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Assessment of methodology for the enzymatic assay of cholesterol in egg noodles

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Abstract

Cholesterol content is generally used as a quality parameter for egg noodles, but some methods for its determination are not specific for cholesterol; moreover, they require numerous organic solvents and corrosive reagents, which have harmful affects on the environment. The present paper suggests an alternative technique for this determination using enzymes, which is cheaper, faster and environmentally safe. This enzymatic method was compared to the provenly reliable and accurate high pressure liquid chromatography (HPLC) method involving direct saponification. The results obtained by the former were significantly higher than those obtained by HPLC, but the correlation was high and a correlation factor was determined: $F = 0.7257 \times$ enzymatic values $- 5.1419$. The values calculated using this correlation factor showed no significant difference from those measured by HPLC.

Keywords: Egg noodle; Cholesterol; Enzymatic method; HPLC; Correlation factor

1. Introduction

Many kinds of pasta are frequently made with eggs, which increase their nutritional value, but industrially there is a need for a rapid method for the determination of the presence of this essential ingredient if the quality of the final product is to be controlled. Since eggs are high in cholesterol, this substance is often evaluated to determine the presence of eggs in the original formula. In the case of egg noodles, various authors have reported such a measurement as a quality parameter (Bragagnolo & Rodrigues-Amaya, 1993; Dumain, Simler, Banner, Quentric, & Pailler, 1988; Fombuena, 1987; Hurst, Aleo, & Martin, 1985)

Unfortunately, many methods for the extraction of cholesterol include reagents which are potentially harmful to the environment. With modern-day concerns for protecting the environment being in vogue, it is clear that safe alternatives should be sought. The most common extraction method today is direct saponification, which is not only environmentally safe, but also rapid and relatively inexpensive.

For some time, colorimetric methods were used for measuring the cholesterol extracted from food (Abell, Levy, Brodie, & Kendall, 1952; Bohac, Rhee, Cross, & Ono, 1988; Searcy & Bergquist, 1960; Sperry & Brandy, 1943; Zlatkis, Bennie, & Boyle, 1953). When the more accurate gas chromatographic (GC) and high performance liquid chromatographic (HPLC) methods were developed, the older colorimetric methods were found to obtain somewhat higher values, ranging from 28 to 200% more (Maurice, Lightsey, Hsen, Gaylord, & Reddy, 1994). These more accurate chromatographic methods involve the use of sophisticated equipment and relatively expensive reagents, however, and various studies have sought ways of circumventing these problems without sacrificing accuracy. One of the possible solutions tried has involved the use of enzymes. Many enzymatic methods using cholesterol oxidase have been introduced. This reaction is not specific for cholesterol, since sterols with a 3 β-OH group also react with this enzyme. The presence of these sterols thus leads to an overestimate of the cholesterol content in foods containing animal or vegetable lipids. When only cholesterol is present, though, it can be just as accurate as the more reliable methods of GC and HPLC.

Various studies have compared the results of the cholesterol obtained in foods from the enzymatic determination

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with those using the more accurate methods of GC (Shen, Chen, & Sheppard, 1982) and HPLC (Nogueira & Bragagnolo, 1998), as well as between enzymatic, GC, and HPLC methods (Jiang, Fenton, & Sim, 1991) and between enzymatic and GC methods (Karkalas, Donald, & Clegg, 1982).

The present study investigated the accuracy of an inexpensive enzymatic method for the determination of cholesterol in egg noodles and compared it with the HPLC method.

2. Materials and methods

2.1. Sample preparation and treatment

The samples of egg noodles for all tests were ground to pass through a 40-mesh sieve. Extraction was carried out by direct saponification according to Jiang et al. (1991), except that heating was carried out for 2 h instead of 1 h. The sample (0.51 ± 0.01 g) was directly saponified with 5 ml of 2% KOH in absolute ethanol for 2 h in a water bath at 50 °C. Water (5 ml) was added and after cooling to room temperature the unsaponifiable fraction was extracted twice with 10 ml of hexane.

2.2. Cholesterol determination by enzymatic methods

The first part of the present work involved the investigation of the equivalence of the results of two different enzymatic methods. For this part of the study, two different enzyme test kits were evaluated, the first being specific for cholesterol determination in food (kit A), and the second traditionally used for blood (kit B).

The enzyme kit A (Boehringer-Mannheim Biochemicals, Germany) involved the use of three solutions, the first containing catalase (approx. 220,000 U) in a solution of ammonium phosphate buffer (pH approx. 7.0) and methanol (2.6 mol/l) and the second was a cholesterol oxidase suspension (approx. 12 U). A third component was acetylacetone (0.05 mol/l), methanol (0.3 mol/l) and stabilizers. The actual reagent was obtained by mixing the first solution with the third at a ratio of 3:2, while the cholesterol oxidase suspension was added directly to the sample being tested.

Two milliliters of the hexane extract obtained by saponification of the ground egg noodles were dried under N_2 and diluted in 0.4 ml of isopropyl alcohol in a test tube, and 5.0 ml of the reagent mixture was added. Samples were incubated in a water bath at 37–40 °C for 60 min, and then cooled to room temperature. The samples were then submitted to a spectrophotometric evaluation at 405 nm. The concentration was calculated according to the manufacturer's instructions.

The second test kit (Laborlab, Brazil) involved the use of two colur reagents (0.025 mol/1 of 4 aminophenazone and 0.055 mol/l phenol), and a reactive solution of Lipase (300 U/mol, COD 3 U/ml, and POD 20 U/ml), with the final reagent consisting of a 1:1:0.4 solution of these three dissolved in 38 ml of water. The reaction here involved a relatively inexpensive method for the determination of the presence of cholesterol, frequently used in routine determinations of cholesterol level in human blood.

Exactly 5 ml of the hexane extract obtained by direct saponification were dried under N_2 and diluted in 1 ml of isopropyl alcohol in a test tube; 3 ml of the reagent mixture was added, and the samples incubated for 10 min in a 37 °C water bath, and then cooled to room temperature. The absorbance of the samples at 499 nm was determined in relation to a control containing only isopropyl alcohol and the reagent mixture. The cholesterol concentration of sample solutions was determined using a standard curve constructed by graphically plotting the absorbance vs μg/ml cholesterol (200, 400, 600, 1000, and 2000 μg in isopropyl alcohol). The slope of the standard curve obtained was always greater than 0.99.

Enzymatic reactions were measured using an UV-visible spectrophotometer (Philips model PU8620, England).

Recoveries were checked by the addition of 0.03 and 0.05 mg of a cholesterol standard to the samples. The accuracy and precision was demonstrated using whole egg powder (SRM 1845, NIST, USA).

2.3. Cholesterol determination with HPLC

The equipment for HPLC consisted of a liquid chromatograph (Pharmacia, Sweden) equipped with a LiChrocart C_{18} column (125×4 mm, 5 μm; Merck, Germany) and a LiChrocart guard column (4×4 mm, 5 μm; Merck, Germany), as well as LKB 2248 pumps, a UV-visible detector (LKB VWM 2141) and a 20 μl loop injector. The mobile phase used was acetonitrile–isopropyl alcohol (70:30 v/v) at a flow rate of 1.0 ml/min, with detection set at 210 nm. Each run took 15 min.

Exactly 5 ml of the hexane extract obtained by direct saponification were dried under N_2 flow and diluted with a portion of 3 ml of the mobile phase; the peak area remained in the center of the standard curve.

Quantification was carried out by external standardization. The relationship between area and cholesterol concentration, in the range from 0.01 to 1 mg/ml, was linear, with a correlation coefficient of 0.9958. Recoveries were checked by the addition of 0.05, 0.1 and 0.15 mg of cholesterol standard to the samples. The accuracy and precision were demonstrated using whole egg powder (SRM 1845, NIST, USA).

Table 1
Comparison of two enzymatic test kits for the determination of cholesterol in egg pasta (mg of cholesterol/100 g)

N^a	Kit A[b]	Kit B[c]
1	64.5a	64.6a
2	66.5a	65.3a
3	65.7a	64.6a
4	72.9a	64.7a
5	77.0a	64.3a
Mean±S.D	69.3±5.4	64.7±0.4
%C.V.	7.8	0.5

S.D., standard deviation; C.V., coefficient of variation. Values with the same letter were not significantly different at $P \leqslant 0.05$.
[a] Number of determinations.
[b] Boehringer-Mannheim.
[c] Laborlab.

2.4. Statistical analyses

The results obtained using the various methods tested were submitted to analyses of variance (ANOVA), considering significance at a 5% level of confidence.

3. Results and discussion

3.1. Cholesterol determination by enzymatic methods

The first experiment involved the use of egg noodles made with fresh eggs, to compare the results obtained by the two enzymatic methods. Five determinations using each of the enzymatic test kits were made, and the average of these was used to compare the results (Table 1).

The results showed that the quantity of cholesterol measured was similar whether based on kit A, specific for food use, or kit B, routinely used in blood tests. The former, however, is quite expensive while the latter is relatively inexpensive.

Using kit B, the cholesterol content of certified reference material (SPM 1845, whole egg powder) was found to be 19.06±0.19 mg/g, compared to the certified value of 19.0±0.2 mg/g. The recovery percentages were 99.4±0.4% and 99.4±0.7%, respectively, when 0.03 and 0.05 mg of cholesterol were added.

3.2. Experiment comparing enzymatic and HPLC methods

Since the two enzymatic methods gave similar results, the inexpensive enzymatic method was compared with the HPLC method.

The recovery percentages for the HPLC method were 98.2±2.2, 97.1±1.4 and 98.1±2.2% when 0.05, 0.1 and 0.15 mg of cholesterol were added. The result obtained for the certified cholesterol in whole egg powder (SRM 1845, NIST, USA) was 19.06±0.08 mg/g, nearly identical to the certified content 19.0±0.2 mg/g.

In this second experiment, homemade egg noodles were prepared with egg powder (30 g egg powder; 47 mg cholesterol) since industrial noodles are made with this rather than fresh egg. This egg powder was mixed with 970 g of wheat flour and 300 ml of water. The noodles were dried in an oven until the moisture content reached 8%. The average of three analyses on this dough gave a mean of 50±1 mg of cholesterol/100 g of sample for the HPLC method and 74±2 mg of cholesterol/100 g for the enzymatic method.

The amount of cholesterol determined by HPLC was found to be quite similar to that calculated on the basis of the amount of egg powder added to the pasta, but these results were somewhat lower than those obtained by the enzymatic method. This difference is due to the fact that the former procedure is able to separate the

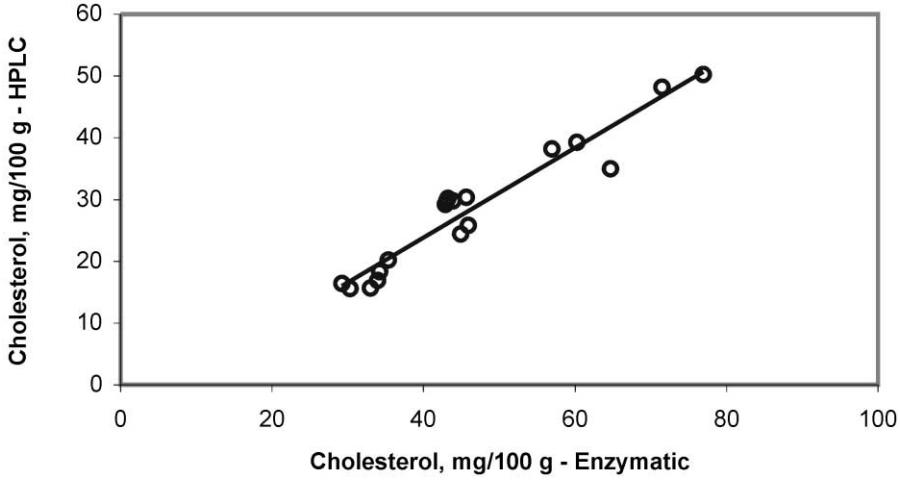

Fig. 1. Linear correlation between cholesterol contents (mg/100 g of egg pasta) determined by HPLC and enzymatic method with kit B (Laborlab).

Table 2
Comparison of HPLC and enzymatic methods, after application of correction factor for commercial brands of egg pasta (mg of cholesterol/l00 g)[a]

Samples	HPLC	Enzymatic kit B[b]	Corrected values (enzymatic)
1	31.1a	54.0b	34.0a
2	17.5a	40.8b	24.5a
3	40.5a	46.9b	28.9a
4	21.1a	46.6b	28.7a
5	15.1a	26.5b	14.1a
6	26.5a	42.7b	25.8a
7	39.6a	57.3b	36.4a
8	29.1a	46.7b	28.8a

[a] Mean based on average of two determinations. Values with the same letter in the same line were not significantly different at $P \leqslant 0.05$.
[b] Kit Laborlab.

cholesterol from the phytosterols in the wheat flour. The failure to distinguish between cholesterol and phytosterols led to an overestimate of the cholesterol content by the enzymatic method.

The results obtained by the enzymatic method were 33.3% higher than the HPLC results, and differences among individual values were constant, showing good correlation.

3.3. Experiment to establish the correlation factor

Given the good correlation between the results obtained by the enzymatic and HPLC methods and the advantage of lower costs for the enzymatic method, in comparison to the HPLC method, an attempt was made to obtain a correlation between the readings obtained by the two methods.

For this experiment, 26 samples of commercial brands of egg pasta (purchased in Campinas, SF, Brazil) were taken. Eighteen of these samples were used for the determination of the correlation factor (F), and the other eight for its confirmation. The linear regression of the correlation factor is presented in Fig. 1. The correlation factor established was $F = 0.7257 \times$ enzymatic values -5.1419. The results obtained using this correlation factor for the correction of the eight samples are presented in Table 2, and were not significantly different from those obtained by HPLC.

4. Conclusions

The enzymatic method routinely used in blood tests is a fast, accurate and inexpensive method for the determination of cholesterol in egg noodles, when using the correlation factor.

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