Guidance Document

Best Analytical Practices for TRP Studies

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Best Analytical Practices for TRP Studies Including Guidance on Method Validation

General Guidance

Special care must be used in sampling and analysis to avoid problems with accuracy and precision of analyses, especially when determining non-volatile fluorinated compounds at ppb levels.

For more accurate and reproducible analytical determinations the use of a dual-label, at a minimum, ^{13}C-internal standard is highly recommended to alert the analyst of interferences from co-eluting peaks.

To verify that unacceptable levels of carry-over contamination do not occur from run-to-run and that contamination is not introduced during sample preparation, multiple sample solvent blanks and at least one reagent blank is recommended for each sample set.

Individuals should take the following precautions when handling samples:

1) Only previously washed clothing should be worn when preparing, shipping or analyzing these samples.
2) Always wash hands thoroughly with warm water and soap prior to working in sample preparation and designated lab areas.
3) Use of nitrile gloves when preparing, shipping and analyzing samples is highly recommended.
4) Avoid exposure to pre-packaged foods when in contact with designated working areas or when working with samples.

Aluminum foil should be avoided at this time. New boxes of zip-lock bags have been found to be free of contamination. In addition, Post Its® should not be used in sampling area or to label samples, and blue ice should not be used with samples.

Analytical Guidance

I. Sampling

Before sampling, define work area for sampling and clean area with a high purity organic solvent; methanol, acetonitrile or isopropanol are suitable. Work area can be swipe tested before and/or after cleaning to test for contamination.

Before using, evaluate all labware, including containers and their covers, measurements devices or implements that will come in contact with samples for PFOA and/or analytes of interest.
See separate discussion on containers.

When sampling, focus full attention on sampling until work is complete. Avoid moving into other areas of the workplace during sampling.

Field blanks may be required and evaluated for the study.

II. Solvents

Check all solvent lots used during sample preparation and analysis for background to avoid false positives. This includes water when used as a reagent for low level, i.e. ppt, testing as well as a mobile phase modifier in HPLC methods. Certain lots of solvents have been found to be unacceptable for use. Solvents should have background significantly lower than the LOQ for a study e.g. < 0.1 LOQ.

III. Containers

To avoid contamination from sampling equipment and containers, fluoropolymers should be avoided. Carefully cleaned polypropylene and polystyrene sample containers and centrifuge tubes have been satisfactorily used for the analysis of fluorinated compounds.

However, all containers that come in contact with treated and untreated samples must be confirmed to be free of PFOA. Containers that are not free from PFOA should be triple washed in high purity organic solvent, i.e., methanol, acetonitrile or isopropanol, and reconfirmed to be free of PFOA before use.

The use of disposable labware is recommended, i.e., pipets.

IV. Reagent Blanks

Run reagent blanks early in the evaluation process of method development as well with each sample set analyzed. That is, conduct complete sample work-up and analysis on sample solvent, following current method protocol. Reagent blanks should have background significantly lower than the LOQ for a study e.g. <0.1 LOQ.

V. Sample Preparation for Analysis

Sample preparation area(s) should be washed extensively with high purity organic solvents, e.g. methanol, isopropanol or acetonitrile, before and after sample preparation. Conduct all sample preparation work on disposable towels or pads.

Weigh and prepare samples in a separate location from standards – more important for ppb determinations. Optimally, the weigh station and balance should be swipe tested periodically and the swipes extracts analyzed for the test substance to ensure that contamination does not exist.

Wear nitrile gloves when handling samples and change gloves often.

The use of disposable labware is recommended, i.e., pipets.

Ensure that containers used to store samples and solvents used in sample preparation are free of contamination –see discussion on containers, solvents and reagent blanks.

VI. Standards and Standard Preparation

Each standard needs a certificate of analysis with its purity and analytical methods used during its determination. Preferably, the purity of the standard should be evaluated using the same methodology used to analyze samples.

At least five standards should be used to generate the calibration curve, bracketing expected levels in samples. The lowest standard should be at the LOQ for the method.

Standards must be analyzed with each set of samples and are generally injected at start of a sample set - from low to high concentration – and standard analyses are generally repeated throughout and/or at the end of the sample set. Analysis of instrument and reagents blanks should precede standard injections, see example sample sequence on page 8.

If background levels in untreated samples are unacceptable, > 20% LOQ, consider raising the LOQ or standards may need to be made up in matrix and not neat solvent.

Standards are a common source of contamination for samples analyzed at a ppb levels.
Some practices to consider when weighing standards and samples at the same balance:

-cover weigh station area with disposable cloths before weighing samples or standards
-bring standards and samples to weighing station in separate tray
-clean weigh station and balance with methanol after weighing
-wipe test balance for contamination routinely

Optimally, standards should be weighed at a different weighing station/balance than samples.

Solvent blanks should be run throughout a sample sequence to ensure that carry-over is not a concern, e.g. inject solvent blank after high standard injection.

See suggested sample sequence included in section on Method Validation.

VII. Sources of Mobile Phase and Instrument Contamination

If the LC mobile phase shows measurable quantities of PFOA that cannot be reduced with the use of another solvent lot or change in solvent manufacturer, consider protecting the analytical column with a Hypercarb pre-column placed after the HPLC pump but before the injector to collect PFOA. At low levels of detection, PTFE or PTFE-lined containers or equipment must not be used, including PTFE-lined vials for the HPLC autosampler.

VIII. Reagent Blanks

A reagent blank should be preferably considerably less than the LOQ, i.e. $\leq$ 0.1 LOQ, and should be included with each chromatographic run.

IX. Filters

Filtration of the sample may be necessary to remove undissolved solids. This should be done only if necessary, since analyte may be lost due to absorption onto the filter and should be experimentally verified. Polypropylene filter media are preferred, since absorption is generally less than for other materials.

Volatile Fluorinated Compounds

Some fluorinated compounds having low water solubility and relatively high vapor pressure, can escape aqueous solutions in the course of hours. For example, after forty eight hours, less than 10% 8-2 telomer B alcohol was recovered from an aqueous solution having an initial concentration of 130 ng/mL; with a 50% loss determined at about 13 hours. As almost no sorption occurred to glass wall, loss to head space and/or parafilm and/or diffusion through parafilm was suspected.

- Aeration and flow-through test systems will accelerate loss of these analytes.
-When dissolved in water, these analytes will most likely be lost to headspace in closed test and assay vessels.
-Also, if an assay for these analytes from aqueous systems is required, back-extract sample into solvent like MTBE at time of sampling or as soon as possible to better reflect the concentration at that sampling time.
-Mix/shake/vortex samples thoroughly immediately before analysis.
-If the aqueous system contains soil or soil-like particles, recoveries can be higher than expected, as these analytes can preferentially sorb to these particles and be subsequently extracted into organic solvent for quantification.

Guidelines for Method Validation for Analysis of Analytes in Test Systems

These guidelines assumes that methods to assay levels of an analyte is a test system can be less stringent than validation criteria used to assay the active ingredient in a drug formulation or to set a U.S. EPA tolerance for a pesticide in a crop. However, in all cases, in order for a method to be valid, the following must be demonstrated:

Linearity
Accuracy
Precision (reproducibility)
Recovery
LOQ and LOD

For a method to successfully meet the above criteria, the chromatography for the analyte should be well-behaved with:

-good peak shape with minimum band broadening
-adequate retention with good retention reproducibility
-signal to noise ratio of 5:1 at LOQ
-minimal interference from other substances (specificity)
-controls, sludge without sample, having area counts of no more than 20% of the LOQ. Analysis of reagent blanks and solvent blanks should be considerably lower than the LOQ, i.e., $\leq$ 0.1.

The GLP method validation protocol should include the following:

I. Linearity and Accuracy

-A calibration curve should be generated for each analyte in the sample. Using five or more standards prepared in the injection solvent, bracket anticipated concentration range of analyte concentration in sample to create calibration curve. *Lowest standard concentration should correspond to the LOQ*. Calibration curve can include a blank solvent sample with or without internal standard. Samples outside the calibration limits should be re-run by diluting a high sample, concentrating a low sample, or expanding the calibration range.

-Use standard curve fitting, by applying the simplest model that adequately describes the concentration-response relationship, e.g. linear regression with $r^2 \geq 0.992$.

-Accuracy: Standard concentrations defined by curve should be within 20% at the LOQ and within 15% for all other points when compared with actual standard concentration. Duplicate standard injections are preferred – see example of sample sequence below.

II. Recovery and Precision

Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery experiments should be performed by comparing the analytical results/analysis for extracted samples at three concentrations (LOQ, medium and high) with the amount applied. At a minimum, triplicate recovery analyses should be conducted at each concentration.

For example, for an analysis with an LOQ of 5 ppb, set the dosing of control matrix at 5 ppb, 50 ppb and 500 ppb, each analyzed in triplicate.

A. 3 control samples (sludge matrix w/o sample) dosed at LOQ or 5 ppb
B. 3 control samples dosed at 50 ppb
C. 3 control samples dosed at 500 ppb

Actual recoveries should be within 70-120% with RSD (precision / reproducibility) of less than 20%.

III. LOD

Use expanded scale of "solvent blank" to determine three times baseline noise and convert to concentration units using calibration curve. LOD can also be calculated; include reference.

IV. Example Sample Sequence

To maintain quality control, the following sequence of samples is proposed for each analytic run:

Example Sample Analysis Sequence

Instrument blank	-Solvent injection to ensure instrument is clear of interfering substances
Reagent blank	-Blank generated from conducting sample workup without sample
Series of standards	-Injected from lowest to highest concentration
Solvent blank	
Three "A" samples	
Solvent blank*	
Three "B" samples	
Solvent blank*	
Three "C" samples	
Solvent blank*	

Check standards could be injected throughout the run or freshly spiked QC samples at LOQ and 10x LOQ. If used, internal standard should be added after extraction to account for LC/MS/MS matrix effects.

*With experience, number of solvent blanks may be reduced.

Report including validation data should be prepared and raw data retained in the study records, according to GLP guidelines.

V. Sample Analysis

According to GLP guidelines, a protocol for the analysis of samples should be written and approved before sample analysis.

Recovery samples should be analyzed with each analysis set at LOQ, medium and high levels to validate data with each run. Also calibrations standards, instrument blanks, reagent blanks and solvent blanks samples should be analyzed as detailed above with each analysis set.

VI. Frozen Samples: Short-Term Stability

If samples are to be frozen before analysis, three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based upon expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

VII. Frozen Samples: Long-Term Stability

Storage time in a long-term stability evaluation should exceed the time between the data of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of sample should be sufficient for analysis on three separate occasions.