

Letter to the Editor

Comments on "A Simplified Experimental Design Approach to Optimization of SFE Conditions for Extraction of an Amine Hydrochloride"

To The Editor:

A recent article in this journal described a method developed for analyzing the toxicant 3-chloro-*p*-toluidine hydrochloride (CPT HCl) in avian diets (1). Bicking's stated objective was to produce a method which allowed for the quantitative analysis of feeds ranging in concentration from 10 to 5,000 $\mu\text{g/g}$. Though data concerning supercritical fluid extraction of polar compounds is important, we believe there are three notable inadequacies within the article.

First, conventional solvent extraction methods were presented as unsuitable for diets containing less than 100 $\mu\text{g/g}$ CPT HCl. This sharply contrasts with the exploratory research conducted in our laboratory. Evaluations of conventional solvent extraction demonstrated that it is most applicable for the quantitative analysis of CPT HCl. Our procedure involves the triplicate extraction of 2 g of the diet with 8 mL of an acetonitrile-water mixture (50:50). The extract is then analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). The mobile phase consists of 60% acetonitrile and 40% water at a flow rate of 1 mL/min. A 4.6-mm \times 250-mm octadecyl silane column provides adequate separation. 10- μL injections are quantified with UV detection at 241 nm.

Seven replicates of a diet fortified with CPT HCl at a concentration of 10 $\mu\text{g/g}$ (which represents the lowest feed concentration of concern) were analyzed for CPT HCl by our method. An 87% recovery of the analyte resulted, with a relative standard deviation of 6%. Furthermore, evaluation at higher concentrations indicated improved analyte recovery at increasing concentrations.

The second inadequacy of the article concerns validation data. While the stated intent of the work was to develop a method that provides quantitative results for avian diets in the range of 10 to 5,000 $\mu\text{g/g}$, validation data (Bicking's Table III) are supplied only for concentrations up to 100 $\mu\text{g/g}$. Application of these results to concentrations as high as 5,000 $\mu\text{g/g}$ would be a radical extrapolation far beyond the range of the data. This same validation data also indicates unsatisfactory recovery even at 100 $\mu\text{g/g}$. As CPT HCl concentration increases from 5 to 100 $\mu\text{g/g}$, decreasing recovery is readily apparent in the data, with a negative correlation (-0.81) between concentration and recovery. Additionally, a 95% confidence interval applied to the 100 $\mu\text{g/g}$ validation recovery data ranges from 57 to 92%. This type of precision is unacceptable for the quantitative analysis of formulated diets.

Lastly, the application of the experimental design in the paper is inadequate. Part of the rationale for applying a factorial design is to investigate the interaction of temperature and pressure on analyte recovery. The described application does not investigate whether recovery follows the same pattern across temperatures at different pressures, and vice versa. Even though the experimental design is simple, only limited data were obtained at each of the four temperature-pressure combinations (three of four combinations are observed only once). Therefore, little insight is provided into the interaction of temperature and pressure. In fact, an analysis of variance (using the GLM procedure in SAS[®] [2] to account for unequal sample sizes) of the data supplied in Table I of Bicking's paper reveals no statistical evidence that any combination of temperature and pressure is superior to any other. The results of the two-way analysis of variance (ANOVA) are given in Table I here. Furthermore, examination of the data (Bicking's Table I) reveal that, had

Table I. Results of SAS[®] GLM ANOVA For Data Supplied in Table I (1)

Source	df	Mean Square	F	P-value
Temperature	1	1,778.7	4.54	0.28
Pressure	1	473.36	1.21	0.47
Interaction	1	457.14	1.17	0.48
Error	1	392		

the recovery of only the first replicate (77%) at the combination of 55°C and 290 atm been observed, without analysis of the second replicate, low temperature and low pressure would have appeared as the best temperature-pressure combination. We find it interesting that the generation of one additional data point produced a major change in inference concerning the optimum conditions, but no subsequent observations were made at any other combinations that may also have shown improvement. Similarly, the recovery

observed for each additional replicate in the validation data (Bicking's Table III) also demonstrated improvement. It would appear that improved recovery is associated more with the analysis of additional replicates than the selection of a particular temperature-pressure combination. Beyond this, the ultimate selection of optimum extraction conditions is well outside the range of the conditions investigated. Thus, there is little basis for considering 45°C and 320 atm as optimal.

In conclusion, we feel that the reported data do not support the intent of the paper to provide a "sensitive and reliable analytical method for the determination of CPTH in avian feed" (1), nor do the data support the contention that supercritical fluid extraction represents a quantitative method for the application in question. On the contrary, we advocate the employment of conventional solvents for effective extraction of CPT HCl from avian diets.

While we endorse the use of a factorial design to optimize extraction conditions for analyte recovery, the application of the elementary experimental design presented in the paper is inadequate to form inferences concerning optimal analytical conditions. A larger factorial design with multiple replications at each combination would serve better to optimize the temperature and pressure parameters.

Bruce A. Kimball and Richard M. Engeman
United State Department of Agriculture
Denver Wildlife Research Center
Analytical Chemistry and Quantitative Sciences
Building 16
Denver Federal Center
Denver, CO 80225

References:

1. Merlin K.L. Bicking. A simplified experimental design approach to optimization of SFE conditions for extraction of an amine hydrochloride. *J. Chromatogr. Sci.* **30**: 358-60 (1992).
2. SAS Institute Inc. SAS® User's Guide: Statistics, Version 6.03, Cary, NC. (1987).

The Author Replies:

Mr. Kimball and Mr. Engeman have listed three issues within my manuscript that they describe as inadequate. In general, I believe their objections reflect a misunderstanding of the scope and purpose of this publication. The title indicates that this manuscript describes a *simplified* experimental design approach. The work was not intended to be a definitive study that results in a final analytical method. As the title implies, the intent was to illustrate a simple optimization strategy. Optimization and initial evaluations are only the first step in developing a reliable method. Much more work is always needed, but the approach described in the article is one way to speed up the initial development work, which is often the most time-consuming. Specific responses to the three issues are provided below.

These workers disagreed with the statement that solvent extraction methods were inadequate at low levels, and cited a method developed in their laboratory. A nearly identical method was developed at TCT during the early phases of our investigations. The poor performance of this method was the reason for evaluating the SFE approach. We were aware of the method developed by scientists at the U.S. Department of Agriculture (USDA), and had extensive discussions with USDA staff about their method. Despite these discussions, we were unable to reproduce the USDA low-level results in our laboratory, although the procedure was effective at higher levels, as noted in the article (page 358, column 1, paragraph 2). The difficulties in transferring methods from one laboratory to another are well known. In addition, this particular analyte-matrix combination was susceptible to other factors such as variabilities in the composition of various batches of feed, particle-size effects, and stability of the analyte. Any or all of these factors could have explained our recovery problems. However, the important distinction is that the SFE method was apparently *not* affected by these problems; generally higher recoveries were obtained at low spiking levels compared to the solvent extraction method. Finally, the solvent extraction procedure was time- and labor-intensive, requiring *three* sequential extractions, followed by centrifugation of the extracts. The SFE method required a single extraction, followed by simple precipitation and filtering.

The second issue involved the validation data. We did not claim that the method was successfully validated up to 5,000 $\mu\text{g/mL}$. Experiments at or above that level, as well as additional replicates at lower levels, would be required before the method could be used over the range indicated. The published data indicated performance at the lower levels only, where recovery problems were most often encountered (solvent extraction could be used at higher levels if necessary). Certainly the lower recovery at 100 $\mu\text{g/mL}$ was less than ideal, as noted in the text (page 360, column 1, last paragraph). Recent advances in SFE restrictor technology would probably eliminate this problem.

The third issue is the validity of conclusions from the experimental design. The 2^2 factorial design does indeed provide information on the effects of both temperature and pressure on recovery, and is an acceptable statistical procedure. Certainly more replicates would always provide better results (page 360, paragraph 2 of "Conclusions"), but this is always the case. However, many workers are faced with other limitations (time, resources, sample availability, and, yes, money) that prevent them from being able to employ a full factorial design. The approach described in this article represented a simple, rapid procedure for optimizing conditions that is readily understood by most chemists. Of course, this simplification is at the expense of the amount of information provided by more complex designs (page 359, column 2, first paragraph). However, many workers do not have the luxury of being able to perform full factorial designs (or the software and training to understand the results).

These limitations are the exact reason why we warned that this approach should not be used for quantitative interpretation (page 360, column, line 3). Indeed, we are only drawing qualitative conclusions from the data. (However, linear regression on the Table I data did produce the same conclusions about the sign of the temperature and pressure parameters.)

The fact that conclusions can change by eliminating a single point is a common problem for small data sets. However, we could have strengthened our argument by eliminating the first replicate and using only the second, but that would be an equally invalid statistical approach. We used all the available data and dealt with the results (less dramatic main effects). Given the better precision demonstrated in Table III, the spread in the Table I replicates is not representative of the precision of the method. Even with different main-effect conclusions, we would still have preferred the low temperature-high pressure conditions because of the advantages in the residue weight of the extracts and the fact that such conditions are quite popular in other SFE applications.

Finally, we wish to again stress the danger in extensive statistical interpretation of small data sets and/or limited experimental designs. The chemist must always employ some statistical "common sense" in interpreting the results of statistical tests. It is this chemical intuition that allows us to draw appropriate conclusions about our data, using statistics as a tool, not a crutch.

In summary, we feel that the conclusions reached from this study are valid, given the interpretive restrictions which are clearly outlined in the text. The experimental design approach described here is useful as a *qualitative* tool only. If more information is needed, more experiments are required.

Merlin K. L. Bicking
TCT Corporation
737 Pelham Boulevard
St. Paul, MN 55114-1776