Short communication on calibration graphs in analytical chemistry: How they work

[1]Gerard Buckhale

[1]Independent Researcher; Canada, Ottawa, e-mail: buckhale.ryr9z@ncf.ca

Abstract. Calibration graphs are a very familiar device routinely used by analytical chemists in their daily work. It may seem silly to ask how they work. This short communication will demonstrate that they are a lot more complex than they appear to be on the surface. Every aspect of the calibration graph is explored in detail.
Key Words: analytical chemistry; chemical analysis; chemical instrumentation; calibration graphs.

Introduction. A calibration graph is a device used between the chemistry side and the instrumental side of a chemical analysis. In a way, it is like the interpreter/translator between two dignitaries who don't speak the same language. On the chemistry side, one has the µg-amount or concentration (µg/ml) of samples and standards. On the instrumental side, one has the response (ultimately electrical) to the µg-amount or concentration (µg/ml) of these samples and standards that are in some way "input" into the instrument. The design of the instrument is always based on some chemical or physical principle of atoms, ions, or molecules that can be exploited in such a way that a particular kind of "output" (usually linear) can be expected from the µg-amount or concentration (µg/ml) of analyte material that was "input" (Skoog & West 1971). In this short communication only calibration graphs based on concentrations of standard solutions (in µg/ml) will be dealt with. A calibration graph based on µg-amount is similar. See Figure 1 at the end of the article for an example of a calibration graph. (µg/ml) is also known as PPM (parts per million).

Discussion. The calibration graph consists of two variables which will be denoted "x" and "y" in the usual 2-coordinate (X0Y) system. The concentration variable (in µg/ml) will be assigned to the "x-axis." The instrument response variable will be assigned to the "y-axis" and specified only as IRV (instrument response variable). The dimensional units, such as AU (absorbance units), XAU (expanded absorbance units), or AREA (area under a peak), for the instrument response will not be given. Only "linear" calibration lines that pass through the origin ("x" = "0", "y" = "0") of the calibration graph will be dealt with. Such linear calibration graphs are usually based on AU, XAU, or AREA. A calibration line in terms of PERCENT TRANSMITTANCE (%T), must pass through the 100 percent point on the (logarithmic) "y-axis" and be linear when plotted versus concentration on the (regular) "x-axis" on semi-logarithmic graph paper. Otherwise, it is recommended to convert the TRANSMITTANCE (T) to ABSORBANCE (A) using the relation ($A = - \log_{10} T$) so as to give a linear plot on regular graph paper (Day & Underwood 1967). There are also "non-linear" calibration graphs, both where a known mathematical function describes the relationship between the two variables and where the mathematical function for the relationship is unknown. These non-linear calibration graphs will not be taken up here.

There are two phases and four dependencies between the two variables of the linear calibration graph and two directions of use. In addition, the "y-axis" variable can either be a non-random variable or a random variable. The "x-axis" variable, while initially a non-random variable, can become a random variable. These characteristics are summarized below in tabular form and will be discussed further, later on. The table will be referred to as the "PHASE 1/PHASE 2 Table."

PHASE 1 (making the calibration graph):

Direction of use: "x" (input) to "y" (output)

"x" is a known concentration, non-random variable.
"y" can be a non-random or random ("y given x") variable.

	Dependency type	Dependency relation
(1)	cause and effect	"y" on "x"
(2)	logical	"y" on "x"
(3)	algebraic	"y" on "x"
(4)	stochastic	"y" on "x"

PHASE 2 (using the calibration graph):

Direction of use: "y" (input) to "x" (output)

"y" will (likely) be an "expanded" composite random variable, containing the random variable ("y given x"), since "y" will have passed through all stages of the analytical chemistry method.
"x" will be a random variable, if, and only if, "y" is a random variable but "x" will never be stochastically dependent on "y".

	Dependency type	Dependency relation
(1)	cause and effect	"y" on "x"
(2)	logical	"x" on "y"
(3)	algebraic	"x" on "y"
(4)	stochastic	"y" on "x"

Let us be concerned, first of all, with PHASE 1 (making or preparing the calibration graph). The chemist or technologist will, first of all, prepare the standard solutions with which to calibrate the instrument usually from an intermediate more concentrated solution of pure primary standard chemical. Nothing more, usually, other than ultra high purity acid, is added to the standards and if this or any other reagents are added to the standards, a "zero-standard" (also called a standard's blank) must be run also. Then, if there is any non-zero reading obtained for the "zero-standard," that reading must be subtracted from the readings for all the other standards. These standard solutions or the equivalent μg-amounts of primary standard chemical used to prepare them, are definitely not to be run through the entire analytical chemistry procedure before making the standard solutions up to volume.

The standards, having been prepared, are "input" into the instrument, and readings ("output") are obtained for them. This is usually done after the regular samples have been run through the analytical chemistry method, their respective extracts made up to volume, and instrument readings obtained for them.   But before any of the concentrations of the regular sample extracts can be determined, the calibration graph must be prepared.

The calibration graph will be prepared, first of all, by entering onto the "x-axis", the known concentrations of three (three, for the purpose of this short communication) standards, not including any zero-standard or standard's blank. Each standard is a concentration constant: each micro-drop is exactly the same concentration as any other micro-drop.   Although there could be a small amount of volumetric error in the volumetric flasks used to contain the standards, it is usually in the order of 0.1 to 0.2 percent and can therefore be ignored (in any case, this volumetric error can be shown to be inherited (Buckhale 2010) in the overall "within-run" variation of the slope).   The instrument is aligned, then a "reading" (IRV) is obtained for each of the three standard

solutions. Each reading is a "y-value" for each of the three standards. The "x" and "y" coordinate points for each standard are then plotted on standard graph paper. After aligning up the points with a straight edge, such as an ordinary ruler, a straight line going through the origin can be drawn best-fitting the calibration points in terms of their nearness to the line. This calibration line, then, is the line that represents the functional relationship between the instrument and the concentration of analyte. This is how calibration graphs were prepared "by eye" for many, many years.

In order to justify the above procedure for preparing the calibration graph, one must know for certain that "y" is varying, in some kind of predictable and quantifiable way, with "x". This is where Beer's law comes in. Beer's law states that the "electrical output" of certain instruments will be linear with respect to concentration (input) "and" pass through the origin ("x" = "0", "y" = "0") of the calibration graph. The word "and" is being emphasized here because sometimes people will use a regression line based on the wrong mathematical model for the graph. The mathematical model, based on Beer's law, where "m" is the slope and "b" is the "y-intercept," should not be "y = mx + b" but "y = mx + 0". The former will usually not pass through the origin of the graph due to the scatter in the points plotted for each of the calibration standards. The origin ("x" = "0", "y" = "0") is a "known point" on the population regression line because Beer's law is being followed (and also due to the design of the instrument) and the two variables are "jointly converging to a mathematical limit of zero." This is why the mathematical model should be "y = mx + 0" and not "y = mx + b".

A few things to note about PHASE 1 are that:

(1) The graph is being entered first on the "x-axis" (direction of use).
(2) "x" is a known concentration and a non-random variable.
(3) "x" is causing "y", the instrument is responding to known concentrations of the ingredient being analysed for (analyte), as it was designed to do.
(4) The logical dependency is in the same order as (3), "x" gives "y" because "x" causes "y".
(5) "x" is the algebraically "independent variable" with "y" dependent on it.
(6) "y" may be a random variable, and if so, it is stochastically dependent on "x".

But the concentrations of the regular sample and subsample extracts cannot yet be obtained from the calibration graph using the readings obtained for them on the instrument (PHASE 2). It must first be ascertained whether or not one can "read" an "unknown pure solution" of pure primary standard chemical, or "unknown standard" as it will be called, and obtain the concentration for it. In order to accomplish this, "x" must somehow become "logically dependent" on "y", that is, "y" must give "x" (the reverse of (4) above). This must also be true for the regular sample and subsample extracts if one is going to be able to use the graph to obtain their concentrations. In preparing the graph (PHASE 1), "x" gave "y" because "x" caused "y". In using the graph (PHASE 2), "y" must somehow give "x". The basic question that has to be answered is this: if "x" causes "y", can "y" somehow give "x"? If all of the four conditions listed below are satisfied, the answer is YES.

Suppose, for the moment, that "y" is a "non-random" variable and that the "unknown standard" has been "input" into the instrument and a "reading" ("y-value") has been obtained for it. The following logical premises are then applied in the form of questions:

(1) Did "x" cause "y"?
(2) Is "x" the only thing that could have caused "y"?
(3) Did anything prevent "x" from causing "y" or change the instrument response (IRV)?
(4) Is "x" causing "y" in some mathematically predictable and quantifiable manner?
(Basically, (1) to (4) mean: Did a particular "x-value" cause a particular "y-value"? If so, the particular "y-value" would "indicate" that a particular "x-value" caused it.)

If the answers to (1), (2) and (4) are YES and the answer to (3) is NO, it is established that under these conditions, "y" gives "x" and it can be concluded that the reading ("y-value") obtained for the "unknown standard" after being divided by the slope of the calibration line is the concentration ("x-value" in µg/ml) of it (PHASE 2). Remember, there was no random variation involved and the calibration line is linear. Alternatively, the calibration graph can simply be "read by eye", entering the graph with the "y-value" (IRV) for the "unknown standard" and obtaining the reading (in µg/ml) from the "x-axis" for it. Under these conditions then, the "logical dependency" seems reversible. Notice that the "algebraic dependency" has also reversed because the "direction of use" has reversed.

The above is an essential first step in developing an analytical chemistry method but what about questions (2), (3) and (4) as regards regular samples? (Note: the answer to question (1) has already been proved: "x" causes "y".) These three questions are constantly on the minds of method developers, analytical chemists, and technologists. They must develop and use the analytical chemistry method in such a way that the answers to these three questions are YES, NO, YES, and this must hold also for the regular samples and subsample extracts. This is not accomplished as easily as it sounds. Various chemicals (reagents) must usually be added to the regular samples and a reagent blank run, and/or several steps of extraction or other forms of physical or chemical separation must be employed to isolate the ingredient being analysed for (analyte), away from the atoms, ions and molecules of the material sample substrate originally containing it. And, in addition to this, stochastic variation is usually picked up at every stage of the analytical procedure along the way, with the instrument reading step being the final stage. But before PHASE 2 can be commissioned for use on regular samples, the effects of possible random variation in the variable "y" must also be considered.

Let us examine the effects of having some random variation in the "output" of the instrument itself when only a known or unknown "standard" is being "input." A "standard" is, of course, a concentration constant (not a random variable), every micro-drop being exactly the same concentration as any other micro-drop, and because of the "purity" of it, one doesn't need to worry about questions (2), (3) and (4). But the random variable "y" will need to be distinguished from the non-random variable "y". This will be done by temporarily changing the name of the random variable "y" to "y given x" (Guenther 1973). The random variable "y", or rather "y given x", as it is now being called, is at a particular measurement level (as determined by its population mean). It's just that now the random variable "y given x" contains some random variation. In contrast to the definition of "algebraic dependency" of "y" to "x", where only one "y-value" is allowed for every single "x-value," the random variable "y given x" can take on more than one "y-value" for every single "x-value." But if it is further decreed that only one population mean of "y given x" be allowed for every single "x-value," then the definition of "stochastic dependency" follows. In regression analysis it is assumed that all population means of "y given x" lie on a straight line called the population regression line (Guenther 1973). The sample regression line which is calculated of the basis of least squares, is an "estimate" or "statistical measurement" of the population regression line in the same sense that the sample standard deviation is an "estimate" or "statistical measurement" of the population standard deviation. It is further assumed that the random variable "y given x" is normally distributed (Guenther 1973). Although regression analysis is not being done here, it is correct to use the assumptions made thus far to determine a sample regression line by using the appropriate regression formula to determine the slope. For now, let it be concluded that, by using the appropriate regression formula to determine the slope and passing a straight line with this slope through the origin, the random variation present in the instrument readings for each of the three calibration standards has successfully been taken into account in determining the calibration line of best fit for the calibration graph.

Now the effects of bringing this and other random variation into PHASE 2 will be investigated. In the previous paragraphs, it was concluded that the reading obtained for the "unknown standard" after having been divided by the slope of the calibration line was

the concentration (in µg/ml) of it, there being no random variation involved. It was concluded that the "logical dependency" was reversible under certain conditions. It was also noted that the "algebraic dependency" had reversed because the "direction of use" had reversed. But the "x" variable was still a non-random variable. The difference now (while in PHASE 2) is that because "y given x" is a random variable, and the direction of use has changed (the graph is input with a "y-value" in order to obtain an "x-value" as output), that "x" must also be a random variable. Statistical theory says that to divide a random variable by a constant, one must divide each outcome of the random variable by that same constant, and the result will be another random variable. But let us not make the mistake of thinking that "x" is somehow "stochastically dependent" on "y" or on "y given x". [PROOF: Each distinct "y-value" will always give the same distinct "x-value."] What is manifest here is that a random variable, "y given x", is being divided by a constant--the slope of the calibration line (this being considered to be a constant for the purpose of doing the calculations) giving us an "x" random variable. This "x" random variable will not be renamed at this point. This kind of thing was not happening when each known standard (a concentration constant) was "input" into the instrument in the original (PHASE 1) "direction of use."   The instrument gave a somewhat variable response "output" for each standard so that there would likely have been more than one "y-value" obtainable for each "x-value."   And so it was concluded that "y" was stochastically dependent on "x" and the "y" variable was renamed to "y given x". But, in the reverse (PHASE 2) "direction of use," there is no time that any more than one "x-value" can be obtained for any single choice of "y-value." Note that the slope of the calibration line here is but one outcome from the "within-run" random variable of the slope. But it is being used in the calculations as though it were a constant. This leads to systematic error being generated "between-runs" (Buckhale 2010). But to simplify the explanations, this matter will not be taken up here.

Now that it can be understood how "x" can become a random variable, one can fully understand PHASE 2 (using the calibration graph). In PHASE 2, "x" becomes a "different" random variable than has just been considered because "y" has become a "different" random variable. "y" has now picked up random variation from every stage of the entire analytical chemistry procedure, including the instrument reading step. [For the purpose of this article, it is assumed there are multiple stages in the analytical chemistry method.] Therefore, "y" is now a bigger random variable than "y given x". To reflect this change, the random variable "y given x" will be further renamed to the overall random variable "Y".   The variances of each of the stages of an analytical chemistry method are "effects-additive."   The overall variance of the analytical chemistry method therefore consists of the "sum" of the variances of each stage, including the final or instrument reading step.   This overall random variable "Y" then contains (by effects-addition) the particular random variable "y given x". [In addition, the population mean of the overall random variable "Y" may now be different from that of the particular random variable "y given x".]   The random variable "x", therefore, will also become a bigger random variable since it has been obtained by dividing the random variable "Y" (and each and every single theoretical outcome of it) by the slope of the calibration line which is being considered to be a constant. To reflect this change, the random variable "x" will now be renamed to the random variable "X".   "Y" is bigger than before, the slope is unchanged, so "X" will be bigger than before also.   The calibration graph will then be entered with a single outcome of "Y" (for each regular sample or subsample extract) in order to obtain a single value of "X" in terms of concentration (µg/ml) for each of the sample or subsample extracts. The particular value of "X" for each "Y" value will then be entered into the numerator of the calculations formula for the particular analytical chemistry method where it will be converted from (µg/ml) to (µg-amount).   Let us summarize the changes that occur in going from PHASE 1 to PHASE 2 in practice:

(1) The "direction of use" has changed.
(2) "Y" has become a bigger random variable than "y given x".
(3) "x" was a non-random variable (PHASE 1). It has now become a random variable (PHASE 2) because "y given x" is a random variable and "Y" is a bigger random

variable. But it has not become stochastically dependent in any sense on "y",
"y given x" or "Y".

(4) Because of (2), "X" has become a bigger random variable than before.

(5) Only the "dependency relations" (2) and (3) of the "PHASE 1/PHASE 2 Table" have
reversed. "X" is now logically and algebraically dependent on "Y".

(6) The mathematical model has changed. The correct mathematical model for PHASE 1
is "y = mx + 0". The correct mathematical model for PHASE 2 is "X = Y/m + 0".

As noted previously, the mathematical model for the calibration graph in PHASE 1 (making the graph), where "m" is the slope and "b" is the "y-intercept," should be "y = mx + 0" not "y = mx + b". The latter would usually not pass through the origin of the graph due only to the scatter in the points plotted for each of the standards. The origin ("x" = "0", "y" = "0") is therefore a "known point" on the population regression line, because of Beer's law being followed (and also due to the design of the instrument) and the "joint mathematical convergence" of the two variables. This is why the mathematical model should be "y = mx + 0" for the graph. This is the case in flame atomic absorption spectroscopy (FAAS) where Beer's law is being followed (linearly) in terms of absorbance (AU and XAU) and also in gas chromatography (GC) where the "equivalent" of Beer's law is being followed (linearly) in terms of AREA ("equivalent" because the design of the instrument is not based on a beam of light). This is not to say that FAAS is not an equilibrium type measurement or that GC is not an integral type measurement, since the linearity of the detector in the gas chromatograph later translates into correctly proportional integrals for the standard solutions being injected. But this matter will not be taken up here at length (Skoog & West 1971). But it should be pointed out that it is not at "high concentration, low variability" that there is a problem in preparing the calibration graph, although at "very high concentration" there can occur a form of non-linearity, called "curvature drop-off" which is due to detector fatigue (electrical saturation of the detector). Some instruments have a built-in non-linear amplifier to do what is called "curvature correction" for the purpose of extending the linearity of the calibration graph at very high concentrations. What needs to be noted here is that the very time that a statistical regression for our calibration line is needed is not at "high concentration, low variability" levels, but at "low concentration, high variability" levels. And this is where a "known point on the population regression line" can really be used. In fact, if one has difficulty "zeroing" the instrument at high expansion (low attenuation) levels, it will be worth it to take the extra trouble (several attempts) to "zero" the instrument while inputting a "zero-standard" (standard's blank) or else to take several readings of the same "zero-standard," the average of which is then subtracted from the readings for each of the other standards. A standard's blank does not lead to the same kind of measurement error as the analytical processing reagent blank since the reading for the standard's blank is only subtracted from the readings for the other standards (not from the readings for the sample or subsample extracts, even though some of the same reagents may have to added to them). Also, the number of reagents required for the standards is usually much less than that required for the regular samples. It should be remembered that the sum total of all the reagents has to be a "microgram constant" for the standard's blank as with the analytical processing blank. Therefore, each reagent has to be dispensed in the exact same proportions to each of the flasks (containing the extracts) in both cases.

And so it can be seen that the correct mathematical model for making the calibration graph (PHASE 1) should be "y = mx + 0" where "m" is the slope of the calibration line and "b", the "y-intercept," is equal to "zero." Also, because the direction of use has changed, the correct mathematical model for using the calibration graph (PHASE 2) is "X = Y/m + 0".

I will reproduce below the three formulas (Armitage 1972) for obtaining "m", the slope of the regression line (calibration line) for "y = mx + 0" depending on how the standard deviation is varying with respect to the concentration levels of the calibration

standards. It is to be understood that "y" in the following formulas is or can be a random variable equivalent to "y given x":

$$(1) \quad "m" = \Sigma_i(x_i y_i) / \Sigma_i(x_i^2)$$

Where the standard deviation (in µg/ml) is constant over the range of concentrations (in µg/ml) afforded by the standards.

$$(2) \quad "m" = (y\text{-bar})/(x\text{-bar}) = \Sigma_i(y_i) / \Sigma_i(x_i)$$

Where the relative variance is constant over the range of concentrations (in µg/ml) afforded by the standards.

$$(3) \quad "m" = \Sigma_i(y_i/x_i) / N$$

Where the relative standard deviation is constant over the range of concentrations (in µg/ml) afforded by the standards.

In these formulas, the summation "$\Sigma_i(x_i)$" is taken over the concentrations (in µg/ml) for each of the standards, not including the origin or zero-standard. The corresponding summation "$\Sigma_i(y_i)$" is taken over each of the corresponding "y-values" (IRV), not including the origin (any standard's blank or average reading for same must have been subtracted first). The same thing is true of the other summations. "(x-bar)" refers to the average of each of the standards (the concentrations thereof in µg/ml) not including the origin or zero-standard. "(y-bar)" refers to the "average" of each of the corresponding "y-values" (IRV), not including the origin (any standard's blank or average reading for same must be subtracted from each before averaging). "N" is equal to the number of standards being run on the instrument, not including any zero-standard or standard's blank (in our case, N = 3). Note that the statistical formulas for the standard deviation of the slope and the standard deviation of "y given x", for example, are different for each of these three slopes and it may be necessary to determine the estimates for these and other parameters by sampling directly from repeated instrument readings rather than by obtaining their values by statistical formulas from the same raw data that was used for calculating the slope of the calibration line.
      It can be shown that formula (2) gives a calibration line of "moderate slope" somewhat "mid-way" in magnitude between those obtained by formulas (1) and (3). The assumption of a "constant relative variance" that applies to formula (2) may more aptly apply to many forms of chemical instrumentation rather than the more "drastic" assumption of a "constant relative standard deviation" that applies to formula (3) which usually allows for a much higher standard deviation of "y given x" at high measurement levels. The calibration line obtained by formula (2) passes through two known points: the origin ("x" = "0", "y" = "0"), (also being the "centroid" here according to the mathematical model "y = mx + 0") and ("x" = "(x-bar)" and "y" = "(y-bar)") which is the point obtained by averaging the concentrations of the standards "(x-bar)," not including the origin or zero-standard, and averaging the corresponding instrument responses obtained for them "(y-bar)," not including the origin (any standard's blank or average reading for same must be subtracted from each before averaging). These two points can be quickly calculated and used as a drawing aid, aligning up the two points with a straight edge (such as an ordinary ruler) and drawing a straight line through them. Or, alternatively, as with any of the linear slopes determined by any of the above formulas, the concentrations of the regular sample or subsample extracts ("X" values) can be obtained by dividing the respective "Y" values (IRV) for each sample or subsample extract by the slope of the calibration line, provided the "goodness of fit" of the line to the calibration points has in some way, graphically or statistically, been demonstrated.
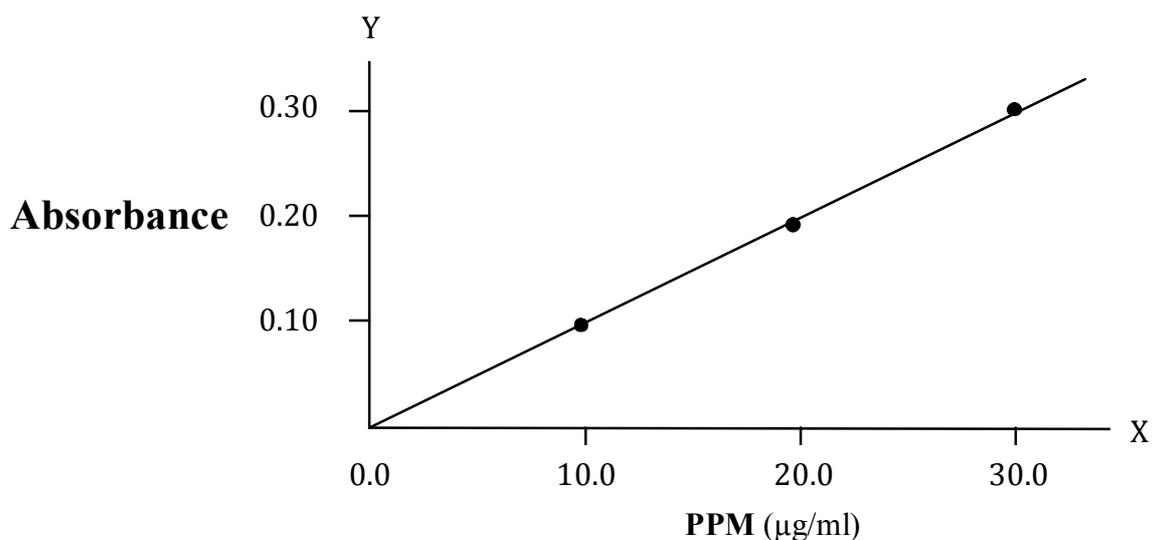
Figure 1. Example of calibration graph.

References

Armitage P., 1972 Statistical Methods in Medical Research. John Wiley & Sons Inc., New York, pp. 278-279.

Buckhale G., 2010 Prototype of a computerized system to automatically supply the parameters needed to compute 95% confidence intervals for chemical (and other) measurements (in both biased and unbiased measurement form). AES Bioflux **2**(2):121-170.

Day R. A. Jr., Underwood A. L., 1967 Quantitative Analysis, 2nd ed, Prentice-Hall, Inc., pp. 296-302.

Guenther W. C., 1973 Concepts of Statistical Inference. McGraw-Hill Book Company, Inc., pp. 1-555.

Skoog D. A., West D. M., 1971 Principles of Instrumental Analysis. Holt, Rinehart and Winston, Inc., pp. 1-710.