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# Review

# Sample preparation for chromatographic analysis of food

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ABSTRACT

Sampling, homogenisation and sample preparation prior to chromatographic injection of food analytes are designed to enhance accuracy and precision. The reduction of inherent errors introduced by these steps requires the analyst's attention as a matter of course. Methods and examples of minimising errors in each step are reviewed.

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## 1. INTRODUCTION

Almost without exception, food is a complex inhomogeneous mixture of a bewildering array of chemical substances. The isolation and measurement of individual chemical compounds in food represents a difficult task. Chromatography is a powerful technique of separation and identification, yet rarely is it possible to directly load a syringe with a food matrix and inject to obtain a sensible result. Perhaps surprisingly, it is not rare to find analytical methods published with precision data reflecting repeated direct injections of standard solutions. This tells the reader little about the practicality of the method to real world samples. Procedures for preparation of the sample should be developed, evaluated and published as an integral part of any analytical method.

There are three steps involved in sample preparation for chromatographic analysis of foods: (1) *sampling*, obtaining a sample for the laboratory; (2) *homogenisation* of the laboratory sample to enable the taking of test portions; and (3) *sample preparation*, physical and chemical manipulation of the test portion prior to injection of the analyte fraction into the chromatograph. It should be appreciated that elements of these steps may occasionally occur in the reverse order or as combined operations. The fourth and final step of the analysis is the chromatography. Paradoxically, although the purpose of each of the three steps is to increase the accuracy and precision of the analysis, each step also introduces inherent errors. The error contributions of these steps for a typical food analysis scheme are shown in Fig. 1. Analyte concentration is limited at one end by detection limit and at the other by overloading of preparation stages or the chromatograph by either analyte or matrix. The significance of the contribu-

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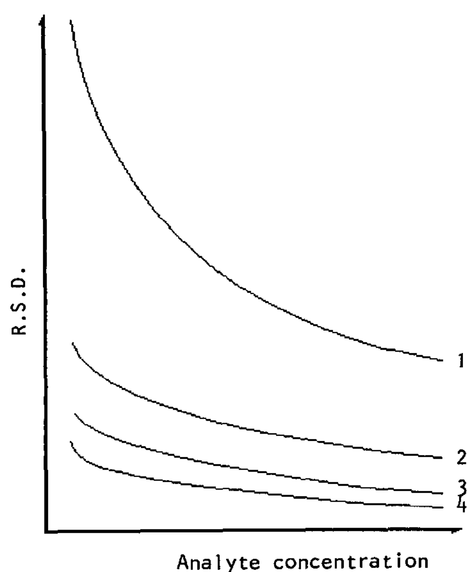


Fig. 1. Typical relative standard deviation of error components for an inhomogeneous matrix. 1 = Sampling; 2 = homogenisation; 3 = sample preparation; 4 = chromatography.

tions of these steps to the total error for the analysis are mathematically described by the relative standard deviation (R.S.D.) relation:

$$\text{R.S.D.}_{\text{total}} = [\text{R.S.D.}^2_{\text{sampling}} + \text{R.S.D.}^2_{\text{homogenisation}} + \text{R.S.D.}^2_{\text{sample preparation}} + \text{R.S.D.}^2_{\text{analytical}}]^{\frac{1}{2}} \quad (1)$$

From the equation it is clear that if any one of the contributing factors is significantly greater than the others, it is futile to attempt to reduce any of the other contributors, as the total error will be disproportionately dictated by the dominant factor being squared. A good example is aflatoxin analysis, where as long as the sampling error contributes 90% of the error there is little incentive to improve the analytical precision [1].

It may often be clear to the experienced analyst what the approximate proportions of the contributing errors are of the total for a familiar analysis. If this is not so, then these may be defined by rigorous assays of replicates and recoveries testing the effects of each successive step of the analysis. This review paper has been divided into three sections to consider problems encountered in each of these respective steps.

## 2. SAMPLING

There has been great concern amongst analysts over the validity of analytical methods. Attempts to rigorously define precision and accuracy of methods include measures such as international collaborative trials. For all the benefits of implementing such expensive measures there is one important oversight; the issue of sampling is not examined. Experience demonstrates that sampling can often be the greatest source of error in chemical analysis, particularly for food matrices (Fig. 1).

> "The classic example of incorrect sampling procedure and its ridiculous consequences is given by the fable of the blind men and the elephant. The consequences are sometimes no less ridiculous for incorrect chemical sampling" (W. J. Blaedel and V. W. Meloche [2]).

Undergraduate texts often form the basis of analysts' future attitudes. The spectrum of emphasis on sampling ranges from serious but brief mentions [3–5] through a good treatment, but at chapters at the end of the book [2], to an integral treatment from the beginning [6].

The best method of sampling in a given situation will depend on such issues as: what information is sought, resources available, accessibility of the target population, is the population heterogeneous? and if so is the variation general, localised or stratified?, required turnaround time, perishability of the food and the analyte, should the population be sampled critically or representatively, randomly or systematically? What are the criteria for acceptability? Are samples to be pooled or replicated? Should analysis be performed separately on different portions of the sample, is the surface to be included in the bulk? What monitoring should occur to prevent contamination and abuse?

Take the example of nutritional analysis of peas: sample variables include size distributions, position of individual peas in their pods, height of pods up the individual vine, individual plant genetics, cultivar, time of planting, efficiency of pollination, watering history, soil type and underlying geology, previous crop history, soil fertilisation, crop maturity, disease and pestilence attack, length of time, handling and storage conditions since harvest. Are the peas to be cooked? If so, how and for how long? Is the analysis to represent nutritional data for a locality, or national database?

These issues are discussed to varying degrees in the sampling literature, are mostly self explanatory or specific to situations. In the latter case discussion is usually found in sections of literature specific to the situation, or remain unrecorded know-how of specialists. These specialists must be encouraged to include such details in their publications.

Literature concerning sampling is well dispersed and generally not easy to locate. A useful survey [7] lists over 60 references of possible use to food analysts, some general references [8–12] being recommended. Several others included various mathematical treatments [13–15] of both general and specific problems. A mathematical consideration [16] of chemical analysis was not included, presumably because it concentrates on assessment of data quality rather than the practical aspects of sample planning. The American Chemical Society has published guidelines [17] regarding sampling. International Standards are complete for sampling of fruits and vegetables [18], meat [19] and oilseeds [20].

More recent discussions of sampling [21–24] have attempted to integrate sampling approaches with laboratory practices, sample characteristics and analytical problems.

The importance of sampling has recently been highlighted by problems encountered in analysis of aflatoxins in peanuts. Several workers have written papers specifically addressing this type of sampling situation [1,25–28]. In these cases, of highly inhomogeneous distribution of analyte in the matrix, the proportion of total analytical error attributable to sampling is commonly greater than 90%.

All too often the analyst has little influence over the taking of the sample. A widening of appreciation of the importance of sampling may serve to rectify this problem. Ideally the analyst should appraise the problem and take the samples personally. Failing that, the analyst should endeavour to thoroughly brief the sampler as to the most appropriate methods for each situation. A specific instance of such difficulty concerned an untrained sampler being assigned to learn about the traditional indigenous foods of Australian Aboriginals. He was also briefed to collect specimens for nutritional analysis, but was little influenced by the analysts. Constraints apart from the lack of training were the hot climate, lack of refrigeration, necessity of taking pocket-size samples, and remoteness from the laboratory, some 3000 km and several days freight away. All of these factors contributed to the degradation of sample integrity. Many of the samples analysed consisted, for example, of three individual thawed fruits totalling 15 g. However while the limitations of the nutritional assays on such unrepresentative samples are obvious, the data are surely indicative and as such useful in this context [29–31], where previously nothing was known of the nutrient content of the foods.

## 3. HOMOGENISATION

The complex structure and composition of food substrates necessitates homogenisation prior to most chromatographic analysis. Variable texture, structure and viscosity, and the presence of immiscible phases, hygroscopic or hydrophobic matter all contribute to the difficulty of this operation meeting with success. The observation that collaborative test results for food materials often show greater coefficients of variation than other matrices [32] is therefore not surprising.

Problems encountered with sampling, particularly for semi-micro combustion analysis [33] led to the author's investigations concerning homogenisation methods used for food samples [34]. This paper surveys nine conventional methods and three cryogenic methods of homogenising numerous food samples, condensed into seven categories of matrix type. Several methods were subjected to more rigorous examination. Other papers have considered more limited ranges of methods and foods [10,35–37]. For assays using test portions of around 1 g several of the conventional methods prove satisfactory with compatible matrices [34]. Many method–food category combinations proved to be incompatible, some unexpectedly so. For assays using smaller test portions of requiring stringent homogeneity of very heterogeneous foods the cryogenic treatments proved well worth the extra effort after a conventional pretreatment and freeze-drying. This dual treatment reduced particle sizes to below 60 $\mu$m (97% below 10 $\mu$m) for one of the most difficult matrices with sufficient mixing to take reproducible test portions of 1 mg. The average R.S.D. of micro-combustion protein assays for a range of foods was 1.33%, performed on test portions of 2–5 mg [34]. The number of particles included in

each test portion was approximately $10^5$.

The required size of the test portion and the sample's characteristics will dictate the degree and type of homogenisation required. If several different assays are to be performed on a sample then whichever has the most stringent requirements will often dictate the homogenisation requirements. Experience shows that it is often prudent to sequentially use two homogenisation techniques. It may be desirable to split the sample after an initial wet-basis homogenisation treatment; analysing the first part for labile vitamins directly; further rigorous homogenising after freeze-drying before subjecting the second part to other analysis. A generalised scheme for homogenisation of samples for nutritional analysis is shown in Fig. 2. The scheme includes approximations for quantities and particle sizes at each stage, and what types of assays are amenable to the products of each stage. However, it must be reiterated that each sample will have different characteristics which will require different homogenisation treatments. The main conclusion that should be drawn is that each sample should be homogenised by methods that have proven effectiveness with the particular matrix, either from experience, literature or by experiment, to a degree that meets the test portion requirements. This must be confirmed by the precision of replicate assays.

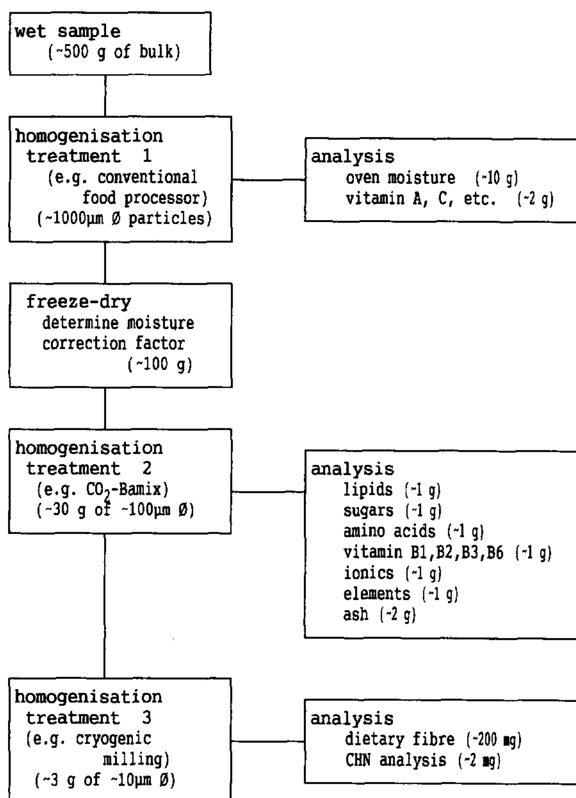


Fig. 2. A sample homogenisation scheme for nutritional analysis of food. ∅ = Diameter.

There are two functions of homogenisation, reduction of particle size and mixing. Reduction of particle size involves cutting, shattering and shearing. The various devices achieve these in different ways to differing degrees. This necessitates judicious choice of homogenisation methods that have demonstrated applicability for use on particular matrices. The efficacy of a method may be observed by microscopic examination or sieving of the product. The importance of particle size reduction is intuitive; quantifying this statistical notion is more difficult. A simplified treatment with graphed relationships [11] is recommended reading for non-mathematical analysts.

Mixing may be more difficult to achieve and examine. Experience suggests visual inspection of colour and texture is very useful, but not necessarily rigorous, especially in the case of a sample consisting of components of similar appearance. There are several means that may prevent adequate mixing: classification, agglomeration and phase separation. Causes include particle shape differences, density differences, electrostatic charging, disruption of stable structures maintaining surface tension, destruction of encapsulating structures, and various hydrophobic–hydrophilic interactions. Typical examples include oil separation in finely ground nuts and classification of whole grain flour. The only way of avoiding such problems is selecting appropriate homogenisation methods through experience, learning, but just as often by intelligent trial-and-error experimentation. The combined effect of reduction and mixing may be examined by performing assays on replicate test portions. For this examination it may be prudent in some circumstances to run simple, cheap assays rather than use the actual target assay. Another method of potential is statistical image analysis of the homogenised test portions under the microscope. It may be necessary to colour-label some components prior to treatment to aid differentiation.

The degree of homogenisation may affect the accuracy as well as the precision of the analysis; in ways that may not be revealed by recovery data. A typical case is the accessibility of the food matrix to enzymatic digestion. The efficiency of enzymatic digestion is proportional to the surface area (or degree of homogenisation) of the food substrate; that is, inversely proportional to particle size. Large particle size may inhibit enzyme access to the whole of the food, specific portions may be encapsulated and not be available for subsequent extraction; such problems have been encountered in thiamin [38], lipid [39] and dietary fibre [40] analysis. Efficient thiamin extractions were only possible after digestion of finely homogenised foods. In the second example, digestion of a well homogenised substrate was required for complete release of lipids; but if the sample was too finely ground, problems with emulsion formation hampered the efficiency of the subsequent liquid–liquid extraction. In contrast, Englyst dietary fibre determinations [40] on poorly homogenised samples give spuriously high results due to incorporation of undigested starch in the fibre fractions. Analysis of total dietary fibre by the AOAC method [41] has different problems: if the sample is too finely ground then there is the risk of low results by losing fibre through the 90-$\mu$m porosity filter, even with the use of filter aids; if the sample is too coarse then there will be high results from insufficient enzymatic digestion of other components, and a loss of precision from poorer test portion sampling of larger particles.

## 4. SAMPLE PREPARATION

Sample preparation includes any operation performed to the test portion prior to injection into the chromatograph; weighing, dilution, cleanup, extraction, digestion, purification, separation, derivatisation etc. ... Descriptions of which are usually included in publications, although frequently lacking background information, "tricks of the trade" finer detail, and rigorous error analysis.

Careful thought and often some background research is required to decide what parts of the sample are to be analysed. For example, is fresh produce to be washed prior to pesticide analysis? If so, how? What is the "edible portion"? Examples of such separations include removal of outer leaves, peels and pips from fresh fruits and vegetables, removal of bones and trimming of excess fat from meat, exclusion of brines from canned vegetables but inclusion of liquid from canned fruit. Many such choices are subject to debate, such as the inclusion or otherwise of seeds in a sample of blackberry jam. It may be desirable to analyse both portions. In any case it is standard practice to weigh the separate portions, analogous to the determination of moisture when drying a sample.

Several authors have emphasized that for nutritional evaluation it is desirable to prepare the food as it is consumed [10,21]. What are the customary methods and times of cooking of meals? What constitutes complete preparation of powdered soups and hot beverages. An instance that comes to mind is the analysis of such beverages for vitamins $B_1$ and C, where the assay of the powder is assumed to be the measure of dietary intake [42,43]. In reality, these vitamins degrade significantly when the powders are stirred into boiling water, especially if the water has been sterilised by iodine agents, as is recommended practice in this context.

The first and practically universal step in the manipulation of the test portion is the weighing step. Fortunately the precision of modern balances is commonly six significant figures or better. Judicious choice of balance can maintain this precision for a large range of test portion size, typically from 10 g down to 1 mg for food analysis. However the analyst must ensure the operation is performed accurately, paying particular attention to eliminating electrostatic charging and moisture variation of food samples. These same errors must be considered when drying samples and determining moisture correction factors. Once dry, samples should be stored in a desiccator.

A frequent operation contributing to the sample manipulation error is the volumetric dilution. For headspace sampling, the size, pressure, equilibration time and temperature of the space are all critical. Volumetric errors are inherently orders of magnitude greater than those for mass measurements.

Digestion, extraction and derivatisation should all be quantitative. Efficiency may be enhanced, for example by application of microwaves [44], but only if sample integrity is maintained. Confirmatory tests should be used in any doubtful cases. A typical simple test is the testing for residual starch with

drops of iodine to confirm completion of amylase digestion [40].

The methods of ensuring good recoveries while using absorption columns prior to liquid chromatography are straightforward. The use of preconcentration methods in gas chromatography [45] can be fraught with complications of volatility differences, reactivity and adsorption. These issues are generally adequately discussed in methodology papers alongside chromatography details.

Degradation of the sample and analyte integrity may take place at any stage, from the taking, transport and storage of the sample, drying, homogenisation and sample preparation to the injection of the manipulated test portion. Addition of contaminants, exposure of samples to heat, warmth (microbiological activity), moisture, oxygen, visible and ultraviolet light, reagent fumes can all compromise accuracy. These problems are considerable in vitamin analysis —consider some examples:

Riboflavin is sensitive to ultraviolet light. Vitamins A, $B_6$, D, E and folic acid are sensitive to light. Laboratory manipulations are usually performed using low-actinic glassware and preferably in the dark [38,46,47].

Ascorbic acid is particularly sensitive to degradation by oxidation, especially when exposed to atmospheric oxygen, heat or high pH. Analysis schemes aim to reduce manipulation and turnaround time to an absolute minimum, making use of various stabilising agents. The tenfold variation in ascorbic acid found in *Terminalia ferdinandiana*, a native plum found in northern Australia rich in this vitamin [29,30] is at least partly due to degradation during lengthy transport on different occasions. A less obvious hazard is contamination by traces of copper, which catalyses the oxidation reaction.

Introduction of metal contamination, by the homogenising device (for example), may be serious beyond the simple raising of metal content. The presence of metal may promote reactions compromising sample integrity, as mentioned above, but may also interfere with extraction, cleanup and enzymatic digestion steps of sample preparation procedures.

Contaminants may be introduced by reagents. The development stages of a method for enzymatic digestive release of lipids [39] revealed that several commercial enzyme preparations contained unacceptably significant amounts of ether-extractable contaminants.

Contamination and the potential complications in sample preparation procedures are highlighted by the gas chromatography of alditol acetates in the Englyst dietary fibre determination. Plasticisers may contaminate food samples at literally any point from the farm to the chromatograph. Pure samples of ubiquitous plasticiser contaminants were found to chromatograph at similar times to some of the analytes [48], but could be resolved from analyte peaks by capillary columns. More recent investigations found that exposing plasticisers to the derivatisation procedure used to form alditol acetates yielded multiple and broad peaks that potentially interfered with the analytes [40]. This is an additional artifact caused by the sample preparation technique fundamental to the analysis. A recent monograph [49] deals specifically with such analytical artifacts, with considerable attention to problems with gas chromatography–mass spectrometry, a technique widely regarded as definitive with respect to analyte specificity.

All elements of the gamut of test portion manipulations performed are potentially significant error contributors. The consistent use of observation, replication of test portions, recoveries and reference materials should highlight problem areas. These may be reduced, or at least quantified, using the skill of the analyst.

## 5. CONCLUSIONS

The analyst wishing for accuracy and precision must focus on all elements in each of the four steps of analysis; sampling, homogenisation, sample preparation and analytical technique. Critical examination should reveal weaknesses where sample integrity may be compromised. The greatest effort should be expended to reduce contributions in the error-dominating steps. Authors should be encouraged to include all experimental details of the first three steps in their publications.

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