

Isolation of Vitamin A, E, D, and Cholesterol by ANKOM^{FLEX} (Method: Vitamins Cholesterol)
Definition

This method is used to isolate Vitamin A, E, D, and Cholesterol within a given sample.

Scope

This method is applicable to food and feed samples.

A. Apparatus

1. ANKOM^{FLEX} Analyte Extractor
2. Digestion Vessels (FLEX54, FLEX55)
3. Magnetic cross stir-bars (9415) – *for use in digestion vessels*
4. Round Bottom Flasks (9364) – *for recovery on the FLEX instrument*
5. Prepacked SPE Columns (FLEX-SPE-01)
6. Vitamin Filters (FLEX-VF)
7. Analytical Balance – capable of weighing 1mg
8. One 100-250ml container with cap – *for preparation of infant formula slurry*
9. Stir Plate and Stir Bar – *for slurry preparation*

B. Reagents

1. Use distilled water (DI) throughout
2. n-Hexane (reagent grade or higher)
3. Ethanol (95% or higher)
4. Pyrogallol (or equivalent)
5. Potassium hydroxide (KOH)
6. Butylated hydroxytoluene (BHT)
7. 2% (w/v) pyrogallol in ethanol: Weigh 10g ± 0.1g pyrogallol into a 500ml volumetric flask. Make up to the mark with ethanol. Mix well.
8. 12.7N Potassium hydroxide (KOH) solution: Slowly add 500g KOH into 500g DI water, while continually mixing. Allow to cool to room temperature.
9. 0.05g/L BHT in hexane: Weigh 0.05g ± 0.005g BHT into a 1L volumetric flask. Make up to the mark with hexane. Mix well.

C. Sample Preparation

Table 1 shows maximum recommended sample sizes, to be run on the ANKOM^{FLEX}. It is important to not exceed the specified sample size as that could result in digestion filters plugging or SPE columns overloading. To evenly disperse vitamin supplements in infant formula, a representative sample must be slurried, before subsampling for analysis on the ANKOM^{FLEX}. For all other sample types, be sure to homogenize, grind, or thoroughly mix a representative sample prior to sampling for analysis on the ANKOM^{FLEX}. If

Table 1. Maximum Recommended Sample Sizes for Analysis on the ANKOM^{FLEX}

Sample Type	Sample Size (g)	Water added with Sample (g)
Oils e.g., melted margarine	2	8
High fat samples (>40% fat) e.g., butter, peanut butter, meat	2	8
Wet pet food	10	0
Dry pet food	5	5
Fresh fruits and vegetables	10	0
Beverages	10	0
Nutritional powders & supplements	0.25-1	9
Infant Formula (slurried) (see procedure in section C-1)	10	0
Animal Feed	5	5
Other	5	5

Preparation of Infant Formula Slurry

1. Weigh 20g, to the nearest 0.1g of infant formula powder into a container (W1).
2. Add 133g, to the nearest 0.5g warmed DI water (60C) to the container (W2).
3. Add a magnetic stir bar and close the container.
4. Place on stir plate and mix the slurry for 15min.
5. The slurry now is ready to be used for subsampling (W3) (see steps D-3).
6. Calculate the weight of sample in the saponified aliquot:

$$\text{Sample weight (g)} = \frac{W3 \times W1}{(W1+W2)}$$

D. Procedure (see the Operator's Manual for more detail)

1. Assemble digestion vessels (digestion vessel + vessel bottom assembly + vitamin filter) and **add a magnetic cross stir-bar** into each digestion vessel before adding sample.
2. Oils and High Fat Samples: Weigh water and sample directly into digestion vessels
 - a. Add water (according to Table 1) into the vessel by squirting water against the vessel glass or dripping onto the stir bar. **If squirted directly onto the filter, the surface tension of the hydrophobic filter will break, and water will leak through the filter.**
Note: It is important to add water first, before adding an oil sample. Since vitamin filters are hydrophobic, hydrophobic samples such as oils will seep into and leak through the filter, if not weighed after (on top of) water.
 - b. Agitate vessel manually until the whole filter is covered with water.
 - c. Weigh sample (see Table 1) into the digestion vessel by slowly adding on top of the stir bar.
Note: When weighing a liquid sample, it is important to drip the sample onto the stir bar. Do not squirt it directly onto the filter, as the action will break the surface tension of the filter and sample will leak through the filter.
Note: It is important to not exceed the specified sample size. Exceeding the sample size could result in digestion filters plugging or SPE columns overloading.
3. Infant Formula Slurry:
 - a. Make sure the slurry is well-mixed before weighing 10g, to the nearest 0.5g of infant formula slurry into the digestion vessel (W3). Do NOT add additional water into the ANKOM^{FLEX} digestion vessels. The slurry already contains the necessary water.
4. All other Samples: Weigh sample and water directly into digestion vessels
 - a. Weigh sample (see Table 1) into the digestion vessel.
Note: When weighing a liquid sample, drip the sample onto the stir bar. Do not squirt it directly on to the filter, as the action will break the surface tension of the filter and sample will leak through the filter.
Note: It is important to not exceed the specified sample size. Exceeding the sample size could result in digestion filters plugging or SPE columns overloading.
 - b. Weigh water into the vessel. The water weight can be calculated as: 10g – sample wt (g). Recommended sample weight and added water volumes can be found in Table 1.
Note: When adding water, do not squirt directly onto the filter as the force will break the surface tension of the filter and the water will leak through the filter. Water can be squirted against the side of the vessel glass or dripped onto the stir bar.
5. Install digestion vessels on the ANKOM^{FLEX} and follow the instructions in the operating manual on how to: Start an Assay
6. Select Method: **Vitamins Cholesterol**
7. After the ANKOM^{FLEX} method has ended, the round bottom flasks in the recovery oven will contain the isolated vitamins. Remove the round bottom flasks, cover the top of each flask with aluminum foil or stopper. Cool each flask under cold running water for ~20 seconds, ensuring water does not enter flask.
8. Reconstitute the isolated vitamins with the appropriate solvent for further quantitation on HPLC.

If limit of quantitation is an issue, please contact ANKOM for analytical support.