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Chapter 34

Spectrophotometric estimation of phytosterols: Principles, methodology, and analytical applications

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1. Introduction:

Cholesterol, obtained through dietary intake and synthesized by liver and intestinal cells, plays an essential role in the biosynthesis of bile acids, vitamin D, and steroid hormones (Harborne, 1998). However, high cholesterol levels increase the risk of vascular accidents and atherosclerosis. Oilseeds in plants are good cholesterol sources. The determination method (Sadasivam, and Manickam, 2008; Allain et al., 1974) involves cholesterol oxidase catalysis. The enzyme reagent in the commercial kit includes specific concentrations of ammonium phosphate, 4-aminoantipyrine, sodium cholate, cholesterol oxidase, horseradish peroxidase, DHBSA, and Triton X-100 (Akinyemi, and Oboh, 2013). The process measures cholesterol enzymatically through reactions that hydrolyze cholesteryl esters, oxidizing cholesterol to produce H_2O_2, which forms a measurable colored complex using a spectrophotometer (Kumari and Jain, 2015).
Cholesterol esterase catalyzes the hydrolysis of cholesterol esters into cholesterol and fatty acids:

a. Cholesterol esterase

Cholesterol ester + H_2O → Cholesterol + Fatty acids

Cholesterol oxidase then oxidizes cholesterol to produce 4-cholesten-3-one and hydrogen peroxide (H_2O_2):

b. Cholesterol Oxidase

Cholesterol + O_2 → 4-Cholesten-3-one + H_2O_2

Peroxidase uses the hydrogen peroxide to convert phenol and 4-aminoantipyrine into a colored complex, quinoneimine, which is measured spectrophotometrically:

c. Peroxidase

$2H_2O_2$ + Phenol + 4-Aminoantipyrine → Quinoneimine + $4H_2O$

2. Materials Required:

1. Oils – Various edible oils such as groundnut, flaxseed, soybean, olive, and rice bran.
2. Cholesterol Estimation Kit – A commercially available kit based on cholesteryl esterase oxidase.
3. Standard Cholesterol – Provided with the estimation kit.
4. Deionized Water – For dilution and sample preparation.
5. Potassium Hydroxide (KOH) – Used for saponification.
6. Petroleum Ether – For lipid extraction.
7. UV-Visible Spectrophotometer – For measuring absorbance during cholesterol quantification.
8. Auto Pipettes – For accurate liquid handling.
9. Analytical Weighing Balance – For precise measurement of sample weights.
10. Incubator
11. Clean and dry test tubes
12. Tissue paper

3. Procedure:

1. Sample Preparation:

a. **Saponification:**
Treat the oil samples with a 150 g/L solution of potassium hydroxide (KOH) to saponify the lipids.

b. **Sterol Extraction:** Extract the resulting sterols using petroleum ether.

c. **Analysis Preparation:**
Use aliquots of the petroleum ether extract for cholesterol estimation via either colorimetric or enzymatic methods.

d. **Test Tube Setup:** Prepare clean, dry test tubes labeled as follows:
   - **Blank (B)**
   - **Standard (S)**
   - **Test (T)**

Tube Label	Enzyme Reagent (mL)	Deionized Water (mL)	Standard (mL)	Vegetable Oil Sample (mg/mL)
B (Blank)	1.0	0.01	–	–
S(Standard)	1.0	–	0.01	–
T (Test)	1.0	–	–	1.0 *(diluted 1:10)*

2. Tube Setup: - Prepare clean, dry test tubes labeled as Blank (B), Standard (S), and Test (T).

3. Incubation: Mix the contents in each tube thoroughly.
Incubate the tubes at 37°C for 5 minutes.

4. Spectrophotometric Analysis:
Adjust the spectrophotometer to 500 nm using the Blank (B) tube.
After the 5-minute incubation, record the absorbance (optical density, OD) for each tube.

Sample	Absorbance at 500 nm
Blank	0.0
Standard	*[Enter Standard OD]*
Test	*[Enter Test OD]*

4. Calculation:

Cholesterol Conc. in mg% = OD of Test × 200 (Conc. of Standard) OD of Standard.

5. Precautions:

1. Allow the spectrophotometer lamps and electronics to warm up for 15 minutes before use.
2. Ensure the correct wavelength is set.
3. Wipe the outer sides of the cuvette with a smooth tissue paper before measuring.
4. Follow the procedure in the correct order.
5. Verify the calibration of the spectrophotometer.
6. Close the cuvette compartment door before recording the OD.
7. Avoid spilling any solution inside the cuvette compartment.
8. Accurately adjust the incubator temperature.

6. References:

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