Isolation of Vitamin A, E, D, and Cholesterol by ANKOM^{FLEX} (Method: VC R4)

Definition

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

Scope

This method is applicable to food, supplements, pet food, and high mineral feeds.

A. Apparatus

1. ANKOM^{FLEX} Analyte Extractor
2. Digestion Vessels (FLEX54, FLEX55)
3. Magnetic stir-bars (9380) – *for use in digestion vessels*
4. Round Bottom Flasks (9364) – *for recovery on the FLEX instrument*
5. SPE Columns (FLEX-SPE-01)
6. Vitamin Filters (FLEX-VF)
7. Diffusers (FLEX-DF)
8. Filter Aid (FLEX-FA2)
9. Analytical Balance – capable of weighing 1mg

B. Reagents

1. Use deionized 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 10 g ± 0.1 g pyrogallol into a 500 ml volumetric flask. Make up to the mark with ethanol. Mix well.
8. 12.7 N Potassium hydroxide (KOH) solution: Slowly add 500 g KOH into 500 g DI water, while continually mixing.
9. 0.05 g/L BHT in hexane: Weigh 0.05 g ± 0.005 g BHT into a 1L volumetric flask. Make up to the mark with hexane. Mix well.

C. Sample Preparation

Table 1 shows recommended sample sizes, to be run on the standard vitamin method: VC R4. Table 2 shows a range of samples to be run on alternative methods (VC R5 – VC R8) (see application notes specific to these methods). It is important to select the correct method and to not exceed the specified sample size as that could result in digestion filters plugging or SPE columns overloading. Homogenize, grind, or thoroughly mix a representative sample prior to sampling.

Table 1. Recommended Sample Sizes for Method: VC R4

Sample	Max Sample Weight (g)	Application Note
Concentrated supplements (tablets, gummies, etc.)	2	1. Supplements
Oils	2	2. Oil
High fat foods, >30% fat (seafood, meat, nut butters, etc.)	2	
Freeze dried protein (fish, meat, etc.)	3	3. Freeze Dried Protein
High mineral mixes (fish meal, cattle mineral)	3-5	4. High Minerals
Dry pet food	5	
Infant Formula (dry)	5	5. Toddler Formula
High fiber foods (psyllium, rice, oat, etc.)	5	6. High Fiber/Starch
High starch foods	5	
Other	5	
Low fat foods, < 30% fat	10	
Wet pet food	10	9. Wet Pet Food
Powdered formulations (protein powder, etc.)	10	7. Protein Powder

Fresh fruits and vegetables	10	
Beverages (milk, juice, etc.)	10	8. Beverage

When analyzing samples from Table 2, please refer to the relevant analytical procedures for VC R5 - VC R8.

Table 2. Samples to be Analyzed by Alternative Methods: VC R5 – VC R8

Sample	Max Sample Weight (g)	Method
Beverages (milk, juice, etc.)	20	VC R5
Compound Feeds (Total Mixed Rations, not incl. pet food)	40	VC R6
	10	VC R7
Difficult-to-drain samples (rice, oat, certain protein powders)	5	VC R7
Premixes (ingredients/formulations that are added to feed or food	5	VC R8

D. Procedure

1. Refer to Table 1 for maximum sample size and application notes that are specific to certain samples/sample groups. Then review the application notes (Section E) prior to continuing with the procedure. Certain samples require the addition of filter aid, diffusers, and/or unique sample treatment.
2. Assemble digestion vessels (digestion vessel + vessel bottom assembly + vitamin filter) and add a stir-bar into each digestion vessel before adding sample.
3. Install digestion vessels on the ANKOM^{FLEX}.
4. Follow the instructions in the operating manual on how to: Start an Assay
5. Select Method: VC R4
6. 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.
7. Reconstitute the isolated vitamins with the appropriate solvent for further quantitation on HPLC. If Limit of Quantitation (LOQ) is a concern, please contact ANKOM for analytical support.

E. Application Notes

1. Supplements: Vitamin gummies often do not dissolve during saponification. This leads to reduced analyte recovery. High fiber tablets often do not drain from the digestion vessels. This leads to drain time out faults.
    a. Vitamin gummies must be reduced in size prior to analysis. Use scissors to cut the sample into small (~1mm) slivers, before weighing it into the FLEX digestion vessels.
    b. High fiber tablets must be saponified with filter aid. Before weighing the sample into the digestion vessel, add 1 g filter aid (FLEX-FA2) into the vessels, on top of stir bars.
2. Oils: When weighing oil samples into FLEX digestion vessels, the sample will leak through the filter. The vitamin filter (FLEX-VF) is hydrophobic and therefore the digestion vessel will not contain oils unless the vessel is first secured in the instrument and then oil is transferred into the vessel through the port. Follow the steps below to weigh oil samples into the digestion vessels.
    a. Assemble and then install empty digestion vessels on FLEX.
    b. Close vessel tops, by pressing the vessel top button on the screen.
    c. Draw oil in a plastic pipette and weigh the filled pipette (W1).
    d. Expel approximately 2 ml oil into the digestion vessel through the port.
    e. Weigh pipette that now contains only residual oil (W2).
    f. Calculate oil weight = - (W2-W1).
3. Freeze Dried Protein: Certain freeze-dried protein samples (e.g., fish or meat) will plug SPE columns, which will result in a drain time-out fault. To allow for better filtration through the SPE column,
    a. Reduce the sample weight, or
    b. Add two diffusers (FLEX-DF) to each of the SPE columns prior to installing it on the FLEX. Hold the column upright and lightly push the diffusers into the column with your fingers until it bottoms out.

4. High Mineral: Certain high mineral content samples (e.g., fish meal and cattle mineral) will plug digestion vessels and/or SPE columns, which will result in a drain time-out fault. To allow for better filtration through the SPE column,
    a. Reduce the sample weight, or
    b. Add one diffuser (FLEX-DF) to the digestion vessel, on top of the vitamin filter, prior to assembling the vessel.
    c. Add two diffusers (FLEX-DF) to each of the SPE columns prior to installing it on the FLEX. Hold the column upright and lightly push the diffusers into the column with your fingers, until it bottoms out.
5. Toddler Formula: Certain toddler formula formulations will plug SPE columns, which will result in a drain time-out fault. To allow for better filtration through the SPE column,
    a. Reduce the sample weight, or
    b. Add two diffusers (FLEX-DF) to each of the SPE columns prior to installing it on the FLEX. Hold the column upright and lightly push the diffusers into the column with your fingers, until it bottoms out.
6. High Fiber and/or High Starch: Certain samples (e.g. psyllium, rice, and oat) will plug the FLEX digestion vessels during filtration and will result in a drain timeout fault. To allow for better filtration through the digestion vessel, you could
    a. Reduce the sample weight, or
    b. Add one diffuser (FLEX-DF) to the digestion vessel, on top of the vitamin filter, prior to assembling the vessel.
    c. Add 1g filter aid (FLEX-FA2) into the digestion vessel, on top of the stir bar, prior to adding sample.
7. Protein Powders: Certain protein powders, when run on the standard method VC R4, will plug the FLEX digestion vessels during filtration and will result in a drain timeout fault. To allow for better filtration through the digestion vessel, you could:
    a. Reduce the sample weight, or
    b. Add 1g filter aid (FLEX-FA2) into the digestion vessel, on top of the stir bar, prior to adding sample.
8. Beverages: Beverages are analyzed on the ANKOM^{FLEX} at a maximum of 10g sample, on Method VC R4. However, certain beverages contain low analyte content and larger sample sizes are required for analysis. If LOQ is a concern, sample sizes up to 20g can be run on Method VC R5. This method accounts for the higher moisture content of the sample by adding no water to the saponification solution post saponification.
    Note: When weighing a liquid sample, drip the sample onto the stir bar. Do not squirt it directly on to the filter, as this action will break the surface tension of the filter and sample will leak through the filter.
9. Wet Pet Food: Certain wet pet foods, at 10g samples size will overload the SPE columns. If this is the case, adjust the FLEX program to add 18ml water after saponification, instead of the standard 23ml.