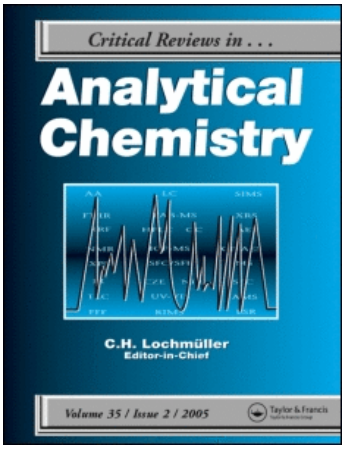

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Review of Univariate Standard Addition Calibration Procedures in Flow Analysis

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Review of Univariate Standard Addition Calibration Procedures in Flow Analysis

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The calibration procedures related to the standard addition method and used in flow analysis are critically reviewed. All examples met in the literature are considered with respect to their facilities for overcoming the interferences. It is disclosed that the flow techniques give a chance to add the standard(s) to a sample by different manners allowing the analytical result to be calculated by either interpolative or extrapolative way. However, from among various calibration procedures those of extrapolative character are distinguished as they are exclusively able to compensate the multiplicative interference effect in wide range of the interferent(s) concentration. It is also shown how the flow standard addition approaches can be employed to solve different analytical problems and—on the other hand—why some of them reveal limited usefulness for calibration purposes. The particular groups of calibration procedures are compared with each other and discussed in terms of their analytical performance.

Keywords analytical calibration, calibration methods, standard addition method, flow analysis

The standard addition method (SAM) is a calibration approach well known in instrumental analysis. In principle, the SAM procedure comprises the addition of known amounts of an analyte into a sample and measurement of the analytical signal for total analyte in each portion. By doing so it is a great chance to compensate the potential interference effects and to obtain the analytical result with greater accuracy than by using the conventional calibration method, i.e., the set of standards method (SSM).

Although SAM has unquestionable advantages over SSM with respect to the overcoming of interferences it is used in laboratory practice only occasionally. There are some reasons for such a situation, including simply the habit to calibrate by SSM. Certainly, one of the serious limitations of SAM is, in contrast to SSM, that the set of working solutions have to be prepared for each examined sample separately hence the calibration procedure is laborious and long-lasting. This drawback is manifested to a special extent in the case of serial routine analysis.

It is known that the calibration procedures can be effectively improved by the use of flow techniques. The reason is that they offer particularly the automation of chemical processing such as sampling, addition of reagents, dilution, and preconcentration. Additionally, in the injection version, they produce the signals in the form of transient peaks that are a potential source of richer information on the chemical system assayed that the steady-state signals obtained when using the batch technique.

In the previous paper it has been shown how the flow techniques can be ingeniously and effectively used for the SSM calibration (1). In this article an attempt is made to review those flow calibration procedures met in the literature, which are related to SAM.

As previously, the calibration is considered here as the reconstruction of the real dependence (calibration dependence) of the analytical signal on the concentration of a substance determined (analyte) in order to transform the signal measured for a sample analyzed into the concentration of the analyte in this sample. Consequently, the calibration procedures reviewed are considered according to two main criteria: how the calibration dependence (theoretical) is *reconstructed* in the form of the calibration graph (experimental) on the basis of measurement data and how the measurement data are *transformed* into the analytical result. Furthermore, all calibration approaches are divided into several categories depending on their ability to overcome the interferences as well as to solve additionally some other analytical problems.

The SAM procedures have been reviewed hitherto in several books (2–5) and articles (6–10). In all those reports they are presented with a special attention paid rather to their preparative and instrumental stages, i.e., to that how the sample and standards are treated, prepared and exposed to measurements.

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The classification proposed here provides better chance of estimating, comparing and discussing the SAM procedures with respect to their analytical performance. In particular, the conditions have been stated how the calibration graph has to be constructed with the use of flow techniques in order to preserve the main advantage of SAM, namely the capability of compensating the interference effects completely. A principle has been also proved that this aim can be achieved exclusively in such a case when the analyte concentration in the sample is calculated in the extrapolative way.

CALIBRATION PROCEDURES OF BASIC APPLICATION

When the interference effect occurs in the analytical system examined, the calibration dependence needed to be reconstructed is a picture of increasing concentration of an analyte influenced by a constant concentration of interferent(s). In such a case the calibration graph constructed on the basis of the analyte only is situated differently from the calibration dependence, as shown in Figure 1. If the interference effect is of multiplicative character, both lines are differently sloped from each other. Consequently, the analytical result is obtained obviously with the systematic error. Thus, the question arises: what is the possibility to reconstruct the calibration dependence accurately?

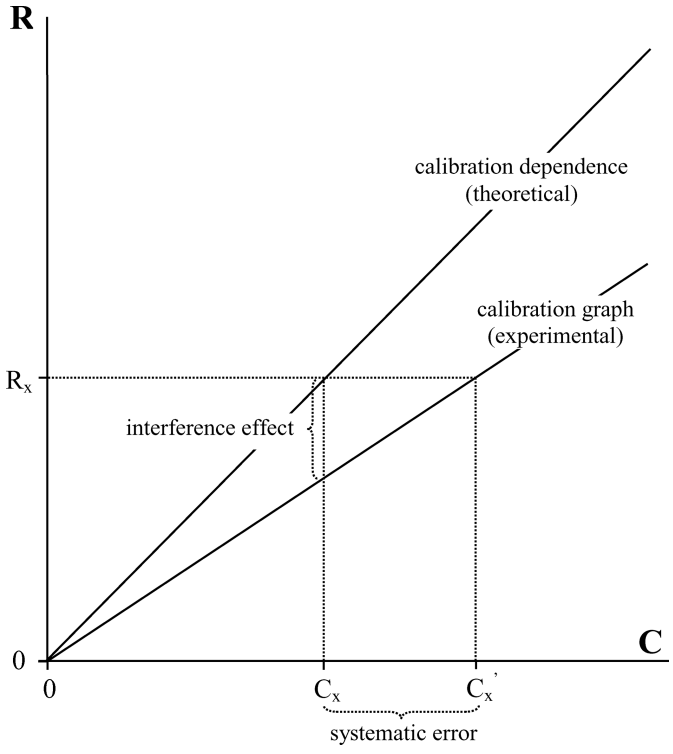

FIG. 1. Main problem of the analytical calibration: If the multiplicative interference effect occurs (caused by certain amount of interferent(s)), the calibration dependence (R vs. C) is not able to be exactly reconstructed by the calibration graph, which is constructed for the analyte only; consequently, the signal measured for a sample, R_x, indicates the analyte concentration, C_x', which is different from true concentration, C_x.

There are two ways for solving above problem by means of compensation of the interference effect: (a) to add the interferents into the standard solutions in exactly such amounts, in which they are present in the sample, and (b) to add the standard solutions into the sample, i.e., to perform calibration by SAM. While the first task is difficult or very often impossible to be realized, the other one is relatively simple and, furthermore, it ensures an ideal matching the sample composition in standards even if this composition is unknown or very complex. However, as results from above, the following condition have to be fulfilled in order to reconstruct exactly the calibration dependence in SAM: *standards have to be added to a sample in such a way to keep the sample components (including interferents) in still the same concentration* (Condition A).

In batch analysis Condition A is easily fulfilled as the calibration solutions are commonly prepared in the separate measurement flasks. The flow techniques enable to merge two solutions by very different means. However, in most cases met in the literature the flow systems dedicated to the SAM calibration are designed in a way enabling to add the standards to a sample in accordance with Condition A. Such reports are just reviewed in this section.

In the examples described either a single calibration graph or a family of calibration graphs is used for transformation process. Furthermore, the measurement data are proposed to be transformed to the analytical result directly or indirectly, i.e., by using the dispersion coefficient.

One-Graph Direct Transformation (ODT) Procedures

In flow analysis the calibration by SAM (like the calibration by SSM) is most often carried out in the manner imitating the batch calibration procedure. At the preparative stage several portions of a sample are placed in the separate measurement flasks in equal volumes, then they are dosed by increased volumes (beginning from the zero volume) of the standard solution and finally all of them are filled up to the mark with the diluent. Each calibration solution is introduced individually into the flow system designed and exposed to the measurement. By doing so a typical single calibration graph based on several experimental points is constructed serving for calculation of the analytical result, C_x, in the extrapolative way, i.e., as shown in Figure 2. The adequate formula is given by:

$$C_x = \frac{\hat{R}_x}{\hat{R}_{x+N\Delta} - \hat{R}_x} \cdot C_{N\Delta} \qquad [1]$$

where $C_{N\Delta}$ is the greatest analyte concentration added to the sample, while $\hat{R}_x$ and $\hat{R}_{x+N\Delta}$ are the signals corresponding to the sample and to the sample with the standard of concentration $C_{N\Delta}$, respectively, both calculated from the linear function fitting the experimental points.

The simplest improvement of the above procedure, used occasionally in continuous flow analysis (CFA), consist in that a

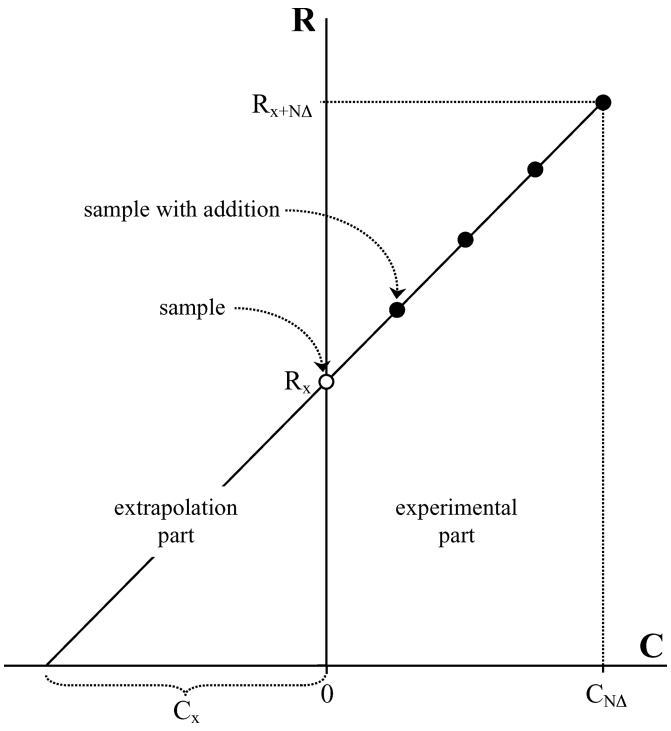

FIG. 2. Calibration by the standard addition method performed according to the ODT strategy: The calibration dependence (compare Figure 1) is partly reconstructed by the calibration graph, which is constructed for the analyte added to a sample; consequently, the analyte concentration in the sample, C_x, is able to be accurately determined in the extrapolative way.

sample is introduced to the flow system through a dedicated tube and merged first with diluent only and then with successive standards flowing one after the other through the other tube (e.g., (11)). The solutions are mixed with each other and directed to the detector. In such a way the calibration solutions can be prepared inside but not outside of the system.

Another simple possibility for preparing the calibration solutions inside the flow system is to apply so called merging-zones technique, which is realizing in the flow injection analysis (FIA) (12). If the FIA system is equipped with two injection valves using for injection of a sample and a standard, both zones have a chance to meet with each other at the same moment before the detector and mixed totally. The calibration procedure should comprise injection of several standards of different analyte concentration (including diluent in order to fulfill Condition A).

In very ingenious approach proposed by Hernández-Córdoba's group the calibration graph is suggested to be constructed with the use of three standard solutions only (13). The chance proved by the authors elsewhere (14) is exploited that when two solutions of the same analyte are propelled toward the detector (FAAS) by two peristaltic pumps running at different turning speeds, the signals recorded for the analyte in the solutions mixed with each other are able to be differentiated by means of the Fourier transformation. In the CFA system one of the pumps is used for propelling two streams of solutions (s_1, s_2) and the other one for propelling of two other streams (s_3, s_4). All streams are merged with each other and mixed before the detector. A sample propelling still as streams s_1 and s_3 is mixed at the first stage of the calibration procedure with diluent (s_2) and with first standard (s_4) while at second stage—with the second (s_2) and third (s_4) standard. The signals corresponding to the sample with diluent and to the sample with three individual standard solutions are generated in a way of the Fourier transformation.

The aforementioned calibration procedures require initial preparation of several (at least three) standard solutions. However, the flow techniques make possible to perform the SAM calibration with the use of a single standard only. In such a case the flow system acts as a tool designed not only for merging a standard with a sample but for prior generating a series of standards of known different analyte concentration.

It has been revealed that for such a purpose the CFA manifold described above can serve (15). In this case one of the peristaltic pumps is running with decreasing turning rate linearly and the second one with increasing flow rate linearly with the same rate. When a sample is propelled in the form of streams s_1 and s_3 and the standard and diluent solutions as streams s_2 and s_4, respectively, a solution reaching the detector always contains the same proportion of the sample while standard solution decreases linearly with time to zero. This produces an multipoint absorbance-time profile with a negative slope, which can be considered as the calibration graph typical for SAM. If it is extrapolated to the time axis, an intersection time, t_s, is obtained, which allows the analyte concentration, C_x, to be calculated from equation:

$$C_x = \frac{t_s - \tau}{\tau} \cdot C_\Delta \qquad [2]$$

where C_Δ is the analyte concentration in the standard solution and τ is time elapsed from the beginning to the end of the pumps operation.

In similar but even simpler CFA system proposed by Frary (16) a sample and a standard solution are propelled to the detector (FAAS) with the use of two peristaltic pumps while a diluent is aspirated through a T-piece. During the calibration procedure the sample flow rate is kept constant but the standard rate is increased step by step allowing the steady-state signals to be measured. The difference between the pumping flow rate and the nebulizer uptake rate is compensated by aspirating diluent. In this case the analyte concentrations in the standard mixed with the sample have to be preliminarily fixed on the basis of values of the standard and sample rates.

For the purpose of single-standard SAM calibration the system designed for discontinuous flow analysis (DFA) with the cam-driven piston pump installed can be also used (17). In this case it is proposed to propel a standard with the aid of the piston pump with increasing rate and to merge it with the sample and diluent streams aspirated through a T-piece. A constant flow rate of the standard-sample-diluent blend is governed by a peristaltic

pump (situated between the merging point and the detector) and the contribution to this rate from the standard varies in a stepwise manner under the control of the piston pump. Consequently, during the single calibration cycle the standard is added to the sample in different well-defined concentrations generating the signals in the form of plateaus.

It has been proved that an instrumental module making the calibration procedures especially simple and effective is the fully rotary valve (FRV) designed in our laboratory (18). The FRV is a doubly-layer eight-channel valve, which is—if compared with conventional two-positional valve—modified in such a way that the rotor, when activated, is capable of not only 45° but 360° rotation in relation to the stator. For the SAM calibration the standard is proposed to be propelled through the FRV by four tubes while a diluent by four other ones (11). When the FRV is rotated gradually through particular eight positions, different parts of the standard have a chance to be mixed and diluted with different parts of diluent and, consequently, the standards of different well-controlled analyte concentrations are prepared. Each standard is then merged with the sample stream flowing with constant rate. It has been proved (11) that so many as eight calibration solutions of different analyte concentrations added (including zero concentration) can be generated by this means. The system is proposed to be used in both the CFA and FIA analysis.

In the approach proposed by Agudo et al. the FIA system is designed with a loop of defined volume allowing a solution to be circulated with the aid of the peristaltic pump (19, 20). In order to provide measurement data for the SAM calibration (20) the loop, initially containing a diluent, is filled up with a sample only and then with still the same volumes of the sample and increased volumes of the standard solution. Every time the sample-standard-diluent mixture is injected to the carrier stream flowing to the detector. The volumes of the sample and standard solutions are fixed by control of either a time or speed of the pumps operation.

Very similar instrumental system designed for typical sequential injection analysis (SIA) has been presented by Silva et al. (21). Preparation of calibration solutions includes introducing known volumes of the sample, standard and diluent solutions into a holding coil of the manifold. To facilitate homogenization of solutions they are aspirated into the coil subsequently, as smaller portions forming a sandwich profile of zones adjacent to each other, until a mixture zone of the assumed volume is formed. The zone is separated from carrier solution of both sides by two small zones of air. To perform the SAM calibration several such zones of equal volumes are prepared, each composed by the same amount of the sample and by variable amounts of the standard, and they are directed to the detector after the other.

One-Graph Indirect Transformation (OIT) Procedures

In all aforementioned SAM procedures the concentrations of an analyte added to a sample could be well known due to either manual preparation of a set of standard solutions beyond the flow system or—in the case of preparation of standards inside of the system from a single standard solution—preliminary measurements of such well-measured instrumental parameters as time, volume or flow rate. Thus, the measurement data obtained for the calibration solutions can be transformed directly to the analytical result.

However, the common case in FIA is to produce a set of different signals for local analyte concentrations which are actually unknown. The auxiliary factor being then most often used in such cases is the dispersion coefficient, D. It is found as the ratio of two signals obtained during the supplementary experiment for a test standard solution: a steady state signal generated by the solution propelled continuously through a FIA system and the transient peaks produced by the solution injected and dispersed in given instrumental conditions. A local analyte concentration in every zone dispersed is then calculated taking into account the corresponding signal and the value of the dispersion coefficient.

The SAM approaches requiring calculation of D-values are the representatives on indirect-transformation calibration procedures as they are based on measurements not only of some instrumental parameters but also of additional analytical signals.

Good examples of the OIT calibration strategy are several SAM procedures proposed by Reis et al. (22–24). In the earliest approach (22) the merging-zones technique is applied coupled with the zone-sampling technique, which has been originally developed by the same group (25). A standard solution is injected to the carrier (diluent) solution and directed to the auxiliary loop. After well-defined time a part of the standard zone and a sample are injected simultaneously from two loops of the second valve to two streams of the carrier solution, they are merged with each other and directed to the detector. Since the standard zone is more and more dispersed in the loop, concentrations of the analyte added to the sample at particular sampling cycles are different from each other but unknown in fact. The exact values of these concentrations are then found from the experiment leading to determination of the dispersion coefficients.

Very similar calibration approach, described several years later (23), is realized with the use of much more sophisticated FIA system working under computer control. In this case the standard zone is also trapped in the auxiliary loop but the sample is injected independently and directed toward this loop. Meeting the merging point the sample zone is dozed by well-defined portion of the standard zone dispersed and then both zones are delivered to the detector. The sample is injected several times and dosed by different analyte concentrations, which are defined again by D-values preliminarily estimated.

The SAM calibration procedure is also proposed by the same authors to be possible of performing during a single cycle of the FIA system operation (24). The valve installed in the system is equipped with two injection loops of different volumes. Two zones of a standard solution are injected simultaneously from these loops in such a way that they have a chance to overlap with

each other. Before the detector they are merged with the sample stream flowing continuously. As a result the double-peak signal is recorded, which is measured for calibration purposes in two maximum points and in minimum point. In order to fulfill Condition A the steady-state signal obtained for the sample merged with the carrier solution only is additionally measured. The local maximum and minimum analyte concentrations in the zones overlapped are calculated from D-values.

Similar SAM procedure has been described by Araújo et al. (26). It is suggested to inject the standard solution to the carrier stream from a single loop, to merge it before the detector with the sample stream, and to store the measurement data from the falling side of this peak at several selected well-defined times after injection. As above, the local analyte concentrations added at selected delay times to the sample are calculated from D-values estimated separately.

The interesting technique which is possible of using for calibration purposes in FIA is the reverse flow technique. It generally consists in injection of a solution (sample, standard or diluent) to the carrier stream of the calibration solution (sample or standard) instead of the diluent stream. As a result the injection peak may appear on the steady-state signal in the form of either positive or negative peak depending on whether the analyte concentration in the solution injected is greater or less, respectively, than the analyte concentration in the carrier stream.

The simplest SAM procedure utilizing the reverse flow technique is consisted of two main stages: first a sample and a standard solution (of the greater analyte concentration than the sample) are injected one after the other to the diluent stream and then the sample is injected from the same loop to the standard solution (27). Two positive and one negative peak obtained are measured in their maximum and minimum points, respectively. It is proved that when the sample is diluted with both the diluent and the standard solution to the same maximum extent, the analytical result can be calculated from all three measurement data and from the adequate D-value.

In another version of above approach the sample is injected first to the diluent stream and then to two standard solutions of different analyte concentration (28). In order to find the analytical result the D-value has to be also additionally estimated.

Quite specific technique possible of performing in FIA is the zone penetration technique, which has been ingeniously applied for the SAM calibration by Fang et al. (29). At the preliminary stage of the calibration procedure the standard solution is proposed to be injected to the diluent stream and two transient signals of equal values corresponding to delay times t_1 and t_2 are chosen in both sides of the peak obtained. Furthermore, the dispersion coefficient is calculated for these signals. At the second stage the sample is injected from the same loop and the sample zone is inserted between the diluent and standard solutions, both flowing continuously. When propelling toward the detector, the standard stream and the sample zone are successively penetrated with each other. Consequently, the signal/time profile is created of two sections, which correspond to the sample (diluted with diluent only) and to the sample with standard additions. If the signals measured again at times t_1 and t_2 are taken from both sections of the profile, the analytical result is able to be calculated from these measurement data and from the D-value determined preliminarily. It is suggested to use several different pairs of t_1 and t_2 in order to complete calibration.

In the FIA system designed by Novic et al. (30) the standard, sample and diulent solutions are pumped independently with defined flow rates by three peristaltic pumps and then merged and mixed with each other before the injection valve. The mixture is injected and directed to the detector. The analyte concentration in the standard stream added to the sample is changed by increasing the flow rate of the pump delivering standard and decreasing the flow rate of the pump delivering carrier simultaneously. Although the authors recommend estimating these concentrations by calculating D-values, the same aim seems to be possible of achieving by experimental determination of relative flow rates of the standard, sample and diluent solutions at each stage of the calibration procedure.

Several-Graph Direct Transformation (SDT) Procedures

The SDT calibration strategy consists generally in construction of a family calibration graphs on the basis of analytical signals measured for two calibration solutions in well-controlled conditions defined by several different values of a selected instrumental parameter. Although such an approach is mainly adapted in flow analysis to the SSM calibration (1), an example proposed to the SAM calibration is also shown in the literature (31).

The calibration procedure is realised with the use of the FIA system with the FRV operated as the injection valve. The valve is equipped with four loops of different well-defined volumes. When it is fully rotated four different signals (peaks) are able to be recorded for a solution injected. The calibration procedure encompass initial preparation of two calibration solutions: a sample and the sample with the analyte added in concentration C_Δ. Both solutions are injected one after the other producing two sets of four measurement data. If both sets are attributed to zero and C_Δ concentration of the analyte added to the sample, respectively, four two-point calibration graphs can be constructed as shown in Figure 3. After extrapolation of each of them the analytical result is able to be estimated by four values of apparent concentrations and the average value is taken as a final measure of the analyte concentration in the sample.

It has been also suggested in the same work (31) to install additional auxiliary loop to the FRV in order to inject the sample and standard solutions sequentially in a given position of this valve. When the FRV of such configuration is rotated from the start position clockwise and inversely, so many as eight different signals for both solutions are able to be produced. This is so because the zones injected are dispersed differently not only due to injection of various volumes but additionally due to different ways of transporting them from the FRV to the detector. Thus, in this case it is possible of constructing so many as eight

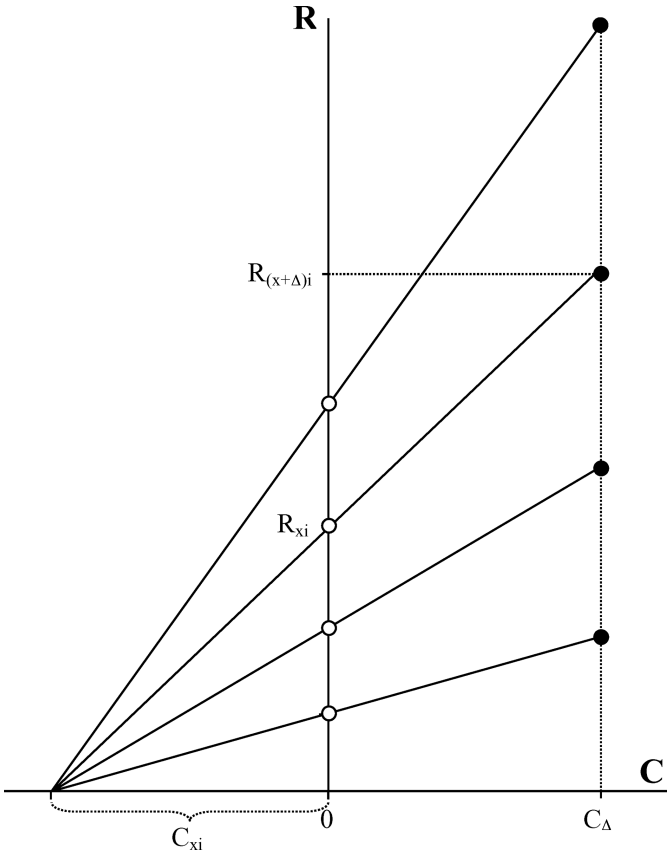

FIG. 3. Calibration by the standard addition method performed according to the SDT strategy: Several two-point calibration graphs are constructed at different values of an instrumental parameter (e.g., volume of the injection loop) by measurements of signals for a sample, R_{xi}, and for the sample with addition, $R_{(x+\Delta)i}$, allowing the analytical result, C_x, to be estimated by several apparent concentrations, C_{xi}.

calibration graphs and calculating eight apparent concentrations for evaluation of the analytical result.

CALIBRATION PROCEDURES OF EXTENDED APPLICATION

Aforementioned calibration procedures have been developed for basic application of SAM: namely to reconstruct properly (i.e., according to Condition A) the calibration dependence, and consequently to compensate this effect completely. However, in flow analysis the SAM procedures have been also developed that allow one to achieve not only this basic goal of the SAM calibration but also some additional aims being very important from analytical point-of-view. Such approaches are specified just below.

Multi-Graph Direct Transformation (MDT) Procedures

The MDT calibration strategy can be perform only when the gradient technique is used, i.e., when the response of an analytical instrument is continuously processed for a solution of progressively changed concentrations of an analyte (1). The total response is then treated as a set of individual signals (measurement points) corresponding to the local analyte concentrations in the solution examined. As already shown (15, 26, 29) such a technique can be realised with the use of both continuous and injection flow systems.

The main idea of the MDT approach related to the SAM calibration is well represented by the gradient ratio-standard addition method developed in our laboratory (32). In this method two calibration solutions, a sample and the sample with standard addition, introduced to the FIA system one after the other are injected to the carrier (diluent) solution from the same loop. Two peaks recorded have to be synchronised with each other with respect to their base-widths. Each pair of transient signals of these peaks, $R_x(t_i)$ and $R_{x+\Delta}(t_i)$ respectively, measured at a defined delay time t_i are believed to represent both solutions being diluted to the same extent (i.e., containing an interferent at the same concentration level). Thus, two sets of measurement data stored from both peaks (e.g., along their falling parts) at different times t_i can be used for construction of many two-point calibration graphs, as shown in Figure 4. Each of graphs, when extrapolated, leads to the apparent concentration calculated from:

$$C_x(t_i) = \frac{R_x(t_i)}{R_{x+\Delta}(t_i) - R_x(t_i)} \cdot C_\Delta \qquad [3]$$

where C_Δ is the analyte concentration in the standard solution.

At the interpretative stage of the calibration procedure the apparent concentrations are presented versus dilution factor, k, then fitted by a function and extrapolated to the value corresponding to the infinite dilution ($k = 0$) of the sample. This value is assumed as the measure of the analytical result, C_x:

$$C_x = \lim_{k \to 0} C_x(t_i) \qquad [4]$$

Considering the shape of the $C_x(t_i)$ vs. k dependence, the following conclusions can be drawn: if it is constant, the analytical system assayed is supposed to be free of interferences, but if it is increasing or decreasing (linearly or nonlinearly), the interference effect is assumed to occur and to be more and more compensated with the sample dilution. Thus, the calibration procedure allows one not only to obtain the analytical result being not biased by interferences (what is the main goal of the SAM calibration) but also *to detect interferences* and *to examine them* in terms of their changes with decreasing concentration of the interferent and with keeping the ratio of the interferent to analyte concentration constant.

In order to avoid preparation of the sample plus standard solution beyond the FIA system, the injection valve with two loops of equal volumes installed can be used for calibration according to the MDT strategy (33). In this case it is proposed to inject two solutions simultaneously from both loops, to merge and mix the zones and to direct them toward the detector. Starting with calibration, at first a diluent and a sample and then a standard and

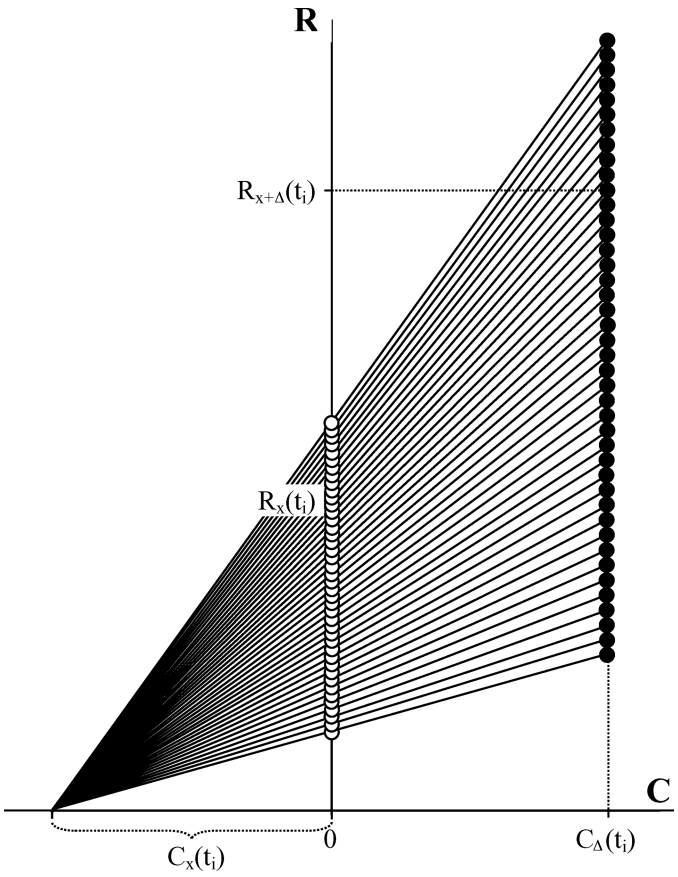

FIG. 4. Calibration by the standard addition method performed according to the MDT strategy: Many two-point calibration graphs are constructed at different values of delay time, t_i, by mesurements of signals for a sample, $R_x(t_i)$, and for the sample with addition, $R_{x+\Delta}(t_i)$, allowing the analytical result, C_x, to be estimated by several apparent concentrations, $C_x(t_i)$.

the sample are injected from the loops. The further procedure is realized similarly to this described above, except that the linear function $C_x(t_i)$ vs. k is taken for consideration only for calculation of the analytical result and examination of interferences.

A specific two-pump CFA system described previously (13, 15) has been also adapted to the SAM calibration in accordance with the MDT strategy (15). The pumps are operated by such means that the solution reaching continuously the detector (FAAS) is the mixture of two solutions of linearly decreasing and increasing amount with time. The calibration procedure encompasses initial preparation of two standard solutions and mixing them one after the other with the sample solution. In such a case the sample is successively diluted with both standards, but not with diluent and with standard as described above. However, the measurement data obtained can be still interpreted in a way presented in Figure 4, provided that each of two-point calibration graphs is based on signals corresponding to the sample diluted to the same extent with the first and second standard solution. It is worth to notice that such a procedure allows one to detect and examine the interferences caused by decreasing concentration of interferents in the presence of an analyte of increasing concentration.

In another CFA system coupled with FAAS as the detector the calibration solution is proposed to be diluted exponentially with a diluent in a dilution flask originally designed (34). In contrast to above MDT approaches, the procedure (named by authors the generalized dilution method) comprises a continuous dilution of three and not two solutions: a sample, the sample with the standard added and the standard solution alone. The absorbance/time profile obtained for the sample is then compared point by point not only with the profile recorded for the sample with the standard addition by also with signals produced by the standard solution. By doing so, two sets of apparent concentrations are able to be calculated in the ways characteristic for SAM (i.e., according to Eq. (3)) and for SSM (i.e., according to the common interpolative formula). As revealed, such an approach is especially effective in terms of examination of the interferences, particularly allowing one to estimate the possibility of not only compensating but also eliminating them with the sample dilution.

Integrated Calibration Method (ICM)

In batch analysis it is occasionally made to compare the calibration graph constructed for the SAM calibration with the calibration graph created for the SSM calibration, i.e., on the basis of the standard solutions only. If both graphs are presented in the same coordinate system, i.e., as shown in Figure 5, the difference between their slopes can be a measure of the multiplicative interference effect occurring in a given case. However, in spite of simplicity such a way leading *to detection and estimation of interferences* is surprisingly not commonly used in routine analysis.

The efforts to develop this approach in flow analysis are constantly made in our laboratory (10, 18, 35–37). As the general idea is to perform calibration according to procedures integrating the SAM and SSM methods, the strategy is called the integrated calibration method (ICM).

In Ref. (18) the simple way is shown how the preparation of the calibration solutions serving in construction of the SAM and SSM two-point calibration graphs can be easily improved with the use of a flow system with the FRV operated as the directive valve. Furthermore, it has been also proved that if the FRV is used in the directive mode adapted to generation of a set of standard solutions of different analyte concentrations (11), two calibration graphs based not on two only but on several measurement points are able to be constructed for the ICM purpose (10). In such a case two reliable analytical results obtained by both SAM and SSM are possible to be calculating hence the difference between their values can be better used for detection end estimation of interferences.

In the further approaches to the ICM method the FIA systems have been designed working on the basis of the merging zones technique (35–37). The first of them is characterized by that the

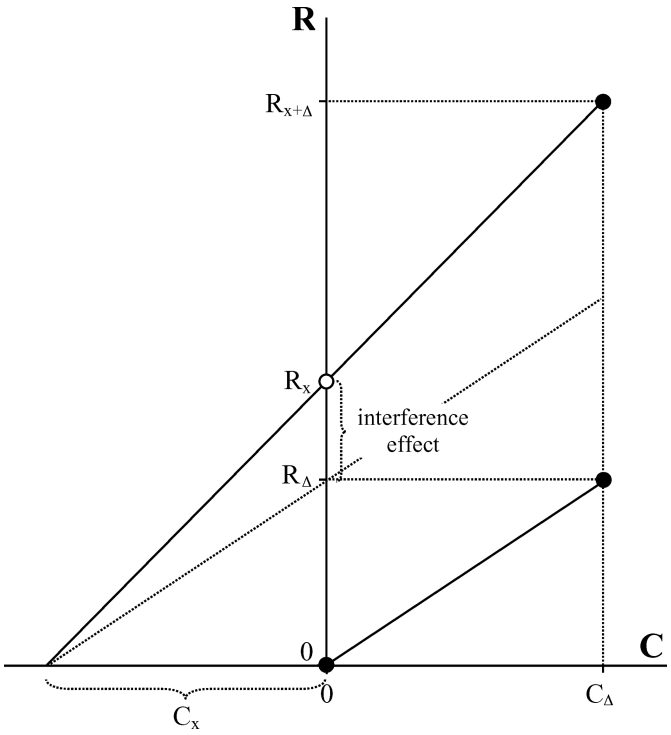

FIG. 5. Calibration by the standard addition method performed according to the ICM strategy: In a single calibration procedure two calibration graphs are constructed by measurements of signals: 0, R_Δ, R_x, and $R_{x+\Delta}$, for solutions of: a diluent, a standard with the analyte in concentration C_Δ, a sample, and the sample with standard addition C_Δ, respectively; consequently, not only the analytical result, C_x, is able to be found, but the interference effect can be detected (compare Figure 1) and examined.

injection loop is divided into two sections of different length which share a common outlet (35). When the loop is initially filled by a diluent and then the sample and standard solutions are introduced to the longer and shorter section, respectively, they have a chance to be merged in the form of two zones partly overlapped and directed in such a form to the detector. If each individual zone is long enough, the double zone consists of three parts of well defined compositions corresponding successively to: the standard diluted with the diluent, the standard diluted with the sample (i.e., the sample with standard addition) and the sample diluted with the diluent. It is revealed that the system, equipped additionally with the FRV as the generator of the standard solutions, allows one to construct two SAM calibration graphs and one SSM calibration graph, all based of several measurement points. Consequently, three apparent analyte concentrations can be calculated as a measure of the final analytical result.

In two other FIA systems dedicated to the ICM calibration the sample and standard solutions are simultaneously injected into the diluent stream from two loops, then both zones are directed to two tubes of different lengths, merged with each other and propelled to the detector (36, 37). In this case a double three-section zone of the structure described above is also able to be constructed. However, in contrast to the previous approach (35), both solutions are proposed to be propelled with two different flow rates and to be merged in two different ratios in each of two positions of the injection valve. Hence, the sample and the standard are diluted with the diluent and with each other in two different ratios creating two three-section zones of different analyte concentration in each their individual section. Owing to this fact the measurement data stored make possible to construct as many as two SAM graphs and two SSM graphs and to evaluate the analytical result by four independent concentration values.

Although both above FIA systems are generally similar to each other, the latter one (37)—owing to some instrumental improvements (e.g., to optimized configuration of the injection valve)—is characterized by some special properties. For instance, it can be closed in this sense that after every injection the standard solution can be directed back to its reservoir. When the valve's position is then changed cyclically, the standard is able to be gradually diluted with the carrier and introduced to the system with the analyte concentration successively changed. As a result, the set of standards is generated. Another useful feature of the system is that the sample and standard solutions in two three-section zones produced during each individual cycle of the calibration procedure are diluted complementarily to each other. Due to this the exact values of the flow rates of the calibration solutions are exceptionally not needed to be known provided that the rates are kept constant.

However, the most important advantage of the systems allowing one to obtain four apparent concentrations for ICM calibration is that *the determinations performed can be verified and controlled in terms of accuracy*. Namely, if all four values are statistically equal to each other, the probability that their average value is an accurate measure of the analytical result is especially great. This is because the apparent concentrations are obtained in different calibration ways (i.e., by SAM and SSM approaches) and in different dilution conditions (i.e., for the sample and standard solutions differently diluted). The scheme how to proceed in this respect when four results are different from each other is also suggested (37). Furthermore, it has been revealed that the cumulative accuracy of the analytical results obtained by four-graph ICM method in a series of analyses tends to be better then cumulative accuracy of the results obtained by either SAM or SSM individually applied (37). The conclusion is that the ICM *can contribute to improvement of analytical accuracy as well*.

CALIBRATION PROCEDURES OF LIMITED APPLICATION

In the literature related to flow analysis the standard addition procedures can be found that are limited in terms of overcoming of interferences. As seen next, most of them do not fulfill Condition A, i.e., although a sample is really dozed by standards, the concentration of interferents in the sample becomes different than this one in the sample with additions. In such cases

the interference effects have a chance to be compensated only if they are great enough to be independent of the concentration of interferents.

The most known of such procedures has been developed by Tyson under the name the interpolative standard addition method (ISAM) (38). The method is based on the reverse flow technique. Namely, it is suggested to prepare initially a set of standard solutions and to inject them one after the other to the sample stream flowing continuously through the FIA system. Consequently, the positive and negative peaks are produced and the differences between their maximum or minimum points, respectively, and the steady state signal measured for the sample are calculated. The results are plotted *versus* the analyte concentration in standards (see Figure 6) and the intercept on the concentration axis is assumed as an estimator of the analyte concentration in the sample.

Due to great simplicity and to similarity to the conventional flow procedure of the SAM calibration the ISAM has found relatively wide analytical application, being realized with the use of not only FIA systems (39–44) but also of the SIA system (45). Furthermore, a chance to use the SAM calibration avoiding extrapolative way for calculation of the analytical result is especially attractive. The point is that—what is well known—the extrapolation process contributes to the worsening of the analytical result in terms of precision. Besides, if the calibration dependence is of different shape in the extrapolation part than in the part reconstructed experimentally, the analytical result found in the extrapolative way can be seriously biased by systematic error (46).

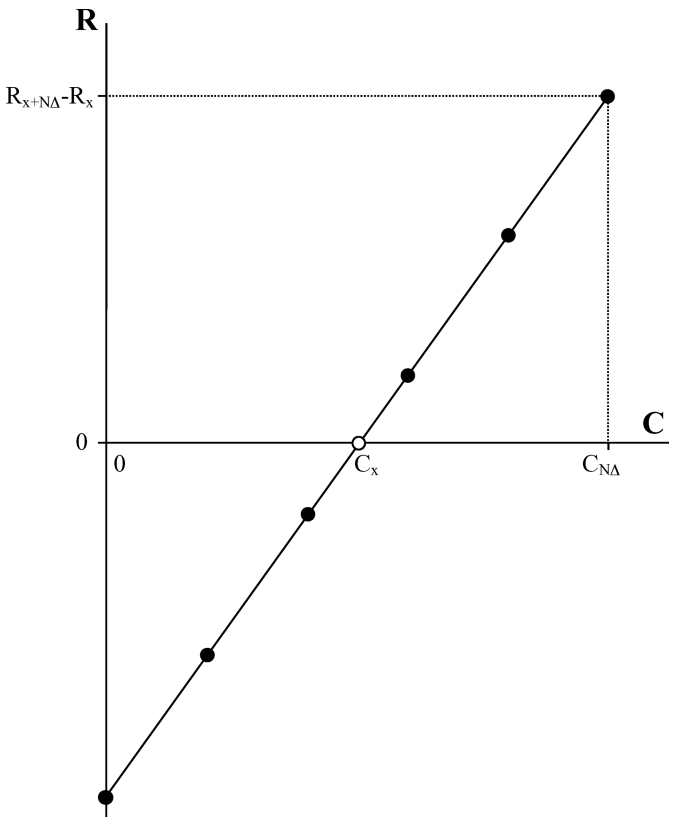

FIG. 6. Calibration by the interpolative standard addition method: Calibration graph based on several experimental points and constructed by measurements of the signal differences (ΔR) serves for transformation of the zero-signal difference to the analyte concentration in the sample (C_x).

Unfortunately, the ISAM calibration procedure is characterized by a week point: the standard solutions injected to the sample stream cause dilution of the sample. Thus, if interferents are present in the sample, their concentration in the sample stream and in these parts of the standard zones, which correspond to maximum and minimum points of the peaks, is different. It has been theoretically and experimentally proved that for this reason the ISAM calibration does not in principle differ from the conventional interpolative calibration (i.e., SSM), requiring additional approaches (e.g., matching a sample in standards) to compensate the interference effect (47). The conclusion is that ISAM—although being interpolative method indeed—can not be considered as SAM in terms of overcoming the interferences.

A modification of original ISAM procedure has been proposed by Israel et al. (48) in the form of so called the sample-to-standard additions method. Namely, it is proposed to use the standard solution (instead of the sample solution) in the role of the carrier stream and to inject two solutions into such a carrier: first a sample and then the blank solution containing all components of the sample matrix. Then, it is suggested to calculate the analytical result in the interpolative way. Certainly it possible of doing so as the maximum concentration of interferents in both zones is the same indeed. However, the problem is that *the interference effect can be compensated only if both kind and concentration of the interferences are known*. Although this condition seems to be very restrictive, it has been revealed by López-Garcia et al. (49) that above calibration approach can be successfully applied when using slurries in FAAS.

Another attempt to use the SAM calibration in the interpolative version has been made with the aid of the FIA system allowing a sequence of three different solutions to be simultaneously injected (50). For the calibration purposes the zones in the sample-standard-sample sequence are proposed to be successively produced with the standard solutions of different analyte concentrations. Each three-section segment is carefully mixed before the detector hence the peaks corresponding to the sample with additions of different analyte concentrations (including zero concentration) are recorded. The calibration graph is constructed on the basis of differences between the heights of above peaks and the height of the peak created by the zones injected in the sample-sample-sample sequence. Since the intercept on the concentration axis is assumed as an estimator of the analytical result, the objection to such an approach is the same as to the Tyson's method (38): maximum concentration of interferents in each segment sample-standard-sample is evidently different than this one in the segment sample-sample-sample.

Two very similar FIA systems dedicated to the interpolative calibration by SAM have been designed by Marshall et al. (51) and by Haghighi et al. (52). In both cases a set of standard solutions is suggested to be successively injected not to the sample stream but to the carrier stream and then merged and mixed with the sample solution flowing continuously. By doing so only positive peaks of maximum points corresponding to the sample with different analyte additions are recorded on the steady-state sample signal. Besides, the sample is injected to the carrier stream and merged with the other stream of the carrier solution. However, in both papers the measurement data obtained are proposed to be interpreted differently: the peak height measured for the sample is related to the calibration graph, which is constructed when the peak heights of the standards are measured either from the steady-state sample signal (51) or from the baseline (52). Although in both cases the analytical result is able to be calculated in the interpolative way, maximum concentration of interferents in the sample zone injected to the carrier stream is different that their concentration in the central part of the standard zone injected to the sample stream.

Apart from those approaches, which are the attempts to make SAM the interpolative method, the extrapolative procedures are also met of limited application in terms of the interference compensation. Such an approach, distinguishing form among others by original interpretation of measurement data, has been developed by Elsholz et al. (53). In the SIA system the calibration solutions are prepared in such a way, that the signals for undiluted sample and for the sample diluted with two standard solutions of different analyte concentrations can be produced. Basing on the signals the calibration graph is constructed but it is taken into account that the analytical result calculated extrapolatively from this graph is the apparent only but not true concentration of the analyte in the sample. The obvious reason is that the calibration solutions contain interferents in different concentrations. In order to obtain the accurate result from the same experimental data the authors recommend applying a mathematical approach: in a few calculation cycles the analyte concentrations added to the sample are successively corrected on the basis of the apparent concentration value obtained one step earlier. The iteration procedure is ended when the difference between two succeeding apparent concentrations ale less that a chosen value.

The same problem with different interferent concentration in the calibration solutions is related to the approach developed by Assali et al. (54). In this case the segments limited of both sides by small zones of air and containing small portions of the sample and standard solutions are created in a loop attached to the solenoid valve. The time interval in which the loop is filled before each injection is increased in order to prepare segments with increasing volumes of the standard and still the same volume of the sample. The calibration graph is constructed from the peaks produced by each segment prepared in such a way. The analyte concentrations added to the sample are calculated from the dispersion coefficients determined separately.

Another questionable approach to the SAM calibration has been realized with the use of a CFA system coupled with FAAS (55). The standard solution is pumped at a linearly increasing flow rate while the other solution—first a diluent and then the sample solution—is aspirated through a T-piece. As a result two absorbance/time profiles are produced. At the interpretative stage of the calibration procedure both profiles are not proposed to be compared point by point but in terms of their slopes. Anyway, *if the signals obtained for the sample with standard addition are related to the signals for the pure standard (but not to the sample), there is evidently no chance to compensate the interference effect*. Realizing that the authors suggest to eliminate interferences chemically (55).

DISCUSSION AND CONCLUSIONS

The calibration procedures reviewed above show that the calibration by SAM is one of analytical domain, which is especially susceptible to improvements by means of flow analysis. The main reason is that the flow analysis in comparison with batch analysis offers much more possibilities of merging two solutions with each other. As presented, the calibration solutions are able to be prepared e.g., by injection the zone to the stream or by merging of two streams, two zones or the stream with the zone. Two zones can be merged flowing by two tubes and then meeting with each other in the connection point or flowing one after the other by a single tube and then being mutually penetrated. Two streams flowing continuously can be propelled by one or more pumps but one of streams can be aspirated as well. In addition, some specific techniques (like the sampling technique), dedicated modules (e.g., FRV, circulation loop) and manipulation instrumental parameters (volume of the injection loop, flow rate, length of tubings) are at disposal to differentiate the analyte concentrations in standards added to a sample.

Unfortunately, so great offer of manners possible to be used at the preparation stage of the SAM calibration procedure can be paradoxically dangerous. The problem is that the manner can not be chosen optionally but—in accordance with Condition A defined above—a sample should be merged with a standard keeping the interferent(s) components in constant concentration. This is necessary if the interference effect occurring in the analytical system examined is expected to be compensated by SAM.

An example of the calibration procedure not fulfilling Condition A is the interpolative standard addition method (38). However, it has been proved that this approach is able to overcome interferences and to provide accurate analytical results when the calibration graph is constructed from absolute values of the signals (peaks) recorded for standards only (including the negative peak obtained for a diluent) but avoiding the steady-state signal measured for the sample (47). The point is that such a graph is typical for SAM, i.e., it is able to reconstruct the calibration dependence in a part only and it needs to be extrapolated in order to find the analytical result.

Thus, the next prerequisite (Condition B) for SAM can be formulated: *if a given calibration procedure encompassing addition*

the standards to a sample is expected to be the SAM procedure (i.e., to overcome interferences), the analytical result has to be calculated in the extrapolative way. In other words: the extrapolation process is inherent attribute of the calibration by SAM.

In order to avoid misunderstandings connected with calibration procedures carried out in flow analysis, the new calibration nomenclature has been recently proposed (10). Namely, it is suggested to call the SAM as the extrapolative method in contrast to the interpolative method (commonly named the calibration curve method or the set of standards method) and to the indicative method (comprising the titration procedures). Such a classification seems to be also more essential as being related to the interpretative but not to the preparative characters of the calibration approaches. It is worth to notice that the procedures reviewed here are subjects to this classification, i.e., (a) all of them of basic and extended application are the extrapolative ones, (b) the interpolative procedures are of limited application, (c) the extrapolative procedures of limited application require some additional treatments (mathematical (53) or chemical (55)).

As far as the extrapolative calibration procedures are considered (Table 1), it is seen that they are typified by different number of both the standards added to a sample and the measurement data possible to be obtained. These features are independent of the strategy represented by particular procedures. However, the common thing is that all of them are considerably improved in terms of preparation of the calibration solutions. Even if the series of the standard solutions are needed to be initially prepared, they are able to be merged with the sample inside of the flow system. In some exceptional cases the sample has to be dozed by the standard beyond the flow system, but only a single calibration solution is proposed to be prepared by this means. The approach especially often recommended in flow analysis is to prepare a single standard solution and to merge it automatically with the sample in a way allowing several calibration solutions of different analyte concentration to be generated and several measurement data to be obtained.

TABLE 1
Characterization of extrapolative calibration procedures at preparation and measurement stages

No. of Standards	Manner of standard addition	No. of measurement points	Strategy	Reference
Several	On-line	Several	ODT	12
1	Off-line	Several	SDT	31
1	Off-line	Many	MDT	32, 34
3	On-line	4	ODT	13
2	On-line	Many	MDT	15
1	On-line	3	OIT	24, 27, 28
1	On-line	4	ICM	18
1	On-line	6	ICM	35
1	On-line	8	ICM	36, 37
			ODT	16, 17, 18, 20, 21
1	On-line	Several	OIT	22, 23, 26, 29, 30
			ICM	37
1	On-line	Many	ODT	15
			MDT	33

Considering each particular group of procedures in terms of analytical performance different factors influencing both precision and accuracy are seen. If the measurement data are not transformed directly (i.e., when the procedures belonging to the OIT strategy are applied), the additional laboratory activities required to estimate the dispersion coefficient certainly make the analytical results worse in terms accuracy and precision. The reliability of the MDT procedures is influenced by the requirements to adjust properly the continuous profiles obtained for the sample and standard solutions and to use very small signals for calculation of the analytical result. On the other hand, the most confident in this respect are the ICM procedures, which allow the analytical result to be calculated directly from the calibration graph and, in addition, to be verified in terms of accuracy.

Some calibration procedures listed above are characterized by own specific sources of both random and systematic errors. These are the approaches being too instrumentally or procedurally sophisticated, e.g., requiring of two injections to be strictly synchronized with each other for the purpose of sampling a zone or expecting of the transient signals to be exactly recorded at defined delay times. Additional problem is that such procedures can in fact be difficult to reproduce to satisfactory extent in a given laboratory conditions. It deals also with all those numerous calibration systems that are dedicated to be coupled with FAAS as the detector: in most cases they are adopted to other FAAS instruments with difficulties and to other detectors—with almost no any chance.

Another general problem is with usefulness of the particular calibration approaches when the calibration dependence is non-linear. In such case only such procedures can be applied which allow this dependence to be reconstructed in the form of several- or multi-point calibration graphs. Thus, the procedures considered are those representing the ODT and OIT strategies but not the SDT and ICM strategies (except the ICM calibration approach realized with the aid of the closed flow system (37)). In the case of the MDT procedures each calibration graph is based on two points only but the non-linearity of the calibration dependence can be taken into account at interpretative stage due to great number of the measurement data collected (32).

Special opportunities to improve the analytical performance are offered by the procedures allowing the calibration dependence to be reconstructed in the form of several or many

calibration graphs, i.e., the SDT and MDT procedures. The point is that the family of graphs of different slopes gives a chance to transform the measurement data from much wider range of the calibration dependence than a single graph. It is also worth to notice that in such particular cases, when the peaks of different heights are obtained for a single standard solution in different experimental conditions, it is not necessary to calculate the corresponding local analyte concentrations with the use of D-values (i.e., to use the OIT strategy) but it is simpler to attribute different signals to the analyte concentration in the standard and to construct several two-point calibration graphs (i.e., to use the SDT strategy).

However, the common case in FIA is to produce a set of different signals for local analyte concentrations which are actually unknown. The auxiliary factor being then most often used in such cases is the dispersion coefficient, D. It is found as the ratio of two signals obtained during the supplementary experiment for a test standard solution: a steady state signal generated by the solution propelled continuously through a FIA system and the transient peaks produced by the solution injected and dispersed in given instrumental conditions. A local analyte concentration in every zone dispersed is then calculated taking into account the corresponding signal and the value of the dispersion coefficient.

As already mentioned, the extrapolative calibration is most often carried out in flow analysis in the manner imitating the batch calibration procedure, i.e., using the set of calibration solutions prepared in the separate measurement flasks. Certainly, the main reason for this situation is simply psychological: If a common procedure is used routinely for a long time it is very difficult to change the habit, even if another attainable way is believed to be better in some respect. Therefore, it can be suspected that from among novel calibration approaches those ones have the greatest chance to be introduced to routine analysis, which are similar to traditional one as much as possible at both the preparative and interpretative stages. Considering this aspect, the procedures allowing a single calibration curve to be constructed and especially those additionally providing a chance to transform the measurement data directly (i.e., those belonging to the ODT strategy) certainly have advantage over the remaining procedures.

On the other hand, it seems to be very important to popularize especially those extrapolative approaches, which are perhaps more sophisticated but are additionally able to solve some essential analytical problems (to examine the interferences, to verify accuracy of the analytical results etc.), i.e., the procedures belonging to the MDT and ICM strategies. Certainly, the scientific examinations and publication of the selected results obtained are not sufficient to do so. The necessary way is to construct dedicated flow calibration manifolds in the form of prototypes, to develop the adequate applications and to try to introduce all of them into analytical practice. It is a great challenge for the analysts involved in flow analysis. Such a line of conduct has been just adapted in our laboratory with respect to the calibration procedures representing the ICM strategy (56).

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