

Chemistry Specifications for Chemistry Services Contractors

National Toxicology Program

Formulations

Final

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1. ***Preliminary Formulation Studies (PFS)***

At the direction of the COR, the contractor shall perform one or more of the following activities:

1. General Requirements

1. The maximum concentration at which solubility will be assessed is assumed to be 600 mg/mL. However, in most cases the COR will specify the highest concentration to be tested, which may differ from 600 mg/mL.
 1. If the test article is not soluble at the specified maximum concentration, and as long as it continues to be insoluble, incremental dilution with additional vehicle will be carried out to a final concentration of 0.50 mg/mL.
 2. If solubility cannot be achieved at a concentration of 0.50 mg/mL, suspendability will be assessed over the same concentration range.
2. One or 2 aliquots of the test article will be used for all solubility and suspendability testing.
 1. For solids to be dissolved in liquids:
 1. One aliquot will be mixed for 30 seconds using vortex mixing.
 2. A second aliquot will be mixed by sonication for 5 minutes.
 3. Both aliquots will be carried through the procedure described in Part 1.2. Solubility Evaluation.
 2. For liquids to be dissolved in liquids
 1. Only one aliquot will be used.
 2. The final volume of the mixed test formulation shall be at least 20 mL.
 3. The aliquot will be mixed for 30 seconds using vortex mixing.
 3. It is acceptable to mix for longer time periods than those specified above with the following stipulations:
 1. Vortex mixing
 1. Times up to 1 minute may be used.
 2. Multiple (repeat) mixing intervals may be used.
 2. Sonication
 1. Times up to 30 minutes may be used

2. Multiple (repeat) mixing intervals may be used.
3. Heating of the sample via sonication must be avoided.
4. When the stability of the chemical or test article is known, formulations may be warmed to a maximum of 60°C, when the stability of the chemical or test article is known, to improve the potential solubility of the constituents. Warmed formulations must be allowed to cool to room temperature prior to the visual assessment of solubility.
5. If the test article is known to have limited solubility in the vehicle, the solubility determination (Part 1.2) may be omitted and the procedure started with the syringeability determination (Part 1.4).
6. All references to suspensions also apply to liquid:liquid emulsions.

2. Solubility Evaluation:

1. Visually estimate the maximum solubility of the test chemical in one or more vehicles, specified by the COR. Vehicles to be tested typically include:
 1. Water
 2. 0.5% aqueous methyl cellulose
 3. 0.2% aqueous methyl cellulose/0.1% Polysorbate 80
 4. Acetone
 5. 95% ethanol
 6. An aqueous emulsifier (e.g., Cremophor™)
 7. Aqueous cyclodextrins
 8. Corn oil
2. Evaluate solubility at room temperature over the concentration range prepared.
 1. Solution Physical Characteristics

Observe and record the physical characteristics of the solution. Note specifically the consistency of the mixture and whether the mixture remains a solution upon standing.
 2. Solution Stability
 1. Store the solution for 24 hours under refrigeration and note any physical changes in the solution.
 2. If precipitation occurs after storage, determine whether warming to

room temperature and mixing can achieve a usable solution.

3. If the test article is soluble at the maximum concentration tested, the Contractor shall evaluate the syringeability of the solution (Part 1.4).
4. If solution takes place after the addition of more vehicle, or at a concentration less than the maximum concentration tested, syringeability (Part 1.4) shall be evaluated at the concentration nearest saturation.

3. Suspendability Evaluation:

1. When the test article has been shown to be insoluble, estimate the maximum suspendability of the test chemical in one or more vehicles, at an initial maximum concentration, specified by the COR (see Part 1.2 for a list of typical vehicles).
2. Evaluate suspensions at the highest suspendable concentration first.
 1. Suspensions shall initially be mixed manually. Once mixed, suspensions shall be stirred with a magnetic stir-bar to maintain the suspension.
 2. Evaluate the resulting mixture, classify it according to the following criteria, and proceed as directed for each suspension class.

1. Paste: Very viscous. Difficult or impossible to stir.

Add more vehicle in small increments until the mixture can be classified as “Thick”, “Dispersed”, or until the test article completely dissolves.

2. Thick: Stirrable, but the chemical is not easily dispersed in vehicle.

1. Prepare a second mixture at the same starting concentration and sonicate for 5 minutes.

1. Evaluate the mixture to determine whether sonication changed the nature of the suspension^{1,2}.
2. Prepare a third mixture at the same starting concentration and mix with a Polytron (or equivalent) for 5 minutes and evaluate the mixture to determine whether the Polytron has changed the nature of the suspension.

3. If sonication or use of a Polytron results in a mixture that can be classified as “Dispersed”, determine the maximum

¹ Liquid:liquid emulsions may not benefit from this treatment.

² Mixing characteristics of the test article in 0.5% aqueous methylcellulose may be improved by the addition of a small amount of Polysorbate 80.

concentration that can be dispensed through a syringe fitted with a gavage needle (Part 1.4).

4. If neither sonication nor use of a Polytron results in a mixture that can be classified as “Dispersed” add more vehicle in small increments to the original mixture until a “Dispersed” suspension is achieved, the test article is completely dissolved, or the lower solubility limit (0.5 mg/mL) is reached.
3. Dispersed: Test chemical is easily dispersed in vehicle giving an essentially uniform suspension with stirring. The mixture may be thick and may settle out rapidly after stirring is stopped. When a formulation is judged to be ‘Dispersed’, determine the syringeability of the formulation (Part 1.4).

3. *Suspension Physical Characteristics*

Observe and record the physical characteristics of the mixtures. Note specifically the consistency of the mixture and the “rate of settling” as fast, average, or slow when the stirring is stopped.

4. *Suspension Stability*

1. If the test article was determined to be suspendable and syringeable after, store the suspension for 24 hours under refrigeration and note any physical changes in the suspension.
2. If settling occurs after refrigeration determine whether warming to room temperature and mixing can achieve a usable suspension.
3. Visually estimate the stability of the dose formulations for 16 days. The COR may direct the Contractor to use a previously developed analytical method to evaluate stability.

4. *Syringeability Evaluation*

1. Syringeability is defined as the maximum gavage formulation concentration that can be dispensed through a syringe fitted with a gavage needle using several needle gauge sizes³.
 1. Syringeability shall be evaluated using 22-gauge gavage needles. If a suspension is not syringeable through a 22-gauge gavage needle, the Contractor shall evaluate successively larger gavage needles until the

³ Syringeability shall only be tested for solutions and “Dispersed” suspensions unless directed by the COR.

solution passes

1. If the mixture is not syringeable through an 18-gauge gavage needle, dilute the suspension with vehicle, stir with a magnetic stir bar, and repeat the syringeability determination with an 18-gauge gavage needle at the lower concentration.
2. If the mixture is still not syringeable, repeat the dilution step until the suspension is determined to be syringeable through an 18-gauge gavage needle and record the concentration.
2. Using the formulation concentration found to be syringeable through an 18-gauge gavage needle, repeat the syringeability determination using 20-, 22-, and 24-gauge gavage needles and record the highest concentration that will successfully pass through all needle sizes tested.
3. A formulation will be judged syringeable if 1–3 mL of the mixture can be drawn and discharged through a gavage needle/syringe assembly while meeting the following specifications:
 1. Time to deliver 1 mL of a formulation shall be \leq 4 seconds.
 2. Formulation must be dispensed within the required time-to-deliver without undue pressure or resistance.
 3. Formulation must be dispensed without noticeable differences in consistency (i.e., test article content) between the original mixture and the withdrawn aliquot, when dispensed within the required time-to-deliver.

5. Additional Requirements

1. Repeat the solubility and suspendability evaluations in Parts 1.2 and 1.3 for each vehicle specified.
2. Record the maximum suspension/solution concentration that gives a stable, syringeable mixture.
3. The COR may direct the Contractor to quantitate any change in formulation concentration resulting from passage through the syringe needle.
4. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

2. Formulation Development (FD)

The Contractor shall design formulation development assignments to support short-term, low cost, toxicology efforts, e.g., 5-day toxicogenomics studies; and

should therefore consider cost as a primary factor.

1. General Requirements

1. When developing formulation methods, the contractor shall be aware that the following vehicles are defaults for each route used in NTP studies:
 1. Drinking water: tap water
 2. Gavage:
 1. Solutions: deionized water
 2. Suspensions: 0.5% methylcellulose in deionized water
 3. Dermal:
 1. 95% ethanol
 2. Acetone/olive oil (4:1) (immunotoxicology, hypersensitivity studies)
 4. IV:
 1. Phosphate-buffered saline (PBS)
 2. Cremophor EL™:ethanol: water, 1:1:8
 5. Corn oil:
 1. Corn oil used for animal studies shall be USP Food Grade or equivalent.
 2. The Contractor shall not use expired corn oil unless approved by the COR.
 6. Feed:
 1. Irradiated NTP-2000 meal (toxicity or carcinogenicity studies)
 2. NIH-07 meal (perinatal, reproductive, or developmental studies)
 3. Lab Diet 5K96 meal (low phytoestrogen, reproductive studies)
 4. The Contractor shall not use expired feed unless approved by the COR.
2. A development plan, including a milestone schedule shall be posted to the NTP IMS and approved by the COR before FD work described in Part 2.2 and following can commence. The development plan shall include estimated cost. The milestone schedule shall include dates for some or all of the following at the direction of the COR.
 1. Commencement of lab work
 2. Completion of lab work

3. Submission of Draft Final report

2. Analysis Requirements

1. At the direction of the COR, the Contractor shall perform the following formulation development work (vehicles include but are not limited to feed, corn oil, acetone, ethanol, drinking water, and methylcellulose solutions) for a chemical or test article.

1. *Method Development*

1. Develop a method for the analysis of dose formulations for a chemical or test article, taking into account the expected dose concentration proposed for a study and its potential degradation products.
2. Typically, analytical ranges vary from 1-500 mg/mL for liquid vehicles to 1-30,000 ppm for feed, with dilution into the analytical range at higher concentrations.
3. For corn oil studies, it must be shown that constituents in the corn oil do not interfere with the analytical method.
4. For dosed feed studies it must be shown that endogenous material from rodent (typically rat) urine and feces at a concentration of approximately 5% (w/w) each does not interfere with the analytical method.
5. The Contractor shall evaluate the linearity of the developed method using a minimum of six vehicle standards prepared in duplicate from two independently weighed stock solutions.
 1. The Contractor shall establish the LLOQ at the lowest concentration with acceptable precision and accuracy, as defined in the development plan. If the LLOQ does not encompass the lowest proposed dose concentrations, the Contractor shall contact the COR before attempting lower the LLOQ.
 2. Standards shall be prepared over the widest possible linear concentration range.
 3. When study formulation samples are expected to fall above the linear range of the method, the Contractor shall confirm that the highest expected dose concentration can be analyzed with precision and accuracy comparable to the vehicle standard curve after it is diluted into the range. Dilution of over-range samples shall be done with vehicle, or a solvent extract of the vehicle.

4. Linearity is confirmed if the correlation coefficient, r is ≥ 0.99 , or the coefficient of determination (weighted curves), r^2 is ≥ 0.98 .
6. The Contractor shall evaluate the recovery of the method using a solvent standard curve prepared at the same concentrations as the vehicle standard curve.

2. *Formulation Development and Homogeneity Evaluation*

1. Formulation Development

1. The Contractor shall develop a method to uniformly formulate a chemical or test article in a designated vehicle.
2. The Contractor shall evaluate the syringeability of solution and suspension formulations, following the requirements described in Section 2.1, Part 1. Preliminary Formulation Studies.
3. Minimum Batch Size Requirements
 1. For liquid vehicles, including suspensions minimum batch size shall be 0.250 L. The Contractor may propose an alternate batch size subject to approval by the COR.
 2. For feed vehicles minimum batch size shall be 2.5 kg. The Contractor may propose an alternate batch size subject to approval by the COR.
4. The Contractor shall avoid the use of serial dilution when preparing dose formulations, unless prior approval is obtained from the COR.

2. Homogeneity Evaluation

1. A dose formulation homogeneity evaluation is required for all dosed-feed formulations and suspensions.
2. Homogeneity shall be evaluated using a validated analytical method, when available. In the absence of a validated method, the method developed under Part 2.2.1 may be used.
3. Calibration for the analysis of homogeneity samples may be performed using a minimum of three independent vehicle standards that bracket the nominal concentration of the study samples (typically: $-\frac{1}{2}X$, X , and $2X$, where X is the nominal concentration of the homogeneity sample).
4. When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diet) are to be used, the Contractor shall conduct formulation

homogeneity studies in both vehicles simultaneously whenever possible, to reduce the time to complete the work and to reduce costs.

5. Homogeneity studies for feed formulations shall be conducted in a V-shell blender. It is acceptable to use alternate mixing procedures for pre-mix preparations.
6. The evaluation shall be conducted at the low concentration standard (LCS) +20% determined for the analytical method (or the lowest dose concentration that is a suspension) and at highest anticipated dose concentration from the supported study(ies) for each chemical or test article in the class according to the following guidelines:
 1. For feed formulations the Contractor shall remove 3 samples for analysis from each of the top right, top left, and bottom ports.
 2. For suspensions the Contractor shall remove 3 samples for analysis from each of the top, middle, and bottom locations within the vessel used to prepare the formulations while it is being continuously stirred.
 3. Samples removed from each port or sampling location shall be analyzed once; replicate analyses are not required.
 4. The Contractor shall calculate the mean, standard deviation, and relative standard deviation for the three samples from each port and for the nine samples together from all the ports.
3. Homogeneity results exceeding the acceptability criteria (Part 2.2.4) Must be approved by the COR prior to commencement of work on other aspects of the dose formulation development assignment

3. *Stability Evaluation*

1. General Requirements

1. Stability studies shall mimic procedures used by the toxicology testing laboratories, and supplies used shall be similar to supplies available at these laboratories.
2. When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diet) are to be used, the Contractor shall conduct storage stability studies in both vehicles simultaneously whenever

possible, to reduce the time to complete the work and to reduce costs.

3. Stability shall be evaluated using a validated analytical method, when available. In the absence of a validated method, the method developed under Part 2.2.1 may be used.
4. Determine the stability for a chemical or test article mixed with the dosing vehicle at a concentration of +20% of the LCS, using the analytical method developed above. The COR may specify that additional or alternate dose concentrations be tested for stability.
5. Calibration for the analysis of stability samples may be performed using a minimum of three independent vehicle standards that bracket the nominal concentration of the study samples (typically – $\frac{1}{2}X$, X , and $2X$, where X is the nominal concentration of the stability sample).

2. Storage Stability

1. The following containers are recommended for each dose vehicle. In some instances, it may be necessary to employ alternate dose vehicle containers if an analytical interference could be introduced into a dosed animal. In these instances, approval must be obtained from the COR.
 1. Feed: plastic bags
 2. Gavage: clear or amber, glass, with Teflon-lined caps
 3. Dermal: glass, amber, Teflon-lined caps
 4. Drinking Water: plastic, polyethylene, and polypropylene⁴
2. Formulation stability shall be determined for up to 23 days under up to three storage conditions, or as directed by the COR.
 1. Samples shall be stored sealed and protected from light under one or more of the following temperature regimes: -20°C , $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and/or ambient. Aqueous formulations shall not be frozen in performance of stability studies unless directed to do so by the COR.

⁴ NOTE: Drinking water container requirements refer to storage containers, not animal drinking-water bottles. Requirements for animal drinking-water bottles are as follows: glass with Teflon-lined screw caps and stainless steel sipper tubes).

2. Reverse stability may be considered with prior approval of the COR.
3. Formulation stability shall be evaluated on days 0, 15±1, and 23±1. An alternate stability schedule may be used, subject to approval by the COR.

3. Dose Simulation

Dose simulation studies are designed to test the stability of the chemical or test article in the formulation, under conditions that approximate the physical, environmental, and handling aspects of dosing. Dose simulation studies are only conducted under specific circumstances, e.g., formulations of volatile test articles, feed formulations for studies of > 5 days, or when specifically directed by the COR.

1. The COR may direct the Contractor to evaluate the stability of a chemical or test article mixed with the dosing vehicle under conditions that simulate the environmental conditions of dosing.
2. Criteria, including volatility, loss on Day 0 of storage stability, reactivity, and light sensitivity, will be used to determine whether a dose simulation evaluation is required.
3. When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diet) are to be used, the Contractor shall conduct dose simulation studies in both vehicles simultaneously when ever possible, to reduce the time to complete the work and to reduce costs.
4. Containers used for these studies shall be similar to those in use at the study laboratories.
5. Individual requirements for each vehicle are given in subparagraphs 1-4 below.
 1. Feed
 1. Duration 7 days.
 2. Use stainless steel powdered diet feeders with followers.
 3. Analyze on days 0, 1, 4, and 7 or 8 in the presence of 5% urine and feces.
 2. Gavage

1. Duration 3 hours.
 2. Use a single screw-cap bottle.
 3. Remove 3, 1-mL replicate samples every 15 minutes for 3 hours. At 3 hours remove 3 replicate 1-mL samples from the formulation remaining in the container.
3. Dermal
 1. “Dosing Bottle” and “Thin Film” stability studies are required.
 2. Dosing Bottle
 1. Samples from each dosing bottle shall be collected as described for gavage dose simulation studies under two storage regimens: (1) left open to air and (2) covered and periodically opened. The residual formulation in the dosing bottles shall be sampled and analyzed without replenishing the vehicle.
 3. Thin Film
 1. Apply a quantitative volume of the dose formulation to three glass Petri dishes. Wait 1, 3, and 5 hours and analyze the amount of chemical or test article remaining after quantitatively transferring the residue to volumetric containers.
 4. Drinking water
 1. Duration 7 days.
 2. All dosed-water animal room stability studies shall be conducted in colorless glass drinking-water bottles with Teflon-lined screw caps and stainless steel sipper tubes.
 3. Samples shall be analyzed on days 0, 1, 4, and 7 or 8.

4. *Acceptance Criteria*

The acceptance criteria shall be set by the COR in consultation with the Contractor at the time of the assignment and documented in the development plan.

3. *Additional Requirements*

1. The Contractor shall obtain COR approval to proceed with homogeneity or

stability evaluations, including dose simulation studies, prior to commencing work on these evaluations.

2. Preliminary indications of inhomogeneity and/or instability shall be reported to the COR as soon as they are found.
3. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

3. Formulation Development and Validation (FDV)

1. General Requirements

1. When developing formulation methods, the contractor shall be aware that the following vehicles are defaults for each route used in NTP studies:
 1. Drinking water: tap water
 2. Gavage:
 1. Solutions: deionized water
 2. Suspensions: 0.5% methylcellulose in deionized water
 3. Dermal:
 1. 95% ethanol
 2. Acetone/olive oil (4:1) (immunotoxicology, hypersensitivity studies)
 4. IV:
 1. Phosphate-buffered saline (PBS)
 2. Cremophor EL™:ethanol: water, 1:1:8
 5. Corn oil:
 1. Corn oil used for animal studies shall be USP Food Grade or equivalent.
 2. The Contractor shall not use expired corn oil unless approved by the COR.
 6. Feed:
 1. Irradiated NTP-2000 meal (toxicity or carcinogenicity studies)
 2. NIH-07 meal (perinatal, reproductive, or developmental studies)
 3. Lab Diet 5K96 meal (low phytoestrogen, reproductive studies)
 4. The Contractor shall not use expired feed unless approved by the COR.

2. A milestone schedule shall be developed and posted to the NTP IMS before the FDV work described in Part 3.1.3 and following can commence. The milestone schedule shall include dates for some or all of the following at the direction of the COR:
 1. Commencement of lab work
 2. Completion of lab work
 3. Completion of Draft Final report
 4. Commencement of QC review
 5. Commencement of QA review
 6. Submission of Draft Final report
3. Formulation development and validation involves three separate, but inter-related activities:
 1. *Method Development (Part 3.2.1) and Validation (Part 3.2.2)*

Develop and/or validate a method for the analysis of dose formulations for each chemical or test article, taking into account the expected concentration of the chemical or test article in the vehicle and its potential degradation products.
 2. *Homogeneity Evaluation (Part 3.2.3)*

Determine the proper procedure for mixing the chemical or test article and vehicle to achieve homogeneous mixtures without introducing artifacts or other contaminants, evaluate homogeneity, and provide specific mixing instructions.
 3. *Stability Evaluation (Part 3.2.4)*

Determine the stability of the chemical or test article mixed with the dosing vehicle at concentrations specified by the COR using a validated analytical method under conditions of long-term storage (*Part 3.2.3.1*) and simulating dosing (*Part 3.2.3.2*).

2. Analysis Requirements

At the direction of the COR, the contractor shall develop and validate an analytical method based on the following requirements.

1. *Method Development*

1. The Contractor shall develop a method for the analysis of a chemical or test article or test article over a concentration range in a vehicle

designated by the COR. The analytical method is expected to cover the dose concentration range proposed for any study that the analysis method would support.

2. Typically, dose concentration ranges vary from 1-500 mg/mL for liquid vehicles to 1-30,000 ppm for feed, with dilution into the analytical range at higher concentrations.
3. For dosed feed studies it must be shown that endogenous material from rodent urine and feces at a w/w concentration of approximately 5% each does not interfere with the analytical methods.

2. *Validation*

1. General Requirements

1. When specified by the COR as part of a FDV assignment, a method validation shall be conducted according to the procedure in Parts 3.2.2.1 – 4). These tests will be conducted over the expected concentration range of the samples to be analyzed, which is typically a concentration equal to the lowest dose that results in an acceptable %RSD value up to at least 110% of the highest dose proposed for the toxicity study. When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diet) are to be used, the Contractor shall develop and validate the analytical method for one type, then cross-validate (Part 3.2.2.3) in the other.
2. The validation must be performed such that it provides the following information:
 1. An indication of the precision, percent relative standard deviation (%RSD), of the method at specified concentrations.
 2. Confirmation by statistical and/or visual inspection that the response versus concentration function is linear, or deterministically non-linear, over the specified concentration range.
 3. An indication of blank vehicle contribution to responses seen in spike vehicle determinations.
 4. An estimate of recovery (percent) of the chemical or test article from vehicle at specified concentrations.
 5. A determination of the accuracy, percent relative error (%RE).

6. Estimates of the measurement limits [i.e. Limit of Detection (LOD), Experimental Limit of Quantitation (ELOQ), and the Limit of Quantitation (LOQ)].
7. A demonstration of the specificity of the analytical method.
3. The vehicle standard curve shall contain a minimum of 6 points, plus a vehicle control.
4. The Contractor shall use a commercially available statistical package to perform the required statistical analysis of the data (Part 3. Calculations).
5. Acceptance criteria are given in Part 3.2.5.

2. Full Validation

A full validation assesses the linearity, recovery, precision and accuracy of the method across the expected dose concentration range, establishes the measurement limits of the analytical method, characterizes any vehicle effects that may influence instrument response and/or recovery, and establishes the blank response for the chemical or test article in the validated vehicle.

1. The contractor shall follow the general procedures found in Part 3.2.2.2.2 – 4, perform the calculations found in Part 3.3, and meet the acceptance criteria in Part 3.4 to validate an analytical method.

2. Solvent Standards Preparation

1. Prepare a series of solvent standards with the same final concentrations (i.e., after any extraction, dilution, or concentration step) as those of the vehicle standards, using two independently prepared stock standards of different concentrations (Stock A and Stock B).
2. Prepare a reagent blank in the same solvent as the solvent standards.
3. Measure the solvent standard responses using the same analytical system used for the vehicle standards.

3. Vehicle Standards Preparation

1. Prepare vehicle standards in triplicate at six different concentrations; using two independently prepared stock standards

of different concentrations (Stock A and Stock B). Use the same Stock A and B standards prepared for the solvent standard curve.

2. Prepare a vehicle blank in triplicate.
3. The concentrations of the vehicle standards shall be arranged so that each standard comes from an alternate stock solution.
4. Measure the response from a single analysis of each vehicle standard and blank.

4. Method Verification

1. When the validated dose formulation concentration range is less than 150% of the highest dose concentration given for the toxicology study, perform a method verification procedure as follows:
 1. Prepare three replicate vehicle standards at a concentration corresponding to the highest expected sample concentration.
 2. Dilute the standards with blank vehicle such that the final concentration lies within the validated analytical sample concentration range. Alternatively, when no vehicle effect has been observed, the standards may be extracted using the validated sample preparation procedure and then diluted with extracting medium until their concentrations are within the validated analytical sample concentration range.
 3. Analyze the diluted method verification standards using the same method as that used for the vehicle and solvent standards that were analyzed for the validation.
2. Calculate the mean concentration, correcting for dilution, the percent relative standard deviation (%RSD), and the percent of the found concentration compared to the nominal concentration. Method verification standards must meet the same criteria as those established for validation vehicle and solvent standards.
5. See Part 3.3. Calculations for required validation calculations and Part 3.4. Acceptance Criteria for typical acceptance values for each calculated parameter.

3. Partial Validation

A partial validation assesses the linearity, recovery, precision and accuracy of the method across the expected dose concentration range,

confirms the method limits found for the fully validated vehicle, characterizes vehicle effects on instrument response and/or recovery, and establishes the blank response for the chemical or test article in the alternate vehicle.

1. The COR may require a full validation to be performed for the chemical or test article in one of the vehicles and a partial validation be performed for the other vehicle(s) when two or more different types of the same vehicle are to be used in a supported study (e.g., NTP-2000, NIH-07 or 5K96 diet).
2. A full validation is run on the primary vehicle first and then once the method passes validation for the primary vehicle (see Part 3.2.5. Acceptance Criteria), the partial validation is run on the alternate vehicle(s) using the procedures given in Parts 3.2.2.3.3 – 5, below.
3. Partial Validation Vehicle Standards Preparation
 1. Prepare three vehicle standards at concentrations bracketing the proposed analytical range (typically > 1 order of magnitude) from a single stock solution and matching the range of the primary vehicle standards.
 2. Prepare 6 replicate standards at a concentration corresponding to the ELOQ for the validated method.
 3. Prepare 3 replicate standards at each of the two higher concentrations.
 4. Prepare a vehicle blank in triplicate.
 5. Sample preparation methods are typically based on those used for the primary vehicle, but may deviate as needed to optimize extraction efficiency and method performance.
 6. Measure the sample response using the same analytical method as the one that was validated for the primary vehicle.
4. Partial Validation Solvent Standards Preparation
 1. Prepare solvent standards with the same final concentrations (i.e., after any extraction, dilution, or concentration step) as the cross validation vehicle standards.
 2. Single replicates of each solvent standard shall be prepared from one stock solution. Use the same standard stock solution (Stock A or Stock B) prepared for the validation (Part 3.2.2.2.2, above), or a

freshly prepared stock at the same concentration, to prepare the solvent standards.

3. Prepare a reagent blank in the same solvent as the solvent standards.
4. Measure the solvent standard responses using the same analytical system used for the cross validation vehicle standards.

5. Partial Validation Method Verification

When the validated dose formulation concentration range is less than 150% of the highest dose concentration given for the toxicology study, perform a Method Verification (Part 3.2.2.2.4).

4. See Part 3.3. Calculations for required validation calculations and Part 3.4. Acceptance Criteria for typical acceptance values for each calculated parameter.

3. *Formulation Development and Homogeneity*

1. Formulation Development

1. The Contractor shall develop a method to uniformly formulate the chemical or test article in a designated vehicle.
2. Minimum Batch Size Requirements
 1. For liquid vehicles minimum batch size shall be 1.0 L. The Contractor may propose an alternate batch size subject to approval by the COR
 2. For feed vehicles minimum batch size shall be 20 kg. The Contractor may propose an alternate batch size subject to approval by the COR.
3. The Contractor shall avoid the use of serial dilution when preparing dose formulations, unless prior approval is obtained from the COR.

2. Homogeneity Evaluation

1. A dose formulation homogeneity evaluation is required for all dosed-feed formulations and suspensions.
2. The analysis shall be done using the validated analytical method; however, Calibration for the analysis of homogeneity samples may be performed using a minimum of three independent vehicle standards that bracket the nominal concentration of the study samples (typically

$-\frac{1}{2}X$, X , and $+\frac{1}{2}X$, where X is the nominal concentration of the homogeneity sample).

3. When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diet) are to be used, the Contractor shall conduct formulation homogeneity studies in both vehicles simultaneously when ever possible, to reduce the time to complete the work and to reduce costs.
4. Homogeneity studies for feed formulations shall be conducted in a V-shell blender. It is acceptable to use alternate mixing procedures for pre-mix preparations.
5. The evaluation shall be conducted at the ELOQ +20% determined for the analytical method (or the lowest dose concentration that is a suspension) and at highest anticipated dose concentration from the supported study(ies) according to the following guidelines:
 1. For feed formulations the Contractor shall remove 3 samples for analysis from each of the top right, top left, and bottom ports.
 2. For suspensions the Contractor shall remove 3 samples from each of the top, middle, and bottom locations within the vessel used to prepare the formulations while it is being continuously stirred.
 3. Samples removed from each port or sampling location shall be analyzed once; replicate analyses are not required.
 4. The Contractor shall calculate the mean, standard deviation, and relative standard deviation for the three samples from each port and for the nine samples together from all the ports.
6. Homogeneity results exceeding the acceptability criteria (Part 3.4), must be approved by the COR prior to commencement of work on other aspects of the dose formulation development assignment.

4. *Stability Evaluation*

1. General Stability Evaluation Requirements

1. Stability studies shall mimic procedures used by the toxicology testing laboratories, and supplies used shall be similar to supplies available at these laboratories.
2. When multiple vehicles of the same type (e.g., NIH-07 and NTP- 2000 diet) are to be used, the Contractor shall conduct storage stability

studies in both vehicles simultaneously when ever possible, to reduce the time to complete the work and to reduce costs.

3. Stability shall be evaluated using the validated analytical method.
 4. Determine the stability for each chemical or test article in the class mixed with the dosing vehicle at a concentration of +20% of the ELOQ established during the limited validation, using the analytical method developed above. The COR may specify that additional or alternate dose concentrations be tested for stability.
 5. Calibration for the analysis of stability samples may be performed using a minimum of three independent standards that bracket the nominal concentration of the study samples (typically $-\frac{1}{2}X$, X , and $+\frac{1}{2}X$, where X is the nominal concentration of the stability sample).
2. Storage Stability
1. The following containers are recommended for each dose vehicle. In some instances, it may be necessary to employ alternate dose vehicle containers if an analytical interference could be introduced into a dosed animal. In these instances, approval must be obtained from the COR.
 1. Feed: plastic bags
 2. Gavage: clear or amber, glass, with Teflon-lined caps
 3. Dermal: glass, amber, Teflon-lined caps
 4. Drinking Water: plastic, polyethylene, and polypropylene⁵
 2. Formulation stability shall be measured on days 0, 7, 14, 21, 35, and 42 ± 1 day for all vehicles.
 1. Samples shall be stored sealed and protected from light at -20 and $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and ambient temperature. Aqueous formulations shall not be frozen in performance of stability studies unless directed to do so by the COR.
 2. An alternate stability schedule may be used, subject to approval by the COR.
3. Dose Simulation

⁵ Drinking water container requirements refer to storage containers, not animal drinking-water bottles. Requirements for animal drinking-water bottles are as follows: glass with Teflon-lined screw caps and stainless steel sipper tubes).

Dose simulation studies are designed to test the stability of the chemical or test article under conditions that approximate the physical, environmental, and handling aspects of dosing.

1. Determine the stability of each chemical or test article in the class mixed with the dosing vehicle under conditions that simulate the environmental conditions of dosing.
2. When multiple vehicles of the same type (e.g., NIH-07 and NTP-2000 diet) are to be used, the Contractor shall conduct dose simulation studies in both vehicles simultaneously when ever possible, to reduce the time to complete the work and to reduce costs.
3. Containers used for these studies shall be similar to those in use at the study laboratories. Individual requirements for each vehicle are given in subparagraphs 1-4 below.

1. Feed

1. Duration 7 days.
2. Use stainless steel powdered diet feeders with followers.
3. Analyze on days 0, 1, 4, and 7 or 8 in the presence of 5% urine and feces (typically rat).

2. Gavage

1. Duration 3 hours.
2. Use a single screw-cap bottle.
3. Remove 3, 1-mL replicate samples every 15 minutes for 3 hours. At 3 hours remove 3 replicate 1-mL samples from the formulation remaining in the container.

3. Dermal

1. "Dosing Bottle" and "Thin Film" stability studies are required.

2. Dosing Bottle

Samples from each bottle shall be collected as described for gavage dose simulation studies under two storage regimens: (1) left open to air and (2) covered and periodically opened. The residual formulation in the dosing bottles shall be sampled and analyzed without replenishing the vehicle.

3. Thin Film

1. Apply a quantitative volume of the dose formulation to three glass Petri dishes.
 2. Wait 1, 3, and 5 hours and analyze the amount of chemical or test article remaining after quantitatively transferring the residue to volumetric containers.
4. Drinking water
1. Duration 7 days.
 2. All dosed-water animal room stability studies shall be conducted in colorless glass drinking-water bottles with Teflon-lined screw caps and stainless steel sipper tubes.
 3. Analyze on days 0, 1, 4, and 7 or 8.

3. Calculations

1. The contractor shall use curve-fitting techniques employing a linear regression model with or without weighting to determine the best fit line for the vehicle and solvent standards from the full validation and/or cross validation study. Under certain circumstances it may be possible to demonstrate that the analytical system is reproducibly non-linear. In these cases, with the approval of the COR, the contractor may utilize a non-linear regression model (e.g., power curve). Once the model has been chosen, the contractor shall perform the following calculations to establish the best fit line:
 1. Calculate the regression equations for the vehicle and solvent standard curves. Do not correct the response for experimental vehicle or solvent blank values. For spectrophotometric (i.e., UV/Vis) determinations, zero the instrument using the solvent only, and then compare the blank value with the Y-intercept obtained from the regression equation.
 2. Calculate the correlation coefficient r for the solvent and vehicle standard curve data. With the approval of the COR, the coefficient of determination, r^2 , may be calculated when it is a better measure of goodness of fit.
2. The contractor shall use the responses from the solvent standards and replicate vehicle standards from the full and/or cross validation study to calculate the precision, recovery, relative error, and measurement limits of the method as follows:

1. To estimate precision, calculate the mean and percent relative standard deviation (%RSD) for each vehicle standard analyzed in triplicate.

$$\%RSD = s_{(n-1)} \div Y_{V\text{Bar}} \times 100 \quad [1]$$

Where $s_{(n-1)}$ is the standard deviation and $Y_{V\text{Bar}}$ is the average of each vehicle standard analyzed in triplicate

2. Determine the percent recovery at each concentration according to the following equation:

$$\%Recovery = [(Y_{V_{ij}} - Y_{V\text{Bar-blank}}) \div (Y_{S_{ij}} - Y_{S\text{Bar-blank}})] \times 100 \quad [2]$$

Where $Y_{V_{ij}}$ is the response for each of the 18 vehicle standards and $Y_{S_{ij}}$ is the response for each of the 6 solvent standards, $Y_{V\text{Bar-blank}}$ is the mean response for the vehicle blank and $Y_{S\text{Bar-blank}}$ is the response for the solvent blank. Do not calculate recovery for blanks.

3. Determine the relative error of each determined (calculated) concentration compared with each theoretical (prepared) concentration as follows:

1. Calculate the concentration from the measured responses for the vehicle standards using the regression equation.
2. Calculate the relative error using the following equation:

$$\%Relative\ Error = [(X_{V_{d_{ij}}} - X_{V_{t_{ij}}}) \div X_{V_{t_{ij}}}] \times 100 \quad [3]$$

Where $X_{V_{t_{ij}}}$ is the theoretical concentration of each vehicle standard and $X_{V_{d_{ij}}}$ is the determined concentration of each vehicle standard calculated from the regression equation.

3. For vehicle standards prepared and analyzed in triplicate, compute the relative error for the mean response at each concentration in addition to that for the individual standards.
4. Determine the measurement limits as defined below:
 1. LOD shall be defined as 3 times the standard deviation of the vehicle blank or the lowest standard, expressed as concentration.
 2. LOQ shall be defined as 10 times the standard deviation of the vehicle blank or lowest standard, expressed as concentration.
 3. LLOQ shall be defined as the concentration of the lowest standard that meets acceptability criteria of the assay (normally $\pm 10\%$ relative error, with a relative standard deviation of $\leq 10\%$).

4. Acceptance Criteria

The following acceptance criteria must be met for an analytical dose formulation method to be considered valid. Deviations from the typical acceptance values listed below require the prior approval of the COR.

1. Validation

1. Linearity or Conformance to the Model

1. Standard curve fitting is determined by applying the simplest model that adequately describes the concentration–response relationship using appropriate weighting and statistical tests for goodness of fit.
2. The correlation coefficient r shall be ≥ 0.99 for linear fits.
 1. When coefficient of determination r^2 is an appropriate measure of goodness of fit, it must be ≥ 0.98 .
 2. To achieve an acceptable fit, a data point may be discarded only after it has been shown to be statistically valid to do so. Normally this involves a Dixon's Q-test of all the replicates at that concentration (http://en.wikipedia.org/wiki/Dixon%27s_Q_test).

2. Accuracy (Relative Error)

1. Relative error shall be $\leq \pm 10\%$ of the nominal value.
2. Higher relative error values may be accepted but will require the approval of the COR.

3. Precision (Relative Standard Deviation)

1. Relative standard deviation shall be $\leq \pm 5\%$ of the mean found value.
2. Higher relative standard deviation values may be accepted but require the approval of the COR.

4. Recovery

1. The method will be considered to be acceptable when recovery at each vehicle-standard concentration is within $\pm 20\%$ of the target value.
2. When recovery is $\leq 80\%$ or $\geq 120\%$ at any vehicle-standard concentration, the COR must be consulted for approval.

5. Measurement Limits

Acceptability criteria for measurement limits are dependent on the study and will be provided by the COR on a case-by-case basis.

2. *Homogeneity*

1. A formulation shall be considered homogeneous if the relative standard deviation of samples taken from an individual blender port (top-right, top-left, and bottom) or sampling locations is less than $\pm 5\%$ and for the nine samples taken together does not exceed $\pm 5\%$.
2. The found formulation concentration of all samples must be within 10% of the theoretical value.

3. *Stability*

1. A formulation shall be considered stable at a particular time point if the found concentration is not statistically different than the Day 0 determined concentration.
2. When the deviation of the found concentration exceeds the statistical limit, the formulation may be considered to be stable if the found value is within 10% of the Day 0 found concentration.
3. When the found concentration on Day 0 differs from the theoretical (prepared) value by $> \pm 10\%$, the COR shall be immediately notified.

5. *Additional Requirements*

1. Preliminary indications of inhomogeneity and/or instability shall be reported to the COR as soon as they are found.
2. The Contractor shall obtain COR approval to proceed with homogeneity or stability evaluations prior to commencing work on these evaluations.
3. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

4. ***Formulation Preparation (FP)***

1. As directed by the COR, the contractor shall prepare dose formulations of chemical or test article in vehicles including but not limited to feed, water, 0.5% aqueous methylcellulose, 95% ethanol, acetone, an aqueous emulsifier (e.g., Cremophor™), acetone:olive oil (4:1), or corn oil, using previously developed mixing methods.
2. If no mixing protocol exists, the contractor shall develop a mixing procedure and submit it to the COR for approval prior to preparing the formulation.

3. The Contractor shall label all prepared formulations with information that clearly differentiates the species and strain for which the dose is intended and each dose concentration; and differentiates dosed vehicle from control. Labels shall employ color-codes and shapes in addition to printed information to aid in identifying each dose.
4. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

5. Formulation Analysis (FA)

1. General Requirements

1. At the direction of the COR, the contractor shall perform analyses of submitted dose formulation samples using previously validated methods and following the requirements in Part 5.2.
2. When samples are to be received from an NTP laboratory or researcher, the COR will provide the sample submission schedule to the Contractor when it is available.

2. Analysis Requirements

1. Formulation Analysis

1. The Contractor shall analyze formulation samples within 1 week of the receipt of the samples.
2. The Contractor shall use a method previously validated over the concentration range of the samples.
 1. If dose formulation concentrations are higher than the range that has been validated, the formulations shall be diluted into the validated analytical concentration range.
 2. If dose formulation concentrations are lower than the range that has been validated, the method shall be revalidated over the new concentration range before the formulations are analyzed.
3. Analysis of dose formulations shall be conducted using the following procedures:
 1. Prepare two standard stock solutions of the test article from independently weighed samples. Use these solutions to prepare six vehicle standards at concentrations bracketing the concentration range expected for the samples such that alternate standards are prepared from each stock solution.

2. If samples are to be analyzed from a single dose concentration, a single stock solution shall be prepared. Use this solution to prepare three vehicle standards at concentrations bracketing the concentration of the sample.
3. Analyze the vehicle standards and a vehicle blank. Duplicate analyses of vehicle standards are not required.
4. Calculate the regression equation and the correlation coefficient. Compare this analysis with all previous analyses and demonstrate that the method is in control. The correlation coefficient (r) must be ≥ 0.99 .
5. Analyze dose formulation samples in triplicate.
6. Use the regression equation to compute the concentration of the unknowns.
7. For chromatographic analyses, only single injections are required.

2. *Referee Analysis*

1. Occasionally it may be necessary to evaluate inter-laboratory variability of a particular analytical method. In such cases the Contractor shall analyze a sample set supplied at the direction of the NTP using a validated method.
2. Within 2 weeks of receipt of the samples, the Contractor shall analyze them following the requirements in the validated analysis method.

3. Additional Requirements

1. Evaluate the suitability of the analytical system used for the analysis relative to precision, theoretical plates, resolution, and tailing factor.
2. The percent relative standard deviation (%RSD) of the triplicate formulation analyses shall be calculated. If the %RSD is $> 5\%$ and an outlier is suspected, Dixon's Q test shall be run. If $Q_{\text{calc}} > Q_{90}$ the outlier can be rejected. If the suspect value cannot be rejected, the Contractor shall analyze a fourth aliquot and report the results of all 4 aliquots to the COR.
3. Results that deviate from the theoretical concentration by $>10\%$ will be considered out of tolerance. There may be cases where a 10% tolerance limit cannot be attained; these will be addressed on an individual basis and must be approved in advance by the COR. If the dose formulation is found to be out of tolerance, the COR and the toxicology laboratory or researcher that submitted the samples shall be immediately notified.

4. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

6. Formulation Preparation and Analysis (FPA)

1. At the direction of the COR, the contractor shall prepare dose formulations of test chemical or test article in vehicles including but not limited to feed, water, 0.5% aqueous methylcellulose, 95% ethanol, acetone, an aqueous emulsifier (e.g., Cremophor), or corn oil using previously developed methods.
2. The Contractor shall label all prepared formulations with information that clearly differentiates the species and strain for which the dose is intended and each dose concentration; and differentiates dosed vehicle from control. Labels shall employ color-codes and shapes in addition to printed information to aid in identifying each dose
3. The Contractor shall remove 2, minimum 35-mL aliquots from each formulation. One aliquot shall be stored frozen or refrigerated (based on minimum storage temperature used in the stability evaluation), and the other shall be designated for analysis.
4. The Contractor shall analyze formulation samples within 1 week of the preparation of the samples, following the requirements listed in Part 5. Formulation Analysis. If animal room samples are anticipated, the Contractor shall analyze them within 1 week, but not to exceed the period of use for the original formulations.
5. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

7. Formulation Preparation, Analysis, and Shipment (FPAS)

At the direction of the COR, the Contractor shall perform one or more of the following: formulation preparation (Part 7.1), formulation analysis (Part 7.2), and/or formulation shipment (Part 7.3).

1. Formulation Preparation

1. At the direction of the COR, the Contractor shall prepare dose formulations for one or more chemicals or test articles in vehicles including, but not limited to, feed, water, 0.5% aqueous methylcellulose, 95% ethanol, acetone, dimethylsulfoxide (DMSO), an aqueous emulsifier (e.g., Cremophor), corn oil, and/or acetone:olive oil (4:1) using previously developed methods.
2. The Contractor shall label all prepared formulations with information that clearly differentiates the species and strain for which the dose is intended

and each dose concentration; and differentiates dosed vehicle from control. Labels shall employ color-codes and shapes in addition to printed information to aid in identifying each dose.

3. The Contractor shall remove 2, minimum 35-mL aliquots from each formulation prepared. One aliquot shall be stored frozen or refrigerated (based on minimum storage temperature used in the stability evaluation), and the other shall be designated for analysis.

2. Formulation Analysis

1. At the direction of the COR, the Contractor shall analyze dose formulations for one or more chemicals or test articles in vehicles within 2 days of the preparation of the formulations. If animal room samples are anticipated, the Contractor shall analyze them within 1 week, but not to exceed the period of use for the original formulations.
2. The Contractor shall use a previously developed method, which covers the concentration range of the samples to be analyzed. The Contractor shall notify the COR if the concentration of the samples to be analyzed differs from the concentration range of the method.
3. Analysis of dose formulations shall be conducted using the following procedures:
 1. Evaluate the suitability of the analytical system used for the analysis relative to precision, theoretical plates, resolution, and tailing factor.
 2. Calibration for the analysis of stability samples may be performed using a minimum of six independent vehicle standards that bracket the nominal concentration of the formulations.
 1. If samples are to be analyzed from a single dose concentration calibration for the analysis of stability samples may be performed using a minimum of three independent standards that bracket the nominal concentration of the study samples (typically $-\frac{1}{2}X$, X , and $+\frac{1}{2}X$, where X is the nominal concentration of the stability sample).
 3. Analyze the spiked vehicle standards and a vehicle blank. Duplicate analyses of spiked vehicle standards are not required.
 4. Calculate the regression equation and the correlation coefficient. Compare the regression equation for this analysis with previous analyses to demonstrate that the method is in control.
 5. Analyze the dose formulation samples in triplicate.

6. For chromatographic analyses, only single injections are required.
7. Use the regression equation to compute the concentration of the dose formulation samples.

3. Formulation Shipment

1. As directed by the COR, the contractor shall aliquot, package, and ship prepared formulations and blank vehicle to a toxicology laboratory or Investigator designated by the COR.
2. The Contractor shall notify the recipient of its intent to ship.
3. Sample information as directed by the COR, shall be provided with all formulations shipped by the Contractor to any NTP laboratory.
4. All shipments shall conform to the requirements given in Section 3. Health and Safety Minimum Requirements and all applicable local, state, and federal regulations.

4. Acceptance Criteria

1. The mean of the determined concentrations for triplicate formulation analyses at a single dose shall be $\leq \pm 10\%$ of the theoretical value.
2. The relative standard deviation (%RSD) of the determined concentrations for triplicate formulation analyses at a single dose shall be $\leq 5\%$.
3. The COR may specify alternate mean and %RSD values to be met, based on the analytical method used.

5. Additional Requirements

1. The Contractor shall report dose formulations that do not meet the acceptance criteria to the COR as soon as the information is known.
2. The COR may direct the Contractor to reprepare formulations that do not meet the acceptance criteria. When a formulation is reprepared, the deadlines for analysis and shipment for that formulation shall be based on its reformulation date.
3. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

8. **Vehicle Analysis (VA)**

1. As directed by the COR, the Contractor shall procure and perform analyses of dosing vehicles used in toxicity studies. These analyses shall be used to determine the identity and purity of the vehicles used in dose formulations.

2. Vehicle specifications:

1. Corn oil:

1. The Contractor shall procure and analyze batches of corn oil that will be used in the Toxicology Testing Program.
2. Each batch of corn oil shall be analyzed for peroxides upon receipt at the Contractor's laboratory and at 12 ± 2 -week intervals thereafter, while it is in use. Corn oil with a peroxide value ≥ 3.0 meq/L may not be used for any studies performed under, or supported by this contract.
3. Corn oil labeled with a Prop 65 warning label (<http://oehha.ca.gov/prop65.html>) shall be analyzed for metals content using an approved, Prop 65 analysis method, or equivalent.
4. Corn oil must be purchased in batches no smaller than 2 gallons (7 kg) per chemical or test article for which it is the dose vehicle, unless substantially less than this quantity is being used monthly. The COR must approve purchase of lesser quantities.
5. Corn oil shall be stored at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$ protected from light.

2. Olive oil:

1. The Contractor shall procure and analyze batches of olive oil that will be used in the NTP Testing Program.
2. Each batch of olive oil shall be analyzed for peroxides upon receipt at the Contractor's laboratory and at 12 ± 2 -week intervals thereafter, while it is in use. Refined olive oil with a peroxide value ≥ 5 meq/kg oil shall not be used for any studies performed under or supported by this contract.
3. Each batch of olive oil shall be analyzed for free acidity, expressed as oleic acid, upon receipt at the Contractor's laboratory and at 12 ± 2 -week intervals thereafter, while it is in use. Olive oil with a free acidity value of > 2 g/100 g oil may not be used for any studies performed under, or supported by this contract.
4. Olive oil shall be stored at $4 \pm 2^{\circ}\text{C}$, under nitrogen headspace, protected from light. Olive oil may be stored at these conditions as long as the measured peroxide and free acid values remain within $\pm 10\%$ of their Day 0 values.

3. Other vehicles:

1. The Contractor shall procure and analyze ethanol, acetone, methylcellulose, or other vehicle that will be used in the Toxicology Testing Program. Each lot shall be analyzed on receipt at the Contractors' laboratories and at 6-month intervals thereafter, while it is in use.
 1. Ethanol used in this program shall be 95% ethanol:water or anhydrous synthetic ethanol, dried over molecular sieves.
 1. The benzene content of ethanol used in this program must be confirmed by an independent analysis of the material (Part 8.7.3. Benzene Screening Analysis).
 2. The preferred concentration for ethanol to be used as a vehicle is 95% ethanol.
 2. Acetone, CASRN: 67-64-1, used in this program shall be USP-NF grade, e.g., Sigma Product No. 650501, Spectrum Product No. HP402, or equivalent.
 3. Methylcellulose, CASRN: 9004-67-5, used in this program shall be 4000 cPs cellulose methyl ester, e.g., Sigma Product No. M0512, Spectrum Product No. ME137, or equivalent.
 4. Feed, used in this program shall be irradiated, NTP 2000, NIH-07, or Lab Diet 5K96 meal for dosed feed studies. The COR may require that feed be analyzed for exogenous materials, including but not limited to, metals, nitrosamines, dioxins and related compounds, mycotoxins, and/or environmental estrogens.
3. Regular shipments of aliquots of vehicles to the toxicology laboratory may be required [see Functional Activity: Shipment (SHIP)].
4. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.
5. Suggested analysis protocols are described below. Deviations from the suggested protocols require prior approval of the COR, which will require the Contractor to demonstrate the precision, accuracy and linearity of the alternate approach.
 1. *Corn Oil Peroxide Analysis*
 1. General Requirements

1. The following method is a standard analytical procedure, derived from the official method (Cd 8-53) of the American Oil Chemists Society (AOCS). (1972)⁶
2. An alternate method may be used, but the COR must approve its use.
3. The method measures all substances that oxidize aqueous iodide under the conditions of the test. These substances are generally assumed to be peroxides or other similar products of fat oxidation.
4. Results of the method are reported in units of milliequivalents (meq) of peroxide per 1000 g of sample.
5. The method is highly empirical and any variation in the procedure described below may adversely affect the results.

2. Procedure

1. Quantitatively transfer a 5.0-g sample of the corn oil to be analyzed to a 250-mL titration vessel and add 30 mL glacial acetic acid:chloroform (60:40 v/v).
2. Stir the mixture until the corn oil has completely dissolved.
3. Add 0.50 mL of a saturated aqueous potassium iodide solution.
4. Stir the test solution thoroughly and allow it to stand for exactly 1 minute.
5. Immediately add 30 mL distilled water.
6. Potentiometrically titrate the iodine liberated by the peroxides in this solution using standardized 0.005N sodium thiosulfate solution, stirring vigorously to ensure thorough mixing.
7. Use an automatic titrator with a platinum disk working electrode and a silver-silver chloride reference electrode to perform the titration.
8. Titrations shall be run in triplicate.
9. A reagent blank shall be titrated on the same day the oil sample is analyzed.
10. If a reagent blank titration consumes > 1.0 mL of 0.005 N sodium thiosulfate standard solution, the peroxide determination must be repeated.

⁶ The AOCS method uses 0.1N sodium thiosulfate and a colorimetric endpoint. Note: AOCS method Cd 8-53 has been replaced with Cd 8b-90, which replaces chloroform with iso-octane.

11. Peroxide number, expressed in meq of peroxide per kg oil (meg/kg), is calculated as shown in equation 1:

$$[(V - B) \times N \times 1000] \div W \quad [1]$$

where:

V = volume (mL) of thiosulfate solution required for the titration of the oil sample

B = volume (mL) of thiosulfate solution required for the titration of the reagent blank.

N = normality of sodium thiosulfate solution
W = mass of the oil sample in grams

3. Additional Requirements

1. The Contractor shall develop a SOP based on this methodology, which shall be in force prior to the first use of the method. The SOP shall be submitted to the COR in accordance with the requirements given in Section 4. Reporting Requirements
2. Under no circumstances will rancid corn oil be administered to any animals used in this Contract.

4. Acceptance Criteria

1. Corn oil with a peroxide number ≥ 3.00 meq/kg shall be considered rancid for the purposes of this Contract and must be replaced immediately.

2. *Olive Oil Peroxide Analysis*

1. General Requirements

1. The following method is a standard analytical procedure, derived from the official method (Cd 8-53) from the American Oil Chemists Society (AOCS). (1972)⁶.
2. An alternate method may be used, but the COR must approve its use.
3. The method measures all substances that oxidize aqueous iodide under the conditions of the test. These substances are generally assumed to be peroxides or other similar products of fat oxidation.
4. Results of the method are reported in units of milliequivalents (meq) of peroxide per 1000 g of sample.

5. The method is highly empirical and any variation in the procedure described below may adversely affect the results.
 2. Procedure (Refer to Part 8.5.1.2, peroxide analysis for corn oil).
 3. Additional Requirements
 1. The Contractor shall develop a SOP based on this methodology, which shall be in force prior to the first use of the method. The SOP shall be submitted to the COR in accordance with the requirements given in Section 4. Reporting Requirements.
 2. Under no circumstances will olive oil that fails to meet the acceptability criteria be administered to any animals used in this Contract.
 4. Acceptance Criteria
 1. Corn oil with a peroxide number ≥ 3.0 meq/kg shall be considered rancid for the purposes of this Contract and must be replaced immediately.
3. *Olive Oil Free Fatty Acid Analysis*
1. General Requirements
 1. The following method is a standard analytical procedure, derived from the official method (Cd 3d-63) from the American Oil Chemists Society (AOCS), 2009.
 2. An alternate method may be used, but the COR must approve its use.
 2. Apparatus and Reagents
 1. Glass electrode: Calomel electrode pH meter.
 2. Alkali: Potassium hydroxide (KOH), 0.1 M—Reagent grade KOH having a carbonate specification of 0.5% max, or 0.1 M KOH with NIST traceable standardization to ± 1 part in 1000 in water, methanol, or ethanol.
 3. Solvent mixture: Equal parts by volume of isopropyl alcohol and toluene.
 3. Procedure
 1. Weigh 15.00 g of well-mixed olive oil into a 250 mL beaker.

2. Add 125 mL of the solvent mixture to the olive oil in the flask. Be sure that the test portion is completely dissolved before titrating. It is acceptable to warm the sample to achieve complete dissolution.
 3. Mount the beaker in the titration assembly (titrator) so that the electrodes are half immersed.
 4. Start the stirrer and operate at speeds that will give vigorous agitation without splashing.
 5. Titrate with standardized alkali. After each addition of alkali, wait until the meter reading is essentially constant, then record burette and meter readings graphically.
 1. Limit incremental additions of alkali so that changes in the meter readings are $\sim \leq 0.5$ pH units. When inflections in the titration occur, add alkali in 0.05 mL increments.
 6. Perform a blank titration using 125 mL of the neutralized solvent mixture.
4. Calculations
1. Acid value, mg KOH/g of olive oil $[(A - B) \times M \times 56.1] \div W$ [2] Where: A = volume, mL of standard alkali used in the titration
B = volume, mL of standard alkali used in titrating the blank
M = molarity of the standard alkali
W = mass of the oil sample in grams
 2. To express the acid value in terms of free fatty acids as percent oleic acid, divide the acid value obtained from Equation 2 by 1.99.
5. Acceptability Criteria
- The acid value, express as oleic acid (%m/m) shall be $\leq 1.0\%$.
6. Additional Requirements
1. The Contractor shall develop a SOP based on this methodology, which shall be in force prior to the first use of the method. The SOP shall be submitted to the COR in accordance with the requirements given in Section 4. Reporting Requirements.
 2. Under no circumstances will olive oil that fails to meet the acceptability criteria be administered to any animals used in this Contract. Olive oil with an acid value ≥ 1.0 shall be considered rancid for the purposes of this Contract and must be replaced immediately.

4. Ethanol Analysis

1. Identity by Infrared Spectrophotometry

1. Prepare a thin film of the sample by placing 1–2 drops between silver chloride plates.
2. Obtain an IR spectrum of the sample from 600–4000 cm⁻¹ using a suitable infrared spectrophotometer.

2. Purity Assessment by Chromatography

1. Sample Preparation

1. Prepare 2 test article (ethanol) solutions with an appropriate internal standard (e.g., cyclohexane) and an internal standard blank.
 1. Solution A: Prepared at ~0.5% ethanol v/v in water
 2. Solution B: Prepared at ~99.5% ethanol v/v in water
 3. Internal Standard Blank: ~0.5% cyclohexane v/v in water

2. Analysis

1. Evaluate the analytical system used for the assay for precision, theoretical plates, resolution, and tailing factor, according to USP guidelines.
2. Analyze solutions A and B, the internal standard blank, a portion of the neat test article, and a water blank using the instrument and parameters described below.
3. Instrument System and Parameters

Instrument: Gas chromatograph with autosampler

Column: DB-Wax, 30 m x 0.53 mm ID, 1-mm film thickness, fused silica

Temperature Program: 40°C (5-min hold) to 220°C (5-min hold) at 10°C/min.

Injection volume: 1 µL Mode: Direct

Detector: Flame ionization Attenuation: 32 x 10⁻¹¹

Temperatures: Inlet: 150°C
Detector: 220°C

Flow Rate: 10 mL/min

Makeup gas:	Nitrogen	Flow Rate: 20 mL/min
	Air	Flow Rate: 300 mL/min
	Hydrogen	Flow Rate: 30 mL/min
Retention times:		
	Ethanol:	2.3 minutes
	Internal Standard:	9.9 minutes

4. Set the attenuation so that a 60–80% pen deflection is obtained for the internal standard peak (approximately 32×10^{-11} AFS).
5. Use the chromatograms from injection of the neat ethanol sample and Solution A to correlate observed peaks with their respective retention times.
6. Use the chromatograms from the neat ethanol sample and the water blank to determine that there are no interferences on the internal standard peak.

3. Calculations

1. Calculate the response factor (RRF_A) for the ethanol peak observed from Solution A using equation 2:

$$RRF_A = (\text{Peak area of ethanol} \times \text{dilution factor}) \div \text{Peak area of internal standard} \quad [2]$$

2. Calculate the relative response factors (RRF_i) for each impurity observed from an injection of Solution B using equation 3:

$$RRF_i = (\text{Peak area of impurity}) \div \text{Peak area of internal standard} \quad [3]$$

3. Calculate the relative concentration of each impurity in the sample of ethanol using equation 4:

$$\text{Relative concentration (\%)} = (RRF_i \div RRF_A) \times 100 \quad [4]$$

3. Benzene Content Screening Analysis

Ethanol used in this program must be screened for benzene content.

1. Prepare a series of benzene standards at concentrations of 1–10 ppm in a suitable solvent.
2. Analysis
 1. Evaluate the analytical system used for the assay for precision, theoretical plates, resolution, and tailing factor, according to USP guidelines.

2. Analyze the benzene standards and a sample of the neat test article using the instrument system described below.
3. Instrument System and Parameters

Instrument: Gas chromatograph with autosampler

Column: DB-5, 30 m x 0.53 mm, 1.5 mm film thickness, fused silica

Temperature program: 40°C (3-min hold) to 200°C (3-min hold) at 10°C/min.

Injection volume: 1 mL Mode: Direct

Detector: Flame ionization

Temperatures: Inlet: 150°C Detector: 220°C

Carrier gas: Helium Flow Rate: 10 mL/min

Makeup gas: Nitrogen Flow Rate: 20 mL/min

Air Flow Rate: ~300 mL/min

Hydrogen Flow Rate: ~30 mL/min

4. Acceptability Criteria

1. Chromatographic purity of ethanol used in this program must be $\geq 99.9\%$, excluding the water content.
2. Benzene content of ethanol used in this program shall be < 0.01 ppm.
3. The IR spectrum of ethanol used in this program must match a library reference IR spectrum for ethanol.

5. *Acetone Analysis*

1. Identity by IR Spectrophotometry

1. Place 5–6 drops of acetone into an IR gas cell equipped with sodium chloride windows and allow the sample to volatilize.
2. Obtain a spectrum for the sample from 600–4000 cm^{-1} using a suitable IR spectrophotometer.

2. Purity Assessment by Chromatography

1. Sample Preparation

1. Prepare and analyze two test article (acetone) solutions (A and B), including an internal standard.

1. Prepare Solution A by volumetrically pipetting 0.5 mL of test article and 0.5 mL cyclohexanone (internal standard) into a 100-mL volumetric flask. Dilute the contents of the flask to volume with water and mix by inversion.
 2. Prepare Solution B by delivering a 0.5-mL portion of cyclohexanone into a 100-mL volumetric flask. Dilute the contents of the flask with test article and mix by inversion.
 2. Prepare an internal standard blank by pipetting 0.5 mL cyclohexanone into a 100-mL volumetric flask. Dilute the contents of the flask to volume with water and mix by inversion.
2. Analysis
1. Evaluate the analytical system used for the assay for precision, theoretical plates, resolution, and tailing factor, according to USP guidelines.
 2. Analyze Solutions A and B, the internal standard blank, a portion of the neat test article, and a water blank using the instrument and parameters described below.
3. Instrument System and Parameters

Instrument: Gas chromatograph with autosampler	
Column: DB-Wax, 30 m x 0.53 mm ID, 1-mm film thickness, fused silica	
Temperature program: 40°C (5-min hold) to 220°C (5-min hold) at 10°C/min.	
Injection volume: 1 µL Mode: Direct	
Detector: Flame ionization	
Temperatures: Inlet: 150°C Detector: 220°C	
Carrier Gas:	Helium Flow Rate: ~10 mL/min
Makeup Gas:	Nitrogen Flow Rate: ~20 mL/min
	Air Flow Rate: ~300 mL/min
	Hydrogen Flow Rate: ~30 mL/min
Retention Times:	Acetone: ~1.2 minutes
	Internal standard: 9.9 minutes

4. Use the chromatograms from injection of the neat acetone sample and Solution A to correlate observed peaks with their respective retention times.
5. Use the chromatograms from the neat acetone sample and the water blank to determine that there are no interferences on the internal standard peak.

3. Calculations

1. Calculate the response factor (RRF_A) for the acetone peak observed from Solution A using equation 2:

$$RRF = (\text{Peak area of acetone} \times \text{dilution factor}) \div \text{Peak area of internal standard} \quad [2]$$

2. Calculate the relative response factors (RRF_i) for each impurity observed from an injection of solution B using equation 3:

$$RRF = (\text{Peak area of impurity}) \div \text{Peak area of internal standard} \quad [3]$$

3. Calculate the relative concentration of each impurity in the sample of acetone using equation 4:

$$\text{Relative concentration (\%)} = (RRF_i \div RRF_A) \times 100 \quad [4]$$

4. Acceptance Criteria

1. Chromatographic purity of acetone used in this program shall be $\geq 99.9\%$.
2. The IR spectrum of acetone used in this program shall match a library reference IR spectrum for acetone.

6. *Methylcellulose Analysis*

1. Receipt and Storage of Methylcellulose

1. When the methylcellulose is received, remove 0.5-g portions for each subsequent analysis.
2. Place each sample in an appropriately labeled glass vial equipped with a Teflon-lined screw cap.
3. Tightly close and seal the vial, store at -20°C .
4. Use this material in subsequent analyses, at intervals specified by the NTP, as the reference standard.

5. Store the remainder of the methylcellulose at room temperature (~25°C).
2. Identity by IR Spectrophotometry
 1. Prepare separate potassium bromide discs containing approximately 3% methylcellulose for both the chemical or test article and the reference standard.
 2. Obtain a spectrum for the 2 samples from 600–4000 cm⁻¹ using a suitable infrared spectrophotometer.
 3. Adjust the instrument settings or sample concentration to obtain baselines of about 80% transmission, keeping the largest absorbance values at ≥ 10% transmission.
 3. Purity Analysis by Methoxy Group Determination
 1. Procedure
 1. The analysis (duplicate samples) may be performed by the Contractor or by an independent laboratory.
 2. If an independent laboratory does the analysis, it is the responsibility of the Contractor to verify the quality assurance compliance of that laboratory.
 3. The purity analysis is to be performed according to the USP assay procedure described for methoxy-group determination.
 2. Calculations
 1. Calculate the average determined values (%) for methoxy group content of the material and the reference standard to the tenths place.
 2. Calculate the relative purity (%) for the methylcellulose to the tenths place by dividing the average determined value (%) for the methylcellulose by the average determined value for the reference standard and multiplying by 100.
 4. Additional Requirements

At the Contractor's discretion, and with the approval of the COTR, this assay may be assigned to a subcontractor.

5. Acceptability Requirements

1. Results of the analysis for methoxy group content must be in the range of 26.0% – 33.0%
2. The IR spectrum of methylcellulose used in this program shall match a library reference IR spectrum for methylcellulose.

7. *Feed Analysis*

1. Receipt and Storage of Feed

Upon receipt store feed in a cool, dry location, protected from direct sunlight. Feed may be refrigerated, but should not be frozen unless directed to do so by the COR.

2. Feed Analysis Requirements

At the direction of the COTR, the Contractor shall analyze a specified lot of feed for contamination by exogenous materials such as dioxins and related compounds, environmental estrogens, or mycotoxins using a chemical- or bioassay-based method.

1. Protocols

1. Dioxins: Densison et al., Talanta 63(5): 1123-1133. 2004.
2. Mycotoxins: Mitterbauer et al., Mycotoxin Research 19(1):69-72. 2003.

2. Results Reporting

1. For dioxins and related compounds, the lab shall report values for each analyte; non-detects shall be reported as $\frac{1}{2}$ LOQ. The lab shall report total measured TEQ for all samples. Values shall be reported as total measured TEQ/g and measured TEQ+ $\frac{1}{2}$ LOQ (for non-detects)/g.
2. For all other analytes the lab shall report the m/m concentration, e.g., ug/g.

3. Additional Requirements

At the Contractor's discretion, and with the approval of the COTR, this assay may be assigned to a subcontractor.

4. Acceptability Requirements

1. Dioxins and related compounds

1. Total measured TEQ shall be < 0.5 pg TEQ/g feed.
 2. Total measured TEQ+ $\frac{1}{2}$ LOQ shall be < 1.0 pg/g.
2. Mycotoxins
 1. If present, mycotoxins shall have individual concentrations ≤ 0.10 ppm.
 2. The total concentration of all mycotoxins present shall be ≤ 1.0 ppm.
3. Nitrosamines
 1. N-nitroso dimethylamine concentration shall be ≤ 10 ppb.
 2. Total (volatile) nitrosamine content shall be ≤ 15 ppb.

Appendix 3.1. Characteristics and Approximate Composition of Corn OilPhysical Properties

Calories per gram	8.9
Iodine value	127
Peroxide value (meg/kg)	1.7
Anisidine value	2.3
Saponification value	191
Color	2.3 R/14 Y

Components

Glycerides	> 98.7%
Unsaponifiable matter ⁸	1.25%
Free fatty acids	0.04%
Phosphorous	0.5 ppm
Sodium	0.1 ppm
Calcium	0.1 ppm
Magnesium	< 0.1 ppm
Organo-chloride pesticide residues	< 10 ppb ⁷
Aflatoxin	< 0.5 ppb ⁷
Heavy metals (Pb, Cu, Ni, Fe)	< 0.1 ppm ⁷
Estrogenic activity	ND (< 5 ppb‡)

Fatty Acids (g/100 g corn oil)

<u>Total fatty acids</u>	<u>94.3</u>
C12:0	trace
C14:0	trace
C16:0	9.5
C16:1	0.2
C18:0	2.3

⁷ Limit of detection

C18:1	25.4
C18:2	55.1
C18:3	1.0
All others	0.8
Essential fatty acid (lipoxydase)	56.8
Unsaponifiables ⁸ (g/100 g of corn oil)	–
<i>Phytosterols</i>	> 1.0
Stigmasterol	0.07
beta-Sitosterol	0.8
gamma-Sitosterol (Campesterol)* 0.2	
<i>Tocopherols</i>	
alpha-Tocopherol	0.014
gamma-tocopherol	0.084
delta-tocopherol	< 0.001
Total	0.098
Ubiquinone (Coenzyme Q-9)	0.02
Squalene ⁸	trace
Carotenoids ⁸	trace

⁸ From past historical experience, not analyzed

Appendix 3.2. Characteristics and Approximate Composition of Olive Oil⁹

<u>Fatty acid composition</u> ¹⁰	<u>(%m/m methyl esters)</u>	
Myristic acid	≤ 0.05	
Palmitic acid	7.5 – 2.0	
Palmitoleic acid	0.3 – 3.5	
Heptadecanoic acid	< 0.3	
Heptadecenoic acid	< 0.3	
Stearic acid	0.5 - 5.0	
Oleic acid	55.0 - 83.0	
Linoleic acid	3.5 - 21.0	
Linolenic acid	< 1.0	
Arachidic acid	< 0.6	
Gadoleic acid (eicosenoic)	< 0.4	
Behenic acid	< 0.2 ⁷	
Lignoceric acid	< 0.2	
<u>Trans fatty acid content</u>	<u>(% trans fatty acids)</u>	
	C18:1 T	C18:2 T + C18:3 T
	<u>%</u>	<u>%</u>
Edible virgin olive oils	≤ 0.05	≤ 0.05
Lampante virgin olive oil ¹¹	< 0.10	< 0.10
Refined olive oil	< 0.20	< 0.30
Olive oil	< 0.20	< 0.30

⁹ International Olive Council. Trade standard applying to olive oils and olive-pomace oils. COI/T.15/NC No 3/Rev. 5. November 2010.

¹⁰ Determined by gas chromatography

¹¹ When the oil has a wax content between 300 mg/kg and 350 mg/kg it is considered a lampante virgin olive oil if the total aliphatic alcohol content is < 350 mg/kg or the erythrodiol + uvaol content is < 3.5%.

Crude olive-pomace oil ¹²	< 0.20	< 0.10
Refined olive-pomace oil	< 0.40	< 0.35
Olive-pomace oil	< 0.40	< 0.35

Sterol and triterpene dialcohol composition

Desmethylsterol composition	(% total sterols)
Cholesterol	< 0.5
Brassicasterol	< 0.1 ⁷
Campesterol	< 4.0
Stigmasterol	< campesterol in edible oils
Delta-7-stigmastenol	< 0.5

Apparent beta-sitosterol:

Beta-sitosterol	+
delta-5-avenasterol	+
delta-5-23-stigmastadienol	+
clerosterol + sitostanol	+
delta 5-24-stigmastadienol	> 93.0

Total sterol content

Virgin olive oils, refined olive oil, olive oil	(mg/kg)
Virgin olive oils, refined olive oil, olive oil	≥ 1000
Crude olive-pomace oil	≥ 2500
Refined olive-pomace oil	≥ 1800
Olive-pomace oil	≥ 1600

Erythrodiol and uvaol content

Edible virgin olive oils	(% total sterols)
Edible virgin olive oils	≤ 4.5
Lampante virgin olive oil	≤ 4.5
Refined olive oil	≤ 4.5
Olive oil	≤ 4.5

¹² When the oil has a wax content between 300 mg/kg and 350 mg/kg it is considered a crude olive-pomace oil if the total aliphatic alcohol content is > 350 mg/kg and the erythrodiol + uvaol content is > 3.5%.

Crude olive-pomace oil	≤ 4.5
Refined olive-pomace oil	≤ 4.5
Olive-pomace oil	≤ 4.5

Wax content (C40+C42+C44+C46) (mg/kg)

Edible virgin olive oils	≤ 250
Lampante virgin olive oil ¹¹	≤ 300
Refined olive oil	≤ 350
Olive oil	≤ 350
Crude olive-pomace oil ¹²	> 350
Refined olive-pomace oil	> 350
Olive-pomace oil	> 350

Maximum difference between the actual and theoretical ECN 42 triacylglycerol content

Edible virgin olive oils	≤ 0.2
Lampante virgin olive oil ¹¹	≤ 0.3
Refined olive oil	≤ 0.3
Olive oil	≤ 0.3
Crude olive-pomace oil ¹²	≤ 0.6
Refined olive-pomace oil	≤ 0.5
Olive-pomace oil	≤ 0.5

Stigmastadiene content (mg/kg)

Edible virgin olive oils	≤ 0.10
Lampante virgin olive oil ¹¹	≤ 0.50

Content of 2-glycerol monopalmitate

Edible virgin olive oils and olive oil:

C:16:0	≤ 14.0%; 2P ≤ 0.9%
C:16:0	≤ 14.0%; 2P ≤ 0.9%

Non-edible virgin olive oils and refined olive oils:

C:16:0	≤ 14.0%; 2P ≤ 0.9%
C:16:0	≤ 14.0%; 2P ≤ 1.1%

Olive-pomace oils $\leq 1.2\%$

Crude¹² and refined olive-pomace oils $\leq 1.4\%$

Unsaponifiable matter (g/kg)

Olive oils ≤ 15

Olive-pomace oils ≤ 30