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ALTERNATE EXTRACTION AND CLEAN-UP FOR CALUX® ANALYSES OF FEEDS AND FEED COMPONENTS

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Introduction

Foodstuffs of animal origin contribute approximately 80% of the human exposure to Persistent Organic Pollutants (POPs), while the majority of the animals' burden comes mainly from feeds[1]. Since dietary intake has been identified as the primary source of exposure to POPs[2], the production of quick results when testing for them is of utmost importance in the food and feed industries. Of particular concern with the feed industry is that feed products can be used quite quickly and a random surveillance program results in only a snapshot of feeds that are on hand at the time of collection. Consequently, many feed lots escape surveillance and elevated POPs levels may go undetected.

Routine, rapid screening of the feed supplies could significantly reduce costly discoveries at a much later date. The CALUX® bioassay is one screening technique that can be used to gather TEQ data for a large number of samples in a relatively short period of time. Sample prioritization for GC/HRMS analyses is facilitated by data obtained from using the CALUX® screening method. If elevated TEQs are detected, then congener specific information by traditional GC/HRMS analyses becomes crucial in identifying the POPs source. The congener data are also needed for risk assessment purposes and the building of geographical databases.

Although a large number of results can be obtained somewhat quickly via CALUX®, the process has some drawbacks, including:

- A labor-intensive sonication and column extraction technique, and;
- Time-consuming/rate limiting solvent evaporation steps.

Moreover, limitations exist with the extraction and clean-up method, such as:

- Clean-up process involves full attention, and;
- Recovery method uses a surrogate (additional extract).

To be considered a viable screening technique, several criteria must be fulfilled. These include a quick, inexpensive assay with high precision and less than 1% production of false negatives. Another criterion that should be considered is production of a limited number of false positives.

Method

The traditional means of extraction[3], illustrated in Figure 1-A, for the CALUX® method involves sonication and column chromatography. Room temperature toluene is the solvent typically chosen for non-tissue samples due to the possibility of active carbon in the sample. Care must be taken in this technique to avoid transfer of sample particles that could be placed onto the column. The

transfer of these particles, in addition to other materials extracted from the matrix, have a tendency to slow or even stop solvent flow. The flow rate is not only affected by the material placed onto the column, but the packing of the column as well. The subsequent solvent evaporation is followed by the clean-up method using acid silica gel and carbon columns. Only small volumes of solvent can be placed on the columns, resulting in additional sample preparation hours. The PCB fraction is then eluted with an ethyl acetate: toluene: hexane (10:10:80) mixture. This is followed by the inversion of the carbon column and elution of the dioxin / furan fraction with 15 mL of toluene.

An alternative extraction / clean-up combination (Figure 1-B) uses an Accelerated Solvent Extractor (ASE-300) from Dionex in combination with a less intensive clean-up procedure. The use of a 1:1 hexane : methylene chloride mixture at elevated temperatures and pressure have resulted in acceptable recoveries from a variety of feed matrices. The less intensive clean-up procedure involves adding roughly 100-150 mL of this solvent into a separatory funnel and then allowing gravity flow through the clean-up columns, reducing man-hours. The collection of the PCB fraction and dioxin / furan fraction is similar to that discussed previously.

Listed here are several advantages to using the ASE:

- Allowing larger sample aliquots to be extracted, thus decreasing the detection limit;
- Dividing the extract in half and using it as a clean-up duplicate, checking precision, and;
- Developing an archival process whereby the extract can be assayed without cleanup.

With respect to the third advantage, by concentrating one-half of the extract to dryness and preparing the extract for the bioassay, the time spent on extract preparation could be reduced by nearly one-half. If no response is obtained above the blank level, then no further action would be needed for that sample. If, however, an elevated TEQ were observed, then the archived extract would be ready for further clean-up including removal of the PAHs and separation of the PCBs from the dioxins and furans. By utilizing this technique, only the time involved to prepare and dose plates is lost if a sample extract produces a signal.

Results & Discussion

The use of the ASE reduces the extraction time from roughly 5 man-hours / 20 samples to 2 hours / 24 samples. This time includes the preparation of columns, reagents, etc. The clean-up method takes nearly the same amount of time for each technique, although the alternate method is somewhat automated and thus less intensive. The ASE at elevated temperatures and pressure has resulted in acceptable recoveries using methylene chloride: hexane as an extraction solvent with most feed matrices. If recoveries fall below 50% the matrix is subjected to a toluene extraction. The use of an internal standard[4] to determine recoveries becomes important while utilizing this solvent mixture. This allows appropriate actions (such as re-extractions with toluene) and corrections (for recoveries) to be made as recoveries are determined.

A comparison of sample results using the ASE with a standard multi-column clean-up for GC/HRMS analyses versus the ASE with the traditional CALUX® clean-up is shown in Table 1. These results not only show agreement between the GC/HRMS TEQ and the CALUX® TEQ, but also illustrates that the ASE with the traditional CALUX® clean-up is amenable to the bioassay. The ASE extraction accompanied with the alternate clean-up technique (columns 4 and 5 in Table

1) indicate that the alternate clean-up technique compares well with the traditional multi-column technique for GC/HRMS analyses. Since those data agree, an additional sample was compared by using identical extraction techniques but different analyses, GC/HRMS versus CALUX®.

Table 1. Comparison of various extraction, clean-up and analyses

Matrix – sample	**ASE/MULTI-COLUMN HRMS (TEQ)**	**ASE/TRAD. CALUX® (TEQ)**	**ASE / ALT. HRMS (TEQ)**	**ASE / ALT. CALUX® (TEQ)**
Copper Proteinate	1.50	2.05		
Feed Ingredient	7.82	9.81	7.55	
Proteinate Test			4.84	4.88

Results from a direct comparison between a single mineral sample extracted by the ASE and analyzed by CALUX® are shown in Table 2. The extract solvent was split in half immediately after the extraction. The "A" portion of the extract was cleaned using the traditional clean-up of acid silica gel and carbon. The "B" portion of the extract was not subjected clean-up techniques. The final appearance of the extracts differed dramatically. The solvents from both extracts were ultimately evaporated. Portion "A" was a clear colorless liquid when diluted into 4 mL of hexane, while "B" had a dark yellow appearance when diluted. The clean-up process, in this case, appeared to make no difference in the final TEQ.

Table 2. Comparison of a cleaned and un-cleaned extract.

Project Sample #	**Dilution Factor**	**RLU**	**Result (pg/g)**
A. Mineral w/clean-up	1:100	7403	65.06
B. Mineral w/o clean-up	1:100	7494	68.53

These data indicate promise in speeding up the extraction, clean-up and analyses for an already rapid method. The results obtained appear to be very matrix dependent. As additional information is gained and matrices are identified upfront, educated guesses can be made in order to obtain the most effective means of preparing a sample.

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References:

1. U.S EPA; Dioxin: Summary of the Dioxin Reassessment Science; Information Sheet 1, May 25, 2001.
2. 06-Dec-01, Official Journal of the European Communities, L 321/1.

3. Xenobiotic Detection Systems, Inc. WL-11 Column Extraction for Feed Samples, April 12, 2002.
4. C. Earnheart, L. Pence, M. Halbert, J. Archer; 2003 FDA Science Forum Presentation "A Simplified Recovery Method Used in the CALUX Bioassay"

Figure 1A. TRADITIONAL

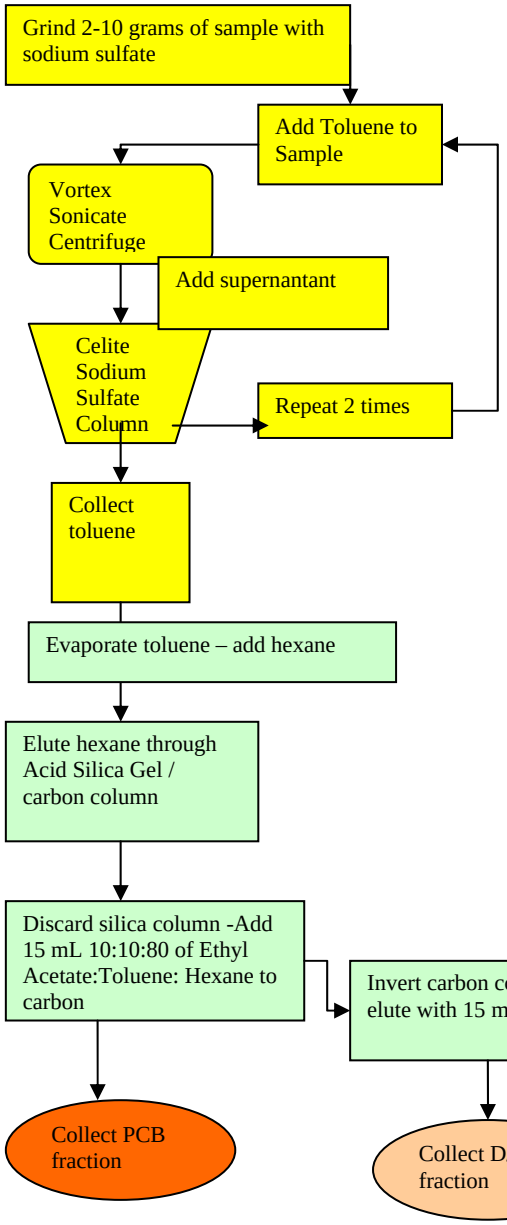

Figure 1B. ALTERNATIVE