expressed mathematically in terms of a non-zero value for b″ in the modified form of eq [1]:

$$A_a'' = k'' . q_a'' + b'' = k'' . (v''/V'').Q_a'' + b'' \qquad [24]$$

where k″ may be interpreted as $(R_a.f_a)$ and b″ is discussed below. Inversion of the calibration relationship given as eq [24], to provide values of Q_a' from measurements of A_a' made on sample extract solutions, gives:

$$Q_a' = (A_a' - b'') (V'/v') /k'' \qquad [25]$$

Conversion of eq [24] to eq [25] is an algebraic triviality, but its implications for the propagation of experimental error are not trivial[5]. This conversion also carries chemical implications whose validity is by no means guaranteed in any particular case. **The values obtained for k″, and particularly for b″, in the calibration experiments (eq [24]) will not necessarily apply to the sample extracts (eq [25]).** For example if b″ is found to be positive, often interpreted in terms of co-eluting interferences, the amount of such interfering substances in the sample extract could well be very different from that in the calibration solution. If such interfering substances were derived from the solvent, different effective values for b″ would pertain, depending upon the total volumes of solvent employed in extracting and dissolving the sample as opposed to dissolving the standard. On the other hand if b″ were negative, corresponding to a positive x-intercept often interpreted in terms of a constant loss of analyte on active adsorption sites in the chromatographic train, this amount of lost analyte could well vary depending upon the quantity of co-extractives from the sample which could compete for these active sites.

The point of the foregoing discussion is to emphasise that non-zero values for b″ usually signal potential uncertainties which are best avoided, if possible, by diagnosing the cause and taking appropriate precautions. Such precautions might involve a more selective analytical method if b″ > 0, e.g. different chromatography and/or increased mass spectrometer resolution, or (for b″ < 0) changing columns and/or silylation of appropriate portions of the chromatographic train, etc. However, if none of these remedies improve the situation, assumption of the applicability of the calibration parameters k″ and b″ (eq [24]), to analysis of sample extracts, gives the working relation as:

$$C_a = Q_a/W_s = (A_a' - b'') \, (V'/v') \, [(F_a' \cdot k'') \cdot W_s] \qquad [26]$$

where the empirical slope parameter k'' may be interpreted as $(R_a.f_a)$. Note that eq [26] is derived from eq [25] plus an <u>assumption</u> of a constant value for the extraction efficiency F_a' (eq [4]). The breakdown of this assumption can also lead to systematic errors, and this possibility is discussed in Section IIIB(iii).

IIIB(ii) Calibration Curve with a Non-Zero Intercept Using Spiked Control Samples.

As in IIIA(ii), the quantities of analyte whose values are known, and for which analytical responses are determined, are the quantities S_a of standard spiked into a (fixed) quantity of blank sample. Here, however, the calibration curve is <u>not</u> presumed to pass through the origin, so that the empirical eq [7] becomes:

$$A_s' = k_s' \cdot (v_s'/V_s') \cdot S_a + b_s' \qquad [27]$$

The non-zero intercept b_s' is subject to a discussion very similar to that for b'' in IIIB(i). Thus, a value $b_s' > 0$ usually implies that a co-eluting interference is contributing to the analytical response A_s', but in this case the interfering compound(s) could arise from the control sample as well as from the solvent. Similarly, if $b_s' < 0$ the corresponding positive value for the x-intercept could now reflect loss of a fixed quantity of analyte on "active sites" of some kind during the extraction and/or clean-up procedures, as well as in the chromatographic train as discussed in Section IIIB(i).

Comments made in IIIB(i), concerning the advisability of diagnosing and removing the causes of non-zero intercepts if at all possible, also apply here. However, if this can not be done inversion of eq [27] to give an expression for S_a, and application of this inverted relationship to analysis of real samples (for which S_a becomes Q_a), is subject to the same comments as applied to the corresponding inversion of eq [24] to [25]. In the present case, the result is eq [28]:

$$C_a = Q_a/W_s = (A_a' - b_s') \, (V'/v') \, (1/k_s') \, (1/W_s) \qquad [28]$$

However, in the present case it is possible to demonstrate how, for simple specific examples, the calibration parameters k_s' and b_s' determined experimentally as the slope and intercept of eq [27] will be in error when applied to determination of Q_a/W_s for the real sample *via* eq [28]. For example, suppose it is found that $b_s' < 0$, corresponding to irreversible fixed loss of analyte on "active sites" somewhere during the analytical procedure <u>prior</u>[25] to the quantitative chromatographic analysis, which is

53

assumed to behave ideally.   That is to say, in the calibration experiments the value of S_a' (the quantity of analyte present in the extract of the spiked blank sample) is assumed to be given by :

$$S_a' = F_s' \cdot S_a - L_s' \qquad\qquad [29]$$

Then, the analytical signal for such a calibration experiment will be given by:

$$A_s' = r_a \cdot q_a' = r_a \cdot (v_s'/N_s') \cdot S_a' = [r_a \cdot (v_s'/N_s') \cdot F_s'] S_a - [r_a \cdot (v_s'/N_s') \cdot L_s'] \qquad [30]$$

so that, for this simple model, the empirical slope and intercept may be interpreted as follows:

$$k_s' = [(R_a \cdot f_a) \cdot (v_s'/N_s') \cdot F_s'] \quad \underline{and} \quad b_s' = [(R_a \cdot f_a) \cdot (v_s'/N_s') \cdot L_s'] \qquad [31]$$

In terms of exactly the same simple (but realistic) model, the corresponding relationships for analysis of the real sample are:

$$Q_a' = (F_a' \cdot Q_a) - L_a' \qquad\qquad [32]$$

$$A_a' = [(R_a \cdot f_a) \cdot (v'/N') \cdot F_a'] \cdot Q_a - [(R_a \cdot f_a) \cdot (v'/N') \cdot L_a']$$

$$= k_s' \cdot (F_a'/F_s') Q_a - b_s' (L_a'/L_s') \qquad\qquad [33]$$

where the second line of eq [33] has made the reasonable assumption (easily corrected for) that $(v'/N') = (v_s'/N_s')$. **Since in general $F_a' \le F_s'$, use of the calibration value k_s' for the slope parameter can result in a proportional error, while a bias error could result if $L_a' \ne L_s'$. This simple example reinforces the recommendation that, if possible, the cause of a calibration curve not passing through the origin should be sought and rectified.**

This same general conclusion holds for another simple realistic example, relevant to the case that $b_s' > 0$ due to presence of a co-eluting interference X in the control (blank) sample. For simplicity, it will be assumed that no constant losses (*e.g.* L_a') occur in this case, so that for the spiking calibration experiments:

$$S_a' = F_s' \cdot S_a ; \qquad Q_{x,s}' = F_{x,s}' \cdot Q_{x,s} \qquad\qquad [34]$$

$$A_s' = (R_a \cdot f_a) \cdot q_a' + (R_x \cdot f_x) \cdot q_x' = [(R_a \cdot f_a) \cdot (v_s'/N_s') \cdot F_s'] \cdot S_a + [(R_x \cdot f_x) \cdot (v_s'/N_s') \cdot F_{x,s}' \cdot Q_{x,s}] \qquad [35]$$

where the double subscript (x,s) refers to the interfering compound x in the blank sample. Then, for this particular example the slope and intercept (eq [27]) may be interpreted as:

$$k_s' = [(R_a.f_a).(v_s'/N_s').F_s'] \quad \underline{and} \quad b_s' = [(R_x.f_x).(v_s'/N_s').F_{x,s}'.Q_{x,s}]$$ [36]

Applying the same simple model to the analysis of the sample itself gives:

$$A_a' = [(R_a.f_a).(V/N').F_a'].\ Q_a + [(R_x.f_x).(V/N').F_x'.Q_x]$$

$$= [k_s' \cdot (F_a'/F_s')].\ Q_a + [b_s' \cdot (F_x'/F_{x,s}') \cdot (Q_x/Q_{x,s})]$$ [37]

where the k_s' and b_s' values in eq [37] are those given in eq [36]. Once again the use of these calibration curve parameters, without correcting for differences in extraction efficiencies, could lead to bias and/or proportional errors, and the situation is best avoided if possible.

IIIB(iii)   *Method of Standard Additions and the Youden Sample Response Curve.*

This Section is that which owes the largest direct debt to the work of Cardone[7-12] and of Youden[13-15]. In its most developed form, this approach[7-15] is a powerful protocol of great generality for the detection of both bias and proportional systematic errors in an analytical procedure. In order to explain the principles involved in a reasonable space, **a particular model for the analytical procedure will be employed:**

(a) the chromatography/detection system used is sufficiently selective that no interferences intervene (as discussed in IIIB(ii), for example); the present discussion could readily be extended to include this effect, if desired;

(b) the detector response is directly proportional to the quantity of analyte reaching it, thus:

$$A_a' = R_a (f_a \cdot q_a' - l_a)$$ [38]

where l_a represents the (assumed fixed) quantity of analyte lost to "active sites" located between the injector and detector, and f_a is the fraction of injected analyte which reaches the detector if l_a is zero:

(c) extraction of analyte from the sample, and the associated selective concentration procedures ("clean-up"), are subject to losses of both the proportional and bias type, such that the proportional loss may vary according to whether the analyte is that originally present or is spiked into the sample, but the fixed loss (possibly on "active sites") is assumed to be the same:

$$Q_a' = F_a' \cdot Q_a + F_e' \cdot S_a - L_a'$$ [39]

While the model specified in (a) - (c) is not completely general, it does cover the majority of circumstances which can lead to significant error in quantitative trace analysis.

Youden[13-15] realised that a general approach to determining whether or not bias errors were present was to determine a response curve in which the only variation in quantity of analyte Q_a was due to controlled variations in sample size W_s:

$$Q_a' = F_a' \cdot (Q_a/W_s) \cdot W_s - L_a' \qquad [40]$$

Eq [40] is a special case of eq [39] with $S_a = 0$ (no spiking) and written so that W_s becomes the independent variable. The implicit assumption that $C_a = (Q_a/W_s)$ is constant amounts to ensuring that the sample is effectively homogeneous over the range of values for W_s to be used. Then, under the assumptions leading to eqs [38] and [40], it is straightforward to derive the corresponding functional form of the Youden Sample Response Curve:

$$A_a' = R_a \cdot (f_a \cdot q_a' - l_a) = R_a \cdot f_a \cdot q_a' - R_a \cdot l_a$$

$$= [R_a \cdot (v'/V') \cdot F_a' \cdot f_a \cdot (Q_a/W_s)] \cdot W_s - R_a \cdot f_a \cdot (v'/V') [L_a' + l_a(v'/V')/f_a] \qquad [41]$$

$$= k_y \cdot W_s + b_y$$

where k_y and b_y are the slope and intercept, respectively, of the Youden Sample Response plot. Note that, under assumptions (a) - (c), only negative or zero values for b_y are predicted. If b_y terms out to be positive, the most probable cause is that the bias errors are dominated by co-eluting interferences as discussed in IIIB(ii). A statistically significant non-zero value for b_y indicates the presence of bias errors in the analytical procedure, although this alone can not determine whether these errors arose during the extraction and clean-up steps or in the chromatographic train. Under the present assumptions (a) - (c), this question could be approached by determining the dependence of b_y upon (v'/V'):

$$b_y = -R_a \cdot [L_a' \cdot f_a \cdot (v'/V') + l_a] \qquad [42]$$

A value for (Q_a/W_s) can be obtained from the Youden slope k_y (eq [41]) if it is assumed that the proportional error factors F_a' and f_a are both unity, and if R_a can be measured. This is not generally feasible, and **the Youden method is usually applied as a diagnostic approach to the detection of bias errors** (but see eq [46] below).

The Method of Standard Additions, described in IIIA(iii) for the simple idealised case, can be adapted to the more realistic model defined by assumptions (a) - (c). In such

56

a procedure, eq [39] applies with the restriction that Q_a is fixed (via a fixed value for W_s), and S_a is the independent variable. Then, eqs [38] and [39] may be combined to give:

$$A_a' \quad = \quad R_a.f_a.(v'/V').Q_a' - R_a.l_a$$

$$= \quad [R_a. (v'/V'). f_a.F_e'].S_a + R_a.(v'/V').[F_a'.f_a.Q_a f_a.-L_a' -(V'/V')l_a] \qquad [43]$$

$$= \quad k_{msa} . S_a + b_{msa}$$

Comparison with eq [41] shows that:

$$b_{msa} \quad = \quad [(R_a.f_a) . (v'/V') . F_a' . Q_a] \; + \; b_y \qquad\qquad [44]$$

$$k_{msa} \quad = \quad k_y . (F_e'/F_a') / (Q_a/W_s) \qquad\qquad [45]$$

where Q_a in the context of eqs [44] and [45] denotes the quantity of analyte in the fixed quantity of sample used in the present Method of Standard Additions. It is evident, from this special but entirely realistic model treatment, that Cardone's claim[7-12] that the Youden and MSA plots may be viewed as simple extensions of one another is valid only if $F_e' = F_a'$. This condition is mostly readily fulfilled if both recoveries are close to 100%, as can frequently be achieved for the relatively simple matries found in the pharmaceutical preparations of concern to Cardone[7-12]. However, it is seldom that this condition can confidently be claimed to be fulfilled in the case for complex environmental matrices.

The most reliable value of (Q_a/W_s) from the Method of Standard Additions is probably that obtained by combining eqs [44] and [45]:

$$C_a = (Q_a/W_s) \; = \; [\, (b_{msa} - b_y)/k_{msa}] . (F_e'/F_a')/W_s \qquad\qquad [46]$$

As before the ratio of analyte recovery efficiencies, for spiked and original analyte, is difficult to determine experimentally and is always a major source of uncertainty. The use of the Youden intercept b_y as the appropriate correction for b_{msa}, rather than e.g. the intercept b_b' of the Standard Calibration Curve (eq [27]), is in accord with the more general conclusions of Cardone[7-13]

IV.   Methods Involving Internal Standards.

Internal standards are used in quantitative chromatography for two reasons: to remove or reduce the dependence of the final analytical result on the values of volumes of solutions, and to provide some measure of the fractional recovery of analyte from the sample into the extract. A compound used for the first purpose is often referred to as a **volumetric internal standard,** and is usually added to the extract solution itself. A **surrogate internal standard,** on the other hand, is added in known quantities to the sample at as early a stage as possible. The fractional recovery of the surrogate internal standard can provide some information on the efficiency of recovery of the native analyte.

The principles upon which these two objectives can be achieved, and their limitations, are discussed below. **The desirable properties for an internal standard are summarised in the main text.**

IVA.   Volumetric Internal Standards in Conjunction with External Standards.

The simple case of a Standard Calibration Curve will be worked through in some detail (Section IVA (i)), in order to establish principles of more general applicability and also the inherent limitations of the approach. The general intent of a volumetric internal standard is to avoid the uncertainties associated with volumes such as v' and V' (see Section III above).

IVA(i).   Standard Calibration Curve.

As for any procedure incorporating a calibration curve, the important characteristics of the latter must be determined, particularly the question of whether or not it encompasses the origin, and also the extent of the linear dynamic range. In the present context, a suitable calibration procedure could involve the following steps:

(a)   using accurate weighings and appropriate dilutions (best not serial dilutions, for which errors accumulate), prepare a series of standard solutions of the analyte; however, before filling to the calibration mark on each standard volumetric flask, add a fixed volume V_{is} of the solution of internal standard, of concentration C_{is} (see general comments under Section IV A). If a volatile solvent is used, it is important to perform all of these additions at the same time, so that each solution contains the same quantity of volumetric internal standard ($Q_i'' = V_{is} \cdot C_{is}$).

(b) using weighings and dilutions, prepare a series of standard solutions of the internal standard alone.

(c) conduct the quantitative chromatography experiments on all solutions prepared in (a) and (b), including as many replicate injections of each solution as are feasible.

The following treatment will assume the validity of a linear response curve with a (possibly) non-zero intercept. Then, the detector response to the analyte in these calibration experiments is assumed to be analogous to eq [38]:

$$A_a'' = R_a (f_a \cdot q_a'' - l_a) = R_a \cdot f_a \cdot (v''/V'') \cdot Q_a'' - R_a \cdot l_a \tag{47}$$

where $b_a'' = (-R_a \cdot l_a)$ is determined as the y-intercept of the plot of A_a'' vs. Q_a''. Similarly, analysis of the series of solutions of pure internal standard leads to the analogous relationship:

$$A_i''' = R_i \cdot f_i (v'''/V''') \cdot Q_i''' - R_i \cdot l_i \tag{48}$$

where $b_i''' = (-R_i \cdot l_i)$ is measured as the y-intercept. The main purpose of these experiments, summarized in eqs [47] and [48], is to determine the intercepts. If possible the physical and/or chemical reasons, underlying statistically significant non-zero values for these intercepts, should be sought; it is preferable, as noted previously, to reduce these intercepts to zero.

Now the data from the analyses of the standard solutions of analyte, spiked with a constant quantity Q_i'' of internal standard, are analyzed in a fashion different from that summarized in eq [47]. The responses A_a'' and A_i'' are measured for each chromatogram, and interpreted in terms of the ratio of eqs [47] and [48]:

$$(A_a'' - b_a'') / (A_i'' - b_i''') = [(R_a \cdot f_a)/(R_i \cdot f_i) / Q_i''] \cdot Q_a'' \tag{49}$$

In each individual analysis of this kind, the volumes v'' and V'' are the same for both analyte and internal standard, so the cancellation is <u>exact</u> in each individual case, regardless of random error (note that V'' is the <u>original</u> volume of each solution, in which the quantities Q_a'' and Q_i'' were dissolved). It is this manipulation which results in exclusion of the errors in the volumes from the final analytical result. The quantity $[(R_a \cdot f_a)/(R_i \cdot f_i)/Q_i'']$ is determined experimentally as the slope of the plot of the corrected intensity ratio (left side of eq [49]) vs. Q_a''. This plot should exhibit a zero intercept, if the corrections b_a'' and b_i''' have been determined properly.

This completes the proposed calibration procedure. **Application of such a calibration to quantitative analysis of sample extracts requires that these extracts be spiked with the identical quantity of volumetric internal standard as**

was used in the calibration experiments, *i.e.* **$Q_i' = Q_i''$.** (Alternatively, it is possible to arrange that $Q_i' = n . Q_i''$ (n integral) by using more than one volume from the same digital dispenser used to spike the calibration solutions). Note that all of these spiking procedures (calibration solutions plus sample extracts) should be done at the same time, to minimise drift in the values of V_{IS} and/or of C_{IS}. Then an equation analogous to eq [49] applies to analyses of the sample extracts:

$$Q_a' = [(A_a' - b_a') / (A_i' - b_i')] / [(R_a.f_a)/(R_i.f_i)/Q_i''] \qquad [50]$$

where $[(R_a.f_a)/(R_i.f_i)/Q_i'']$ was determined experimentally as the slope of the plot corresponding to eq [49]. In addition, evaluation of eq [50] requires the additional assumption that the intercepts b_a' and b_i', pertinent to the sample extract solutions, are given by the experimental values b_a'' and b_i''' from the calibration experiments.

It is not possible to generalise about the validity or otherwise of this assumption, and this emphasises yet again the importance of investigating the causes of any non-zero intercepts with a view to remedial action to reduce them to zero. If this can in fact be done, the long calibration procedure (a) - (c) can subsequently be shortened to a **single-point calibration procedure,** involving just a single calibration solution containing an accurately known quantity Q_a'' of analyte standard, and a reproducible but not necessarily accurately known quantity Q_i'' of volumetric internal standard. Then eq [49] applies, with b_a'' and b_i''' both zero, and the calibration factor $[(R_a.f_a)/(R_i.f_i)/Q_i'']$ can be determined (mean value and standard deviation) from repetitive injections of this standard calibration solution.

The way in which a volumetric internal standard corrects for systematic errors associated with uncontrolled evaporation of volatile solvent (effectively uncertainties in V') can now be appreciated. This procedure uses $Q_i' = Q_i''$ as the normalising parameter, rather than solution volumes. It is thus extremely important that the volumetric internal standard itself be involatile, and that it be added in precisely equal quantities to the analyte (external) standard solution(s) and to the sample extract(s).

The effect of a volumetric internal standard on the overall analytical precision is less clearcut, however. If the random error is dominated by that in the injection volume V', as is commonly the case for GC analyses, it might be expected that use of a volumetric internal standard would improve the precision. However, modern sample loop injectors for HPLC can achieve a precision of injection of 0.05%; it seems inherently unlikely that use of a volumetric internal standard could improve on this, and indeed the question arises as to whether it might impair the overall precision. This question has been considered in detail by Haefelfinger[26], in an interesting analysis of the propagation of error in such experiments; this statistical analysis was illustrated by real-life examples, including at least one in which the random error associated with the manipulations of the volumetric internal standard were large enough to significantly decrease the overall precision, relative to that achievable using only the simple

external standard procedure (Section IIIA(i)). Clearly, an informed assessment is required in each individual case[26].

IVA(ii).   Calibration Using Spiked Control Samples.

In this Section, we discuss modifications to the method described in IIIB(ii) appropriate to incorporation of a volumetric internal standard. In general, the instrumental response to an injected quantity q_a'' of analyte will be given by:

$$A_s'' = R_a \cdot (f_a \cdot q_a'' - I_a) \qquad [51]$$

where A_s'' has been used instead of A_a'' to denote the analytical signal, since the latter symbol has also been used for the case of standard solutions of pure analyte. It turns out that non-zero values of I_a result in algebra which is too intractable to be of practical use. Accordingly, **it is assumed in this treatment that analyte losses, associated with "active sites" in the chromatography train, have been reduced to negligible values.**

However, the corresponding assumption is <u>not</u> made for the extraction/clean-up procedure:

$$q_a'' = (v''/V'') \cdot S_a'' = (v''/V'') \ (F_s'' \cdot S_a - L_s'') \qquad [52]$$

Combining eqs [51] and [52] gives eq [53]:

$$A_s'' = [(R_a.f_a) \cdot F_s'' \cdot (v''/V'')] \cdot S_a - [(R_a.f_a) \cdot (v''/V'') \cdot L_s''] \qquad [53]$$

where F_s'' is the fractional recovery of the spiked analyte from the blank sample matrix, and L_s'' is the associated constant loss. The quantity of analyte spike S_a is the independent variable.

In this context, a fixed quantity Q_i'' of volumetric internal standard would be used to spike all such <u>extracts</u> from the blank samples, in a fashion analogous to that described in IVA(i) for the analyte standard solutions. The analytical response for the internal standard is given by an equation analogous to eq [51] **with I_i assumed to be zero** (see above):

$$A_i'' = R_i \cdot f_i \cdot (v''/V'') \cdot Q_i'' \qquad [54]$$

The peak areas (or heights) A_s'' and A_i'' are measured in the <u>same</u> chromatographic analysis of the <u>same</u> sub-volume v'' of spiked sample extract of volume V''. **Thus, dividing eq [53] by eq [54] to give eq [55] implies that (v''/V'') cancels <u>exactly</u>,**

for each individual experiment regardless of statistical variations in v'' from experiment to experiment. This is, of course, the whole point of using a volumetric internal standard.

$$A_s''/A_i'' = [(R_a.f_a/R_i.f_i).(F_s''/Q_i'')] .S_a - [(R_a.f_a/R_i.f_i).(L_s''/Q_i'')] \qquad [55]$$

$$= k_{s,i}'' . S_a + b_{s,i}''$$

The least-squares-fit parameters $k_{s,i}''$ and $b_{s,i}''$ are now <u>assumed</u> to be directly applicable to analyses of real sample extracts, also spiked with volumetric internal standard:

$$A_a'/A_i' = k_{s,i}'' . Q_a + b_{s,i}''$$

$$C_a = Q_a/W_s = [(A_a'/A_i') - b_{s,i}''] / [k_{s,i}''. W_s] \qquad [56]$$

In order for eq [56] to be valid within the assumptions made to this point, the interpretation in terms of the model shows that all of the following conditions must also be satisfied:

$$Q_i' = Q_i''; \quad F_s'' = F_a'; \quad L_s'' = L_a' \qquad [57]$$

The first of these conditions may be satisfied experimentally by careful attention to control of both C_{IS} and V_{IS}, as discussed in IVA(i). The recovery efficiency F_s'', for analyte standard spiked into a blank sample, is likely to be an upper limit for F_a' but the discrepancy can be minimised if the recovery efficiencies are developed to the extent that both approach 100%. The extraction/clean-up losses L_s'' and L_a'' are not necessarily constants (see discussion in Section IVB(i)), and should preferably be reduced to zero if possible. The advantage of the spiked blank calibration procedure, over that employing standard solutions of pure analyte (IVA(i)), is that the two assumptions (eq [57]) concerning equality of parameters describing recovery efficiency are less stringent than those required in IVA(i), *viz.* $F_a' = 1$ and $L_a' = 0$.

A different comparison involves the precision achievable using the method described here, and the analogous method (IIIB(ii)) which does not use a volumetric internal standard. This same question was discussed in IVB(i), and need not be repeated here except to emphasise that an informed decision must be made in each case[26].

IVA(iii)   Method of Standard Additions with a Volumetric Internal Standard.

In this section we consider the modifications to Section IIIB(iii) brought about by incorporation of a volumetric internal standard into the sample extracts, as discussed

above. The intrinsic advantage of the Method of Standard Additions is that it supplies its own internal calibration procedure.

Again, in order to keep the algebra tractable, **it will be assumed here that the quantitative chromatography is well-behaved in the sense that I_a in eq [38] (Section IIIB(iii)) is zero to within experimental uncertainty.** An analogous assumption must be made for the internal standard. However, eq [39] will be used without further simplification, *i.e.* the possibility of a bias error, arising during the extraction and clean-up steps, is not excluded. Then, eq [42] still applies to the Youden Sample Response experiments, but with $I_a = 0$, and the corresponding expression for the analytical response for the volumetric internal standard is:

$$A_i' = R_i \cdot f_i \cdot (v'/V') \cdot Q_i' \qquad [58]$$

where Q_i' is the fixed reproducible quantity of internal standard added to each extract solution, of volume V' before the first aliquot volume v' is removed for analysis. On dividing eq [41] by eq [58] under the stated conditions, the volume ratio (v'/V') again cancels exactly despite random variations in v', to give the theoretical expression for the Youden Sample Response curve:

$$A_a'/A_i' = [(R_a \cdot f_a /R_i \cdot f_i) \cdot (F_a' /Q_i') (Q_a/W_s)] \cdot W_s -(R_a \cdot f_a/R_i \cdot f_i) (L_a'/Q_i') \qquad [59]$$

$$= k_{y,i} \cdot W_s + b_{y,i}$$

As discussed in IIIB(iii), the main practical purpose of this procedure is to determine whether or not a bias error exists, *via* a value for $b_{y,i}$ which is (or is not) statistically different from zero. If random errors in v' dominate those for the overall procedure, use of the volumetric internal standard will generally improve the precision[26], and the degree of confidence in evaluating $b_{y,i}$ will increase correspondingly.

With regard to the analyses corresponding to the Method of Standard Additions itself (fixed mass of sample W_s, vary amounts S_a of pure analyte added), eq [43] with $I_a = 0$ is the relevant theoretical expression for the analyte response. Now, however, these extract solutions have been spiked with the same fixed quantity Q_i' of volumetric internal standard, whose analytical response is given by eq [58]. On dividing eq [43] by eq [58], we obtain a relationship independent of the volume ratio (v'/V'):

$$A_a'/A_i' = [(R_a \cdot f_a/R_i \cdot f_i) (F_e'/Q_i')] \cdot S_a + (R_a \cdot f_a/R_i \cdot f_i) (F_a' \cdot Q_a - L_a')/Q_i' \qquad [60]$$

$$= k_{msa,i} \cdot S_a + b_{msa,i}$$

Comparison of eqs [59] and [60] gives:

$$b_{msa,i} = (R_a \cdot f_a/R_i \cdot f_i) \cdot (F_a'/Q_i') \cdot Q_a + b_{y,i} \qquad [61]$$

63

$$k_{msa,i} = k_{y,i} \cdot (F_e'/F_a') / (Q_a/W_s) \qquad [62]$$

As in Section IIIB(iii), the most reliable value of Q_a/W_s, obtainable from the combined Youden Sample Response and Method of Standard Additions, is probably given by combining eqs [60] and [61]:

$$C_a = Q_a/W_s = [(b_{msa,i} - b_{y,i}) / k_{msa,i}] \cdot (F_e'/F_a') / W_s \qquad [63]$$

with the necessary assumption that $F_e' = F_a'$. If the Youden Sample Response intercept is not determined, it is necessary to assume in addition that $b_{y,i} = 0$, *i.e.* $L_a' = 0$.

The same comments, concerning the likelihood that use of the volumetric internal standard will improve or impair the overall precision[26], apply here also. Note, however, that if volatile solvents are used the incorporation of a non-volatile volumetric standard will always provide insurance against bias errors introduced by uncontrolled solvent evaporation from the final sample extract. Even if a volumetric internal standard is included for this latter reason, there is of course no subsequent requirement to evaluate the data using eqs [59] - [63]. The internal standard can be ignored, if it is judged to be wise to do so[26], and the methods of Section IIIB(iii) used instead.

IVB. Methods Exploiting Surrogate Internal Standards.

The ideal surrogate internal standard is an isotope-labelled version of the target analyte, with sufficient isotope labels in each molecule that the natural abundance of this species in the sample, is negligible (condition (g) in the introductory comments to Section IV). **The mass spectrometric responses A_a' and A_i', corresponding to analyte and internal standard in the sample extract, do not then interfere with one another, and this simplification is assumed to be valid in the entire discussion of this section.**

IVB(i).   Use of a Surrogate Internal Standard with No External Standard.

The simplest procedure is to add a measured quantity Q_i of the surrogate internal standard to a measured quantity W_s of the sample. The spiked sample is than taken through the extraction and clean-up steps, and analyzed for both native and isotope-

labelled analytes in the same chromatography experiment. In the present treatment it will be necessary to assume that no bias errors exist in the chromatographic quantitation of either pure analyte or pure internal standard, *i.e.* that I_a in eq [38] is zero, and similarly for I_i in the analogous expression for the internal standard. If this is found experimentally to be not so, it is strongly recommended that the causes be identified and the situation remedied; otherwise the algebraic expressions become intractable.

Then, the expressions for the chromatographic responses for analyte and surrogate internal standard are:

$$A_a' = R_a . f_a . q_a' = [R_a . f_a . (v'/V') . F_a'] . Q_a - R_a . f_a . (v'/V') . L_a' \qquad [64]$$

$$A_i' = R_i . f_i . q_i' = [R_i . f_i . (v'/V') . F_i'] . Q_i - R_i . f_i . (v'/V') . L_i' \qquad [65]$$

On dividing eq [64] by [65], the volume ratio (v'/V') cancels exactly for each individual analysis, despite random variations from case to case, since analyte and surrogate internal standard are present together in the same homogeneous extract solution.

$$(A_a'/A_i') = (R_a . f_a/R_i . f_i) (F_a'/F_i') [(Q_a - L_a'/F_a') / (Q_i - L_i'/F_i')] \qquad [66]$$

In order to deduce a value of Q_a from the measured values of Q_i and of (A_a'/A_i'), *via* eq [66], additional information and/or approximations are required. The ratio of chromatographic response factors $(R_a . f_a/R_i . f_i)$ can be measured using solutions containing known quantities of both analyte and internal standard. **Frequently, in the case that the surrogate internal standard is an isotopically labelled version of the analyte, it is. assumed that these response factors are equal for mass spectrometric detection.** While this assumption is probably valid for most cases when applied to total ionisation yields, it can break down due to kinetic isotope effects if molecular ions are monitored and are subject to appreciable ion fragmentation. A dramatic example of this effect has been published[28]. It is good pratice to establish whether or not such an effect is operating by comparing mass spectra of the analyte and its isotope-labelled analogue, as a function of ion source temperature, ionizing energy, *etc.*

The use of eq [66] to deduce values for Q_a also requires knowledge of, or assumptions regarding, the recovery parameters F_a', F_i', L_a', L_i'. Some experimental information on this question can be obtained if sufficient sample is available that several aliquots can be analyzed using varying quantities Q_i of surrogate. Then eq [65] indicates that a plot of A_i' *vs.* Q_i should provide a value of (L_i'/F_i') as the ratio of the intercept to the slope. Estimates for F_i' and L_i' separately can be obtained from the same plot if independent information on $(R_i . f_i)$ and on (v'/V') is available. (Note that, ideally, F_i' should be equal to F_e', pertinent to the Method of Standard Additions). The most usual procedure, however, is to assume that $F_i' = F_a'$ (in most cases F_a' will

be less than F_i'), and that the constant loss parameters L_a' and L_i' are both zero. **Note that it is possible that non-zero values of L_a' and L_i' may interact with one another, due to competition for the "active sites". This potential complication is related to the so-called "carrier effect", whose importance has been the subject of considerable debate[20,29,30].**

Finally, it is worthwhile to add a few comments about the quantity Q_i of surrogate internal standard, to which eq [66] directly relates the value of Q_a. The best accuracy and precision are achieved when Q_i is determined by direct weighing of a sample of known purity (both chemical and isotopic). However, such internal standards are generally scarce and expensive, and it is common practice to dispense Q_i as a measured volume V_{IS} of a solution of concentration C_{IS}. In this case the calculated value for Q_a depends directly upon the product $C_{IS} \cdot V_{IS}$ (no cancellation, as in the methods using volumetric internal standards described in Section IVA). Thus, errors in the dispensed volume V_{IS} and in the concentration C_{IS} are reflected directly in the value deduced for Q_a.

In summary, the simplest and most commonly used method employing a surrogate internal standard, uses eq [67]:

$$C_a = Q_a/W_s = (A_a'/A_i') \cdot C_{IS} \cdot V_{IS}/W_s \qquad [67]$$

Apart from the disadvantages associated with explicit dependence on C_{IS} (potential systematic errors due to solvent evaporation) and V_{IS}, eq [67] implies assumptions concerning equal chromatographic response factors (usually, but not invariably[28], valid), equal recovery efficiencies ($F_a' = F_i'$), and zero constant losses in both the extraction/clean-up sequence
($L_a' = 0 = L_i'$) and in the chromatographic train ($l_a = 0 = l_i$).

IVB(ii). *Use of a Surrogate Internal Standard in Conjunction with an External Standard.*

In this method, the surrogate internal standard is used to spike both the raw sample (as in IVB(i)) and an external standard solution(as was done for the volumetric internal standard in IVA(i)). As a result, the quantitation is done by measuring Q_a relative to Q_a'' (a weighed quantity, rather than to $Q_i = C_{IS} \cdot V_{IS}$ as in IVB(i)), while the surrogate internal standard plays a dual role of correcting (partially) for extraction efficiency and also that of a volumetric internal standard. This dual role will become apparent in the following treatment.

For the external standard solution spiked with surrogate internal standard, the following relationships hold within the linear dynamic range of the analytical method:

$$A_a'' = R_a \cdot f_a \cdot (v''/V'') \cdot Q_a'' - R_a \cdot l_a \qquad [68]$$

$$A_i'' = R_i \cdot f_i \cdot (v''/V'') \cdot Q_i'' - R_i \cdot l_i \qquad [69]$$

The constant chromatographic losses l_a and l_i must be determined experimentally, by calibration experiments using solutions of different concentrations or possibly different injection volumes v'' of the same solution. It is always best to take precautions to ensure that the effects of such chromatographic "active sites" are zero, but this is even more important in cases like the present one where competition for any "active sites" can occur, i.e. the effective values for l_a and l_i will not be constant, but will vary with the absolute values of q_a'' and q_i'' in the injected volume. Therefore, it is assumed in what follows that experimental precautions have been taken to ensure that the "active sites" have been deactivated to the point where $l_a = 0 = l_i$. Then the situation is equivalent to that of a **single-point calibration**, so dividing eq [68] by [69] gives the calibration factor required in the analysis of the sample extract:

$$(R_a \cdot f_a/R_i \cdot f_i \cdot Q_i'') = (A_a''/A_i'') / Q_a'' \qquad [70]$$

where Q_a'' is measured by weighing the pure unlabelled standard, and the ratio (A_a''/A_i'') can be measured and its precision estimated by replicate injections.

Analogous considerations apply to the extracts of the sample spiked with surrogate internal standard. Now, however, the problem of constant losses L_a' and L_i', occurring during extraction and clean-up, must be faced. As for the corresponding losses l_a and l_i these will interact with one another in a manner related to the question of the carrier effect[20,29,30]. Thus, the only hope for an accurate and precise analysis is that these losses are reduced to zero by appropriate experimental precautions. Under these conditions the analytical responses, for extracts of the spiked samples, are:

$$A_a' = R_a \cdot f_a \cdot (v'/V') \cdot F_a' \cdot Q_a \qquad [71]$$

$$A_i' = R_i \cdot f_i \cdot (v'/V') \cdot F_i' \cdot Q_i \qquad [72]$$

On dividing eqs [71] and [72] the volume ratios cancel exactly, and combining the result with eq [70] gives eq [73], on condition that $Q_i'' = Q_i$:

$$C_a = Q_a/W_s = (A_a'/A_i') \ (A_i''/A_a'') \ (F_i'/F_a') \cdot (Q_a''/W_s) \qquad [73]$$

Note that the absolute values of Q_i and Q_i'' need not be known. The only condition required is that they be equal, a condition met experimentally by using a good-quality digital dispenser (good precision for V_{is}), and by spiking the sample extract and the external standard solution at the same time (same value for C_{is}, not necessarily known accurately). This feature (lack of dependence on the absolute value of C_{is}) is particularly important when a volatile solvent must be used. Since surrogate internal standards are usually scarce and expensive, they are usually not available in quantities sufficient that a quantity Q_i can be weighed out accurately and precisely each time (a minimum of several milligrams for most analytical balances). The same restriction does not usually apply to the unlabelled standard, so sizable quantities Q_a'' can be weighed out, and external standard solutions made up fresh, each time. This is a considerable advantage of the present method over that described in IVB(i).

In addition, the procedures described here permit an estimate of the recovery efficiency F_i', of the surrogate internal standard, to be made. Dividing eq [72] by eq [69] (with $I_i = 0$) gives eq [74]:

$$F_i' = (A_i'/A_i'') (v''/v') (V'/V'') \tag{74}$$

provided $Q_i = Q_i''$. Such an estimate of F_i' is subject to combined errors in the volumes, and in any event provides only an upper limit to F_a'. However, practical application of eq [73] requires the assumption that $F_a' = F_i'$, and this is most likely to be valid when F_i' is close to unity. Thus, estimation of F_i' via eq [74] provides a check on the internal consistency of this procedure.

IVB(iii)   *Analysis Using a Surrogate Internal Standard in Conjunction with Both a Volumetric Internal Standard and an External Standard.*

This procedure is identical to Method IVB(ii) except that a volumetric internal standard is also used, as described below, in order to provide more reliable measurements of F_i' (fractional recovery of the surrogate spiked into the raw sample). Monitoring F_i' is essential for quality control of the overall analytical procedure, and is possible with very little additional effort over and above that required for Method IVB(ii). However, in cases where precision of injection volumes or detector drift are <u>not</u> major problems (compare discussion in IVA(i)), Method IVB(ii) is adequate (eq [74]) and may even be superior[26] to the approach described below.

It is necessary to use two different symbols to denote the two internal standards. Subscript i will denote the surrogate, as for Method IVB(ii), while a subscript j will denote the volumetric internal standard.

In practice, the surrogate internal standard is used to spike the raw sample and the external standard solution, exactly as in Method IVB(ii). However, prior to analysis by

68

(usually) chromatography with mass spectrometry, both the sample extract and the external standard solution are also spiked with a volumetric internal standard. (A realistic example would involve analysis for a specific PCB congener, using a surrogate internal standard which is an all-^{13}C isotopic variant of the specific congener, and using octachloronaphthalene as the volumetric internal standard which also serves as a retention time reference point).

The volumetric standard does not enter the analytical result Q_a itself; eqs.[68]-[73] apply under the same restrictions, including an assumed validity of a single-point calibration, as in Method IVB(ii). Under these restrictions **eq [73] is valid as the working relationship for the present method**, but can be applied only if the fractional recovery F_a' is assumed equal to F_i'. Note that, in particular, eq [73] assumes that the surrogate spike quantities Q_i and Q_i'' are equal; this condition is readily fulfilled experimentally, as discussed above.

However, the chromatograms obtained for the sample extract and external standard also contain peaks corresponding to the volumetric internal standard, and this information permits reliable measurement of F_i'. If these chromatograms are regarded as analyses for the <u>surrogate</u> internal standard (subscript i) by Method IVA(i), using the external standard in conjunction with the volumetric internal standard (subscript j), the relationships derived for Method IVA(i), but with the subscript substitutions **a --> i** and **i --> j**, apply here.

By assuming that the relative response factors (R_{ij} . f/R_j . f$_j$) are equal for the two chromatographic runs (sample extract and external standard), the following result is readily obtained:

$$Q_i' = (A_i'/A_j') . (A_j''/A_i'') . (Q_j'/Q_j'') . Q_i'' \qquad [75]$$

If it was arranged by experiment that the volumetric internal standard quantities were equal, *i.e.* $Q_j' = Q_j''$, eq [75] is simplified. If in addition the surrogate internal standard quantities were also equal, *i.e.* $Q_i'' = Q_i$, eq.[75] further reduces to:

$$\boxed{Q_i'/Q_i \equiv F_i' = (A_i'/A_j') . (A_j''/A_i'') \qquad [76]}$$

In this way, reliable measurements of F_i' can be obtained from two peak area ratios, obtained from the same chromatograms as those used to quantitate the analyte itself (eq [73]). These values of F_i' are invaluable for quality control of the overall analytical procedure but are upper bounds to F_a' if the surrogate is an isotopically labelled version of the analyte.

69

In summary, Method IVB(iii) has no advantage over Method IVB(ii) as far as the desired analytical result (Q_a/W_s) is concerned (still uncertain due to lack of knowledge of the ratio of fractional recoveries), but provides more reliable measurements of F_i' with little additional effort, in cases where injection volumes v are the limiting factor in the overall precision (usually true of GC methods). In other cases (*e.g.* HPLC loop injection), injection volumes are dispensed with high precision and use of a volumetric internal standard could actually impair the overall precision[26].

IVB(iv)   *Use of a Surrogate Internal Standard which is NOT an Isotopic Variant of the Target Analyte.*

This topic is fully discussed in the main text.

V.   Comments on Non-Linear Calibration Curves.

Although at first sight a situation involving non-linear calibration curves may appear to be mathematically intractable, a simple decision renders it no more difficult algebraically than any other method. As an illustrative example we shall use the method involving a surrogate internal standard in conjubction with an external standard (Section IVB(ii)), since this method is reasonably complicated but provides results of a high degree of reliability.

If the calibration procedure, using external standard solutions spiked with constant amounts Q_i'' of surrogate internal standard, reveals a highly non-linear relationship between (A_a''/A_i'') and (Q_a''/Q_i''), possibly with a non-zero intercept, the most obvious course would be to describe the dependent variable in these calibration experiments, *viz.*, (A_a''/A_i''), as a function of the independent variable (Q_a''/Q_i''). Such a course does lead to algebraic difficulties, basically because in the analyses of the unknown samples the roles of the variables are reversed.

Accordingly the calibration data are best treated, by appropriate curve-fitting procedures, to give the inverse functional relationship:

$$Q_a''/Q_i'' = \Phi_{ai}''\{(A_a''/A_i'')\} \tag{76}$$

where no restrictions upon the form of the function Φ_{ai}'' are required, though in practice a simple power series is often sufficient. The simple response function exemplified by eq[1] is no longer appropriate.

While such an inversion of dependent and independent variables is indeed an algebraic triviality, it carries implications for the propagation of experimental errors as has been emphasised by Miller[5].

It is now necessary to make the crucial assumption that the <u>same</u> functional form applies also to the analyses of the extract solution from the spiked sample. That is, eq.[76] is assumed to apply also to this analysis, with the identical values of the function parameters as were determined experimentally from the analyses of the spiked external standard solutions:

$$Q_a'/Q_i' = \Phi_{ai}''\{(A_a'/A_i')\}$$

[77]

Dividing eqs [76] and [77], gives eq.[78]:

$$Q_a'/W_s =$$

[78]

$$[\Phi_{ai}''\{(A_a'/A_i')\}/\Phi_{ai}''\{(A_a''/A_i'')\}].(Q_i'/Q_i'').(Q_a''/W_s)$$

where each of the two peak area ratios is determined within a single chromatographic run, and each ratio may be evaluated as a mean value of repetitive determinations. The quantities Q_a'' and Q_i'' in eq[78] are measured quantities. However, the desired quantity is Q_a/W_s, not Q_a'/W_s, and Q_i' is not known although the original quantity Q_i of surrogate added to the raw sample <u>is</u> known (generally arranged to be equal to Q_i''). We thus require the relationships between Q_a' and Q_a, and between Q_i' and Q_i.

The only feasible simplification at this point is to <u>assume</u> that the offset losses L_a' and L_i' are zero to within experimental error, so that:

$$Q_a' = F_a'.Q_a \quad \underline{and} \quad Q_i' = F_i'.Q_i$$

[79]

Then, provided that it was arranged experimentally that $Q_i = Q_i''$, and <u>assuming</u> that $F_a' = F_i'$ for a surrogate internal standard, **the working relationship for this method becomes eq.[80]:**

$$Q_a/W_s = [\Phi_{ai}''\{(A_a'/A_i')\}/\Phi_{ai}''\{(A_a''/A_i'')\}].(Q_a''/W_s)$$

[80]

It can be seen that eq[73], the working relationship for Method IVB(ii), is just a special case of eq[80]. Although eq[80] may look forbidding, it merely requires determination of the functional form Φ_{ai}'' from appropriate calibration experiments (e.g. determining the values of the coefficients in polynomial fits to the data), followed by substitution of the experimental peak-area ratios determined for the extracts of the spiked samples, into these functions. Similar treatments can be developed for the other methods if the calibration curves turn out to be non-linear over the desired range. However, the

question of propagation of errors, associated with the inversion of dependent and independent variables[5], will always require considerable attention.