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Perspective

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Quantitation of Sensory-Active and Bioactive Constituents of Food: A *Journal of Agricultural and Food Chemistry* Perspective

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ABSTRACT: The proper procedures for the measurement of amounts of compounds that may occur in a food or other matrices are presented in this perspective. Factors dealt with include sampling, use of standards, advantages and limitations of chromatographic and other techniques for quantitation, and proper presentation and reporting of data. Such factors must be considered at the initial stages of an investigation and incorporated completely into the overall experimental design. These standards are to be employed in determining quantities of such components, and their careful incorporation should result in more favorable evaluation of manuscripts submitted to the *Journal of Agricultural and Food Chemistry*.

KEYWORDS: *measurement, quantitation, gas chromatography, high-performance liquid chromatography, immunoassays, reporting data*

INTRODUCTION

"But taking once a week on a knife's point, the quantity of a grain of mustard of it;..."

Sir Epicure Mammon in "*The Alchemist*" II, i, by Ben Jonson, 1610

Throughout recorded history, plant seeds have been used to define units of mass, thus intimately relating agriculture and food to systems of weights and measures. Mediterranean traders used grains of wheat or barley together with other seeds, such as those of the carob tree (*Ceratonia siliqua* L., Fabaceae).[1] A legacy of this system still exists in the occasional use of the "grain" as a unit of mass in pharmaceuticals and commonly for bullets and gunpowder. By ancient convention, the mass of a carob seed, defined as 1 carat, was equivalent to the weight of 4 wheat grains or 3 barleycorns.[2] Carob seeds were chosen because they were regarded as being very consistent in weight, but the weights of cereal grains can be highly variable, as a consequence of differences between cultivars and the environmental conditions under which they are grown, as well as moisture content, making their use unreliable. As a result, shipments of merchandise could have quite different weights at various points along the delivery route, leading to serious disagreements as to their value. Such problems led to the development of standardized systems of weights and measures, although these still varied from country to country.

With the development of the scientific method, a universal standard needed to be developed, and this resulted in the metric system, which has evolved into the International System of Units (SI; from Système international d'unités).[3] In particular, studies on biological activities of food constituents require exact quantitative data, for example, to address their activity thresholds. In addition, legal requirements with respect to toxicologically relevant compounds, such as contaminants, mycotoxins, or allergens, must also be addressed by means of precise analytical data. The recent development of extremely sensitive equipment has enabled the measurement of quantities at the nanogram, picogram, or even lower levels. In agricultural and food chemistry, the extraordinary sensory ability of humans to perceive the character of taste and/or odor has required determination of levels of components with desirable or undesirable sensory properties in such ranges. Similarly, food contaminants (e.g., mycotoxins and pesticide residues) or foodborne toxicants have come under increasingly strict regulation and often require determinations in the part per billion (ppb; ng/g) range to pass inspection. Such levels are difficult to conceive of in a physical sense and can be appreciated only by analogy. For example, the current tolerance levels set by the European Community of 2 ng/g aflatoxin B_1 for most food products[4] would translate to an error in time measurement of 1 s in 15.8 years!

In 2007 we published a perspective[5] designed to summarize the standards to which authors of manuscripts submitted to the *Journal of Agricultural and Food Chemistry* must adhere in the course of establishing the structures of compounds. The primary motive was to ensure that compounds are securely identified with respect to all aspects of structure and stereochemistry, thereby ensuring that the high standards of the *Journal* are maintained. However, the proper procedures for measurement of amounts of compounds that may be present in a food or other matrices were not addressed. This perspective is designed to set down standards to be employed in determining quantities of such compounds in manuscripts submitted to the *Journal of Agricultural and Food Chemistry*. For a comprehensive discussion of factors relating to analytical laboratory quality assurance. it is recommended that the *AOAC Official Methods of Analysis*, Appendix E, be consulted.[6]

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It should be noted that we employ the term "quantitation" rather than "quantification", because the former generally implies measurement of a quantity with high accuracy and often measurement of uncertainty, as in "quantitative analysis". On the other hand "quantification" is a broader term, having several distinct meanings in both science and logic, and encompasses not only counting and measuring, which may entail accuracy, but can also include classification into broad categories such as small, medium. or large. "Quantitation" therefore fits more precisely the requirements for papers published in this *Journal* and is the preferred term, even though the two terms are often used interchangeably in colloquial English.

The first part of this perspective will deal with procedures, common errors, and misunderstandings with respect to quantitation of components and the second part with specifics as to reporting the measurements.

OBTAINING DATA

"Measure what is measurable, make measurable what is not so"

Galileo Galilei, 1564−1642

Experimental Design and Sampling. Prior to obtaining data, the experimental design should be carefully planned to include sufficient samples for the quantitation to be statistically significant. This requires that the statistical methods to be applied be considered before sampling commences. Attempts to force statistical procedures to fit a data set after sampling can result in conclusions that are not meaningful. Although the number of samples to be analyzed clearly depends on the type of product, that is, liquid or solid, at least a minimum of three samples should be analyzed separately. However, injecting the same sample three times into the chromatographic system will not indicate the standard deviation of the entire method.

Almost all methods of quantitation basically consist of four steps: (i) representative sampling; (ii) isolation of the analyte; (iii) separation by means of chromatography, or use of direct detection methods that are so selective as to not require separation; and, finally, (iv) monitoring of signals caused by the analyte and an internal standard. Because the high sensitivity of modern instrumentation has permitted workup of samples with minimum amounts of foods, inhomogeneity of the sample can be a major cause of analytical errors. It is important that the sample and subsample sizes are sufficient to be completely representative of the material being analyzed. Compound isolation commonly starts with the extraction of the respective food using an appropriate solvent. Alternatively, for volatile isolation, either static or dynamic headspace techniques, such as solid phase microextraction (SPME), as well as steam or vacuum distillation methods, for example, solvent-assisted flavor evaporation (SAFE), may be used. However, it must be kept in mind that, no matter which method is employed, no total (100%) isolation of the target compounds can be achieved. In particular, headspace isolation techniques may lead to a significant discrimination of compounds with high boiling points.

Use of Standards. Accurate measurement of constituents requires the availability of pure standards on which correlations can be based. In the best case, standards will be available for each of the compounds to be quantitated, but usually only a few, or perhaps only a single standard, can be obtained. When a limited number of standards are available, it is important to select structurally analogous compounds for those components for which standards are lacking to minimize any discrepancies that might arise because of differences in response to the analytical method used. If the standards are obtained from outside sources (either commercially or as gifts from colleagues), it is always good practice to establish that the compounds are in fact as labeled and not contaminated with closely related compounds. Also, samples may undergo decomposition or rearrangements if they have not been stored under appropriate conditions. Characterization by mass spectrometry can be particularly misleading in this respect because compounds that are isobaric and structurally similar may have virtually identical fragmentation patterns. Another problem is that the sample may be contaminated with components (e.g., inorganic salts) that are not detectable by methods commonly used for organic compounds, for example, gas chromatography with flame ionization detection (FID).

A common mistake with commercially available standards is to assume that the specified weight is accurate and to prepare standard solutions directly based on the label weight. In practice, a vial that is labeled, for example, "1.0 mg" may contain an amount of compound in the range of 0.95−1.04 mg, with such extremes seriously distorting the concentration of the prepared solution. Standard solutions should be prepared only directly from the supplied sample if the weight of compound is certified by the manufacturer/supplier; in such cases the weight will usually be specified to two or three decimal places. Even then, a precautionary principle dictates that the purity of the sample be checked.

Chromatographic techniques are commonly used to determine purity, but each of these involves advantages and disadvantages with respect to quantitation. Most of these problems arise from the method of detection and are discussed in the following sections.

Compound Separation by Gas Chromatography (GC). Analysis by GC for both type and quantity of constituents is a long-established technique, particularly for volatiles or aroma constituents. It is important to recognize that certain constituents may be insufficiently volatile or too unstable to survive the column temperature and, therefore, be undetected or elute as broad peaks that are not easily distinguishable from baseline drift.

Quantitation by flame ionization is highly reliable because each compound eluting from the column is detected with a linear response, within the dynamic range of the instrument, although individual compounds have different detector response factors when compared one to another. However, this detection method has been replaced to a large extent by interfacing the GC with a mass spectrometric detector (GC-MS), which not only provides structural information but can also be used for quantitation. Unfortunately, the technique has some serious limitations that are often not recognized, or appropriate steps are not taken to surmount them. In particular, MS response is highly instrument specific, varying not only from one model to another, as might be expected, but even between individual instruments of the same model. Results obtained and reported in the literature with an instrument under specific tuning conditions in a particular laboratory may not translate well to the same type of instrument in a different laboratory. Even more serious is the fact that the response of any given instrument can vary considerably not only from day to day but also throughout the working day. It is therefore important to analyze standard samples inserted periodically throughout a series of sample analyses to account for such drift in instrument response.

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Compound Separation by High-Performance Liquid Chromatography (HPLC). Analysis by HPLC is advantageous in that it can be used for a broader range of compound types than GC. In particular, this technique is applicable to insufficiently volatile compounds (or that cannot be made sufficiently volatile by derivatization) such as those that contribute to taste, mycotoxins, or agrochemical residues.

Spectrophotometric (single or multiple wavelength, or diode array) detection, in the UV or visible range, is most commonly used for HPLC because such detectors are relatively inexpensive and provide spectra that can be used to characterize compounds or elucidate structural features. However, compounds that lack a chromophore, or absorb in a region that is not being monitored, will not be detected even though these may comprise a large proportion of the total being analyzed. Furthermore, without specific standards for each compound, or literature values for the absorbance of the compound at the wavelength being monitored, quantitation can be problematic. When standards are not available for all of the compounds being analyzed, it is fairly common practice to assume that all compounds of a particular class have similar absorbance values, but the potential errors in adopting this approach should be recognized and acknowledged in any resulting manuscript.

Mass spectrometric detection (LC-MS) is becoming increasingly available as the cost and ease of maintenance of such detectors have improved. Nevertheless, the same provisos apply as those discussed previously for GC-MS.

Stable Isotope Dilution Assays (SIDA). Most elements show a natural distribution of isotopes; for example, carbon consists of 98.9% ^{12}C and 1.1% ^{13}C. By means of appropriate synthetic approaches, organic compounds (analytes) can be labeled to 100% with a minor isotope in a certain position, for example, ^{2}H, ^{13}C, or ^{15}N. The synthesized compound consequently shows a higher molecular weight than the analyte, but exhibits nearly identical physicochemical properties, such as boiling point, polarity, etc. This makes the so-called "isotopomers'" ideal internal standards for quantitation. Methods based on the use of labeled internal standards are called "stable isotope dilution" assays (SIDA). The term "dilution" refers to the fact that the analyte is "diluted" with its isotopomer, the internal standard.[7]

The benefit of a SIDA approach is that losses in the analyte caused by procedures such as extraction, distillation, or even degradation are fully compensated, if the labeled isotopomer is added to the sample prior to the workup procedure and enough time for equilibration is allowed. Thus, time-consuming recovery and spiking experiments, necessary if structurally different internal standards are used, can be minimized. The quantitation can simply be done by monitoring the target fragment or molecular ion of the analyte and the corresponding isotopomeric internal standard (Figure 1). A shown for the quantitation of 3-methylindole by MS/CI, to improve the sensitivity and the precision of the method, GC/GC-MS (heart-cutting) can be applied without off-line sample purification. However, because deuterated isotopomers are always eluted slightly earlier than the corresponding analyte (Figure 1), this must be taken into account if GC/GC-MS is applied. The SIDA approach can even be used if a derivatization step is needed, for example, in the GC analysis of very polar compounds, such as acids or amines (Figure 2). The major drawback of this approach is the fact that the respective labeled analytes are often not commercially available. Furthermore, the prices for labeled reagents used in synthetic

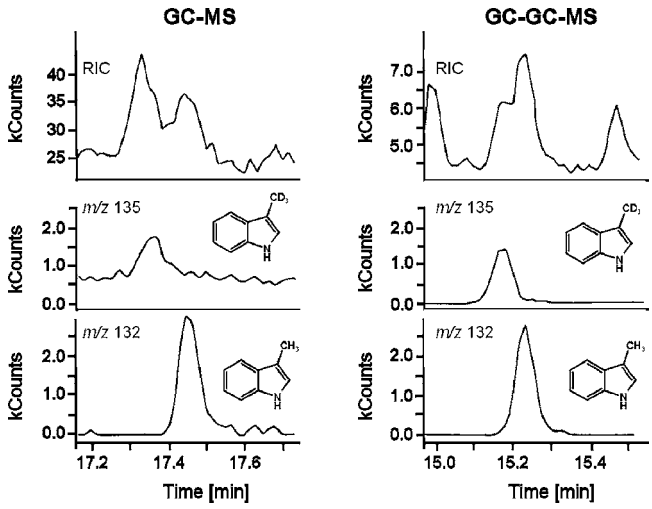

Figure 1. Quantitation of 3-methylindole by stable isotope dilution analysis in spoiled dairy cream: mass chromatograms of [$^{2}H_3$]-3-methylindole and 3-methylindole obtained by either GC-MS or GC/GC-MS (heart-cutting) (Martin Steinhaus and Peter Schieberle, unpublished material). RIC = total ion current chromatogram.

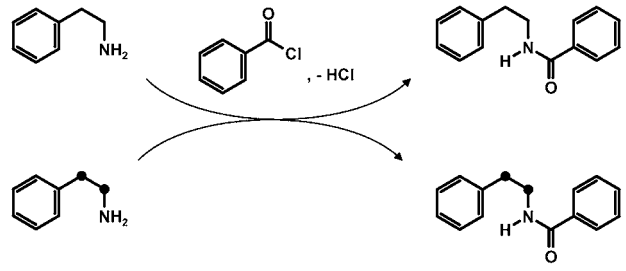

Figure 2. Derivatization of 2-phenylethylamine and ($^{2}H_4$)-2-phenylethylamine with benzoyl chloride applied in the quantitation of biogenic amines: ● = deuterium labeling (Christine Mayr and Peter Schieberle, *J. Agric. Food Chem.*, in press, DOI: 10.1021/jf204900v).

approaches may be quite high. However, because only very small amounts of the standards are usually needed and, thus, the syntheses are usually done in the milligram range, this argument is generally unfounded.

A "cheaper" method offering the same degree of precision is the standard addition technique. In this method, the analyte itself is used as the internal standard. The analyte is administered to the same sample in different concentrations, and the respective signal, usually the intensity of mass fragments, is recorded. By application of linear regression, the analyte concentration can be determined. However, in any case the linear range of the mass spectrometer must be taken into account.

Immunoassay Methods. Numerous immunoassay methods for the detection and analysis of mycotoxins and food contaminants have been developed, and many are commercially available. They may have certain advantages over chemical analysis, such as high throughput, relatively low equipment costs, and less rigorous training for their use. It is important to recognize that such methods often have serious limitations. Depending on the format of the assay, quantitation often represents a range, rather than absolute values. Because many such assays are employed to ensure that samples conform to a regulatory requirement, they may be designed to conform to a

cutoff point beyond which values are unreliable. In theory, a well-designed immunoassay should show no significant cross-reactivity. In practice, when closely related structures are present, cross-reactivity may distort quantitative values. This is particularly the case when only selected cross-reactants are important from the regulatory perspective. It is good practice to accompany immunoassays with chromatographic analysis of periodic samples in a series, particularly when transformations to potential cross-reacting metabolites that were not included in the validation of the assay may have occurred.

Another consideration is that compounds may occur as both free and bound or sequestered molecules,[8,9] with only the former being detected and quantitated. Because immunoassays are designed to be used with only minimal extraction, it is less likely that the bound forms will be regenerated in immunoassay sample preparation than in chromatographic methods. This is because immunoassays are designed with minimal extraction, whereas chromatographic methods typically use more rigorous extraction and purification procedures. Furthermore, immunoassays provide no structural information with regard to nontarget components; consequently, bound compounds are likely to remain undetected. In contrast, methods such as LC-MS have at least the potential, and at best the capability, of identifying these.[8,9] For this reason, it is essential that manuscripts reporting the development of new immunoassays be validated with "real-life" samples, not merely those that have been spiked with known weights of the analyte. It must be remembered that spiking is merely a test of recovery and not an absolute measure of the efficiency of the analytical method.

Essential Oils. Essential oils are volatile plant extracts, commonly isolated by steam distillation. Data on the chemical composition of such extracts were previously used, in particular by botanists, to find molecular correlation markers between botanical species. However, today the biological activity of essential oils and regulatory and quality issues are the focus of their analysis, but relative abundancies of FID signals without response factors or, even worse, simply headspace techniques are still applied. As explained above, such results cannot be accepted as "quantitation".

It must be commented that due to the complexity of essential oils the exact quantitation of all compounds is a difficult task. One option could be a grouping of the components by their functional groups within chemical classes, for example, monoterpenes, and to set up calibration curves with commercially available standards for each group.[10] However, a much better approach would be the characterization of the bioactive components or markers for the assessment of quality to decrease the number of analytes. This was already done two decades ago for sensory active compounds by means of the molecular sensory science approach.[11]

■ REPORTING AND PRESENTATION OF DATA

General. In reporting data, the appropriate format should be used in describing the technique used for the separation and quantitation of mixtures. Different techniques should be separated by a hyphen, whereas variants of the same technique should be separated by a slash. Thus, gas chromatography with electron ionization tandem mass spectrometry detection would be abbreviated as GC-EI/MS/MS, whereas high-performance liquid chromatography with UV and visible detection using a diode array detector would be identified as LC-UV/vis/DAD.

Correlation Curves. It is rarely necessary to include correlation curves as figures in the body of the paper; regression equations in the text are sufficient. At the author's discretion, correlation curves can be submitted as Supporting Information, available electronically as described in the Scope, Policy, and Instructions for Authors.[12] Do not cite these in the text but include a list of all figures or tables included in Supporting Information at the end of the text, preceding the Literature Cited section.

Significant Figures. Instrumental software will often calculate values to three, four, or even five numbers following the decimal point, implying a high degree of accuracy. Authors are advised to use their discretion in reporting such numbers, limiting them only to those that are significant in terms of the capabilities of the instrument. For example, a value measured as μg/g when reported to three decimal places will imply that the instrument has an accuracy in the ng/g range, which might simply not be achievable. Furthermore, the values for chromatographic peaks are highly dependent on the manufacturer's algorithm for determining the inflection points and, consequently, the integrated value.

Percentages are often similarly calculated to several decimal places. There is rarely any justification for reporting percentage compositions to more than one decimal place, and calculated values should be rounded to this level at most or even to whole numbers.

Coefficients of Variation and Standard Deviations. Tables and figures must include coefficients of variation and standard deviations, reported as $\pm$ values or error bars, as appropriate. As outlined earlier, these should be derived by appropriate statistical treatment of the data.

■ EXPECTATIONS

The appropriate acquisition of quantitative measurements and subsequent proper treatment and reportage will not only make the data more accessible to readers but also enhance the reputation of the *Journal of Agricultural and Food Chemistry* in the scientific community. It must be stressed that such factors should be considered at the initial stages of an investigation and incorporated completely within the experimental design. Taken together with the previous perspective on compound identification,[5] the standards set down herein are designed to provide authors with the requirements that are expected for submissions to the *Journal* and should increase the possibility that manuscripts will be favorably evaluated by reviewers and editors.

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Notes

The authors declare no competing financial interest.

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