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Short communication

Determination of cholesterol in food samples using dispersive liquid–liquid microextraction followed by HPLC–UV

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ABSTRACT

A fast, simple, and sensitive sample preparation procedure based on dispersive liquid–liquid microextraction (DLLME) is proposed for the determination of cholesterol in food samples using isocratic reverse phase high performance liquid chromatography (RP-HPLC) and UV detection. The influence of several important parameters on extraction efficiency of cholesterol was evaluated. Under optimized conditions, a linear relationship was obtained between the peak area and the concentration of cholesterol in the range of 0.03–10 $\mu g\,l^{-1}$. The detection and quantification limits were 0.01 and 0.03 $\mu g\,l^{-1}$, respectively. Intra-day and inter-day precisions for the analysis of cholesterol were in the range of 1.0–3.1%. The applicability of the proposed method was demonstrated by analyzing cholesterol in milk, egg yolk and olive oil.

1. Introduction

Cholesterol widely occurs in animal products and is substance of concern due to its important role in developing cardiovascular diseases [1]. There is no sufficient evidences support the link between the disease and dietary cholesterol but it can be oxide to some harmful by-product when exposed to the air [2–4].

Several methods have been reported for the determination of cholesterol in foods, including gas chromatography [1,5–7], high performance liquid chromatography [3,8–11], and spectrophotometry [12]. Gas chromatography and liquid chromatography are the most suitable methods for the analysis of cholesterol. Although direct analysis of cholesterol by liquid chromatography is simple and very sensitive, but there are some drawbacks i.e. long operation time, saponification prior to the analysis, and considerable amount of expensive and environmentally damaging organic solvents for extraction procedures is required [3,13]. In order to control cholesterol content of foods, a reproducible and rapid analytical method is necessary.

It has been suggested that dispersive liquid–liquid microextraction (DLLME) can be used as an alternative to the extraction and clean-up steps in sample preparation. DLLME was developed for the extraction of some organic compounds in aqueous matrices. The main advantages of DLLME are: rapidity, high enrichment factor, high extraction recovery, and simplicity of operation [14–16].

The aim of the present work is the development of a rapid, simple, and sensitive DLLME method and high performance liquid chromatography combined with UV detection for direct determination of cholesterol in food samples.

2. Experimental

2.1. Chemicals and solvents

Cholest-5-en-3β-ol (cholesterol) with purity of >99%, acetic acid, hydrochloric acid, sodium hydroxide, and sodium bicarbonate were obtained from Merck (Darmstadt, Germany). Acetonitrile was supplied from Acros (Belgium). All organic solvents were HPLC grade and purchased from Merck (Darmstadt, Germany). Doubly distilled water was used in all experiments.

2.2. Instrumentation

The HPLC system (model SCL-10Avp) consists of a UV detector (model SPD-10Avp), operating at wavelength of 210 nm, dual solvent pump (model LC-10Avp) and an injection valve (model EIG 001) (Shimadzu, Japan). The analytical isocratic RP-HPLC separation was performed on a shim-pack CLC-ODS-C8 column (6 mm × 150 mm, particle size, 5 μm) with a guard column (CLC G-ODS). The mobile phase was made up of acetonitrile and ethanol (50:50, v/v) and a flow rate of 1 ml min^{-1} was used at room temperature.

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The pH measurements were made with a 780 pH meter (Metrohm, Switzerland) equipped with a combine Ag/AgCl glass electrode. The centurion scientific centrifuge (K280R, UK) was used for centrifuging.

2.3. DLLME procedure

An aliquot (4 ml) of solution containing cholesterol was placed in a 15-ml screw capped test tube with conic bottom. A 0.8 ml of ethanol, as disperser solvent, containing 35 μl of carbon tetrachloride (as extraction solvent) was rapidly injected into the sample solution with a 1.0-ml syringe (Hamilton, USA), and then the mixture was gently shaken for 1 min. A cloudy solution was formed. The mixture was then centrifuged at 5000 rpm for 5 min. Then the dispersed fine droplets of extraction solvent were settled at the bottom of the conical test tube. The sedimented phase was completely transferred to another test tube using a 25.0-μl HPLC syringe (Hamilton, USA). The extract was evaporated to dryness at room temperature, re-dissolved with 4 μl of ethanol, and injected into the HPLC–UV system.

2.4. Sample preparation

2.4.1. Egg yolk

Egg yolk samples were manually separated from the albumen and placed on absorbing paper to remove albumen and homogenized by a food processor. 0.1 g yolk was weighted and added to 10 g doubly distilled water and shaken for 1 min. Yolk suspension was centrifuged at 2000 rpm for 2 min. A 100-μl aliquot of upper aqueous phase was spiked with standard solution of cholesterol, treated with acetonitrile (0.4 ml) and centrifuged at 1000 rpm for 1 min. The upper aqueous layer was transferred to another test tube for the extraction of cholesterol according to the procedure described above.

2.4.2. Milk

An aliquot (100 μl) of milk sample that was previously centrifuged at 2000 rpm for 10 min was spiked with standard solution of cholesterol. The solution was treated with acetonitrile (0.4 ml) and centrifuged at 1000 rpm for 1 min. The upper aqueous layer was transferred to another test tube for the extraction of cholesterol according to the procedure described above.

2.4.3. Olive oil

An aliquot (100 μl) of extra virgin olive oil sample was spiked with standard solution of cholesterol. The solution was transferred to test tube for the extraction of cholesterol according to the procedure described above.

3. Results and discussion

In this study the effects of several important parameters influencing the extraction efficiency of cholesterol including disperser and extraction solvent, pH, and extraction time were investigated. Peak area of cholesterol was used to evaluate and compare the performances at the different set parameters. The identification of cholesterol was done by matching its retention time against that of the standard. For each aspect of study the extraction was repeated three times for statistical analysis. A solution of 1 $\mu g\,l^{-1}$ cholesterol in 20% ethanol was used for the optimization of DLLME procedure.

3.1. Effect of type and volume of disperser solvent

The selection of disperser solvent is a critical factor in DLLME. Ideally, the disperser solvent should be miscible both with extraction solvent and sample. Acetonitrile, ethanol, and acetone were compared in the extraction of cholesterol. Table 1 summarizes the results in the term of listing the percent recovery of cholesterol with different disperser solvents at fixed volume (35 μl) of carbon tetrachloride (extraction solvent). As can be seen, ethanol provided better extraction efficiency than other two solvents.

Table 1
Effect of kind of disperser solvent (0.8 ml) on the extraction recovery of cholesterol ($n = 5$).

Disperser solvent	Recovery (%)	R.S.D. (%)
Ethanol	97.3 ± 0.03	3.08
Acetonitrile	74.2 ± 0.02	2.69
Acetone	43.5 ± 0.01	2.29

The effect of disperser solvent volume on the peak area is shown in Fig. 1. The results show that peak area and recovery increased with increasing disperser solvent volume up to 0.8 ml. At lower volumes of ethanol, the cloudy suspension of CCl_4 droplets is not formed well, resulting in a decrease in the extraction efficiency. At higher volumes of ethanol, the solubility of cholesterol in water increases and the extraction efficiency decreases.

However, the peak area and the extraction efficiency decreased by further increase in disperser solvent volume from 0.9 to 1.0 ml. A 0.8 ml of ethanol was used for the subsequent experiments.

3.2. Selection of extraction solvent

The selection of extraction solvent was based on (a) immiscibility with aqueous phase; (b) the higher density than aqueous phase and (c) good chromatographic analysis. Based on these considerations, carbon disulfide (CS_2), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), and carbon tetrachloride (CCl_4) were performed as extraction solvents to analyze the effect of the solvent on extraction efficiency. It was found that except for carbon tetrachloride–ethanol system, all other combinations of extraction and disperser solvents did not show stable cloudy solution. Based on the above results, CCl_4 and ethanol were chosen as extraction and disperser solvents, respectively. CCl_4 had no good chromatographic behavior and interfered with determination of cholesterol by HPLC. Therefore, the extract was evaporated to dryness. The residue was dissolved in 4 μl ethanol.

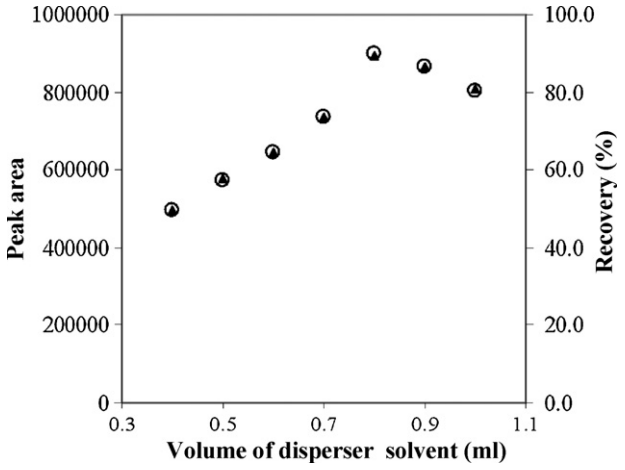

Fig. 1. Effect of volume of disperser solvent (ethanol) on the peak area and recovery of cholesterol. (○) Peak area, (▲) recovery (%). Experimental conditions: volume of extraction solvent = 35 μl, pH 7, time = 0 min.

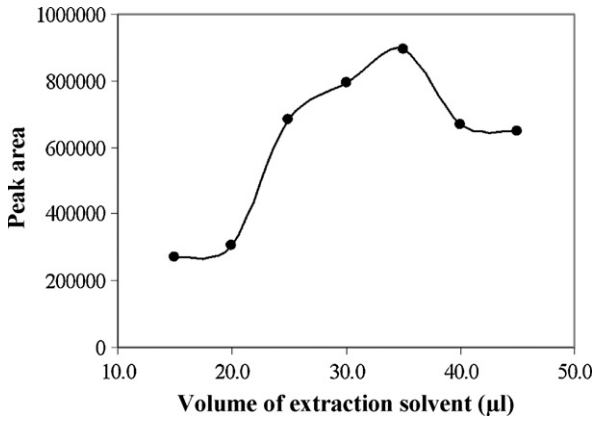

Fig. 2. Effect of volume of extraction solvent (CCl_4) on the extraction efficiency of cholesterol. Experimental conditions: volume of disperser solvent = 0.8 ml, pH 7, time = 0 min.

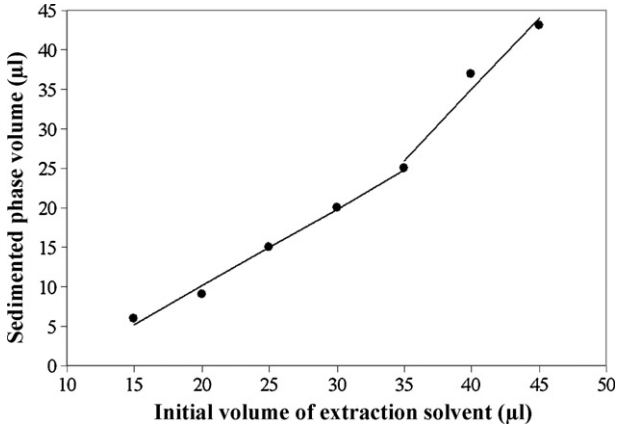

Fig. 3. Relationship between initial extraction solvent volume and sedimented phase volume. Experimental conditions: disperser solvent volume = 0.8 ml, pH 7, time = 0 min.

3.3. Effect of extraction solvent volume

To optimize the effect of extraction solvent volume, a fixed volume of ethanol (0.8 ml) containing different volumes of CCl_4 in the range 15–45 μl was subjected to the same DLLME procedure. The results shown in Fig. 2 indicate that the analytical signal virtually increases with CCl_4 volume in the range of 15–35 μl. However, a further increase in CCl_4 volume from 40 to 45 μl result a decrease in the peak area. This may be attributing to the formation of larger CCl_4 droplets and consequently increases in sedimented phase volume (Fig. 3). Hence, a 35 μl of extraction solvent volume was applied for the subsequent experiments.

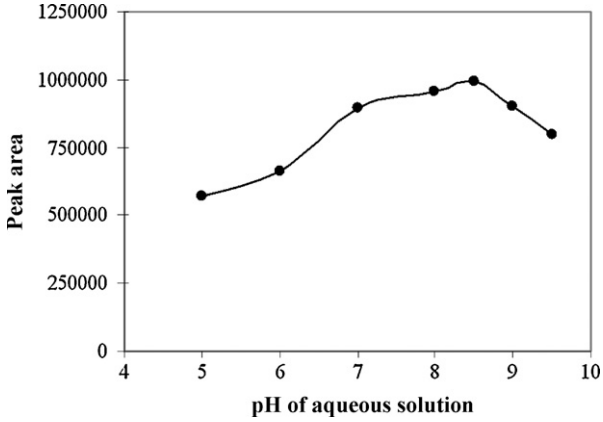

Fig. 4. Effect of pH of aqueous solution on the extraction efficiency of cholesterol. Experimental conditions: disperser solvent volume = 0.8 ml, extraction solvent volume = 35 μl, time = 0 min.

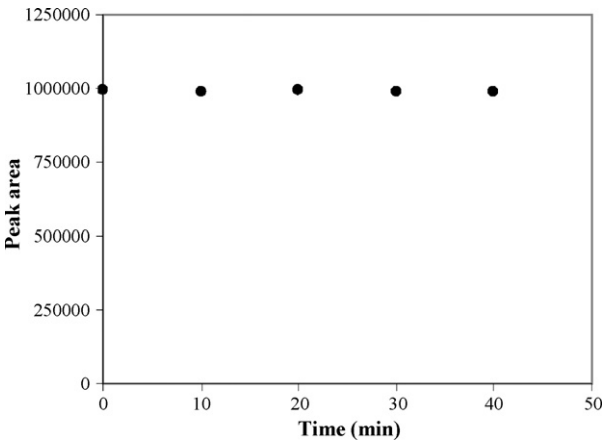

Fig. 5. Effect of extraction time on the extraction efficiency of cholesterol. Experimental conditions: disperser solvent volume = 0.8 ml, extraction solvent volume = 35 μl, pH 8.5.

3.4. Effect of pH

The effect of pH in the range from 5.0 to 9.5 was evaluated. The results, as shown in Fig. 4, demonstrate that the HPLC signal generally increased with pH. For a pH value above 8.5 the extraction efficiency began to decrease. Therefore, a pH value of 8.5 was fixed with the use of acetic acid and sodium bicarbonate.

Table 2
Results from determination of intra- and inter-day precision and accuracy of cholesterol by standard addition method ($n = 5$).

Sample	Added ($\mu g\,l^{-1}$)	Found ($\mu g\,l^{-1} \pm$ S.D.)	R.S.D. (%)	Recovery (%)
Milk				
Intra-day	0.0	2.91 ± 0.03	1.03	–
	0.2	3.12 ± 0.05	1.60	105.0
	1.0	3.94 ± 0.05	1.27	103.0
	3.0	5.90 ± 0.06	1.00	99.6
Inter-day	0.0	2.60 ± 0.03	1.15	–
	0.2	2.80 ± 0.07	2.50	100.0
	1.0	3.58 ± 0.10	2.80	98.0
	3.0	5.40 ± 0.10	1.85	93.3
Egg yolk				
Intra-day	0.0	2.97 ± 0.05	1.68	–
	0.2	3.16 ± 0.09	2.84	95.0
	1.0	3.95 ± 0.10	2.53	98.0
	3.0	5.80 ± 0.14	2.41	94.3
Inter-day	0.0	2.95 ± 0.07	2.37	–
	0.2	3.15 ± 0.08	2.54	100.0
	1.0	3.91 ± 0.11	2.81	96.0
	3.0	5.90 ± 0.15	2.54	98.3
Olive oil				
Intra-day	0.0	1.44 ± 0.02	1.38	–
	0.2	1.65 ± 0.04	2.42	105.0
	1.0	2.44 ± 0.07	2.86	100.0
	3.0	4.34 ± 0.11	2.53	96.7
Inter-day	0.0	1.47 ± 0.03	2.04	–
	0.2	1.68 ± 0.05	2.97	105.0
	1.0	2.47 ± 0.06	2.43	100.0
	3.0	4.39 ± 0.13	2.96	97.3

Table 3
Comparison of different methods for the determination of cholesterol in food samples.

Method	LOD ($\mu g\,l^{-1}$)	LR ($\mu g\,l^{-1}$)	R.S.D. (%)	Time (min)	Recovery (%)	Extraction solvent volume	Reference
Electrophoresis	5	0–1000	≤6.3	30	–	Petroleum ether + diethyl ether (5 ml)	[18]
Reverse micelle	1	5–200	<11	20–40	–	SF-CO_2	[19]
SPE–GC–FID	1	–	≤3.6	<30	96.2	n-Hexane (3 ml)	[20]
MIP–GC–FID	–	–	≤9.6	–	>80.4	n-Hexane + toluene (2 ml)	[1]
HPLC-fluorimetric	10	38.7–773.3	≤5.6	90	>99.1	Toluene (3 ml)	[3]
DLLME–HPLC–UV	0.01	0.03–10	≤3.1	~5	>95.0	CCl_4 (35 μl)	This work

3.5. Effect of extraction time

Another important parameter affecting the extraction efficiency in conventional liquid–liquid extraction procedure is the extraction time. In the present study, the influence of extraction time was also investigated in the range 0–40 min. The results are shown in Fig. 5. By increasing the extraction time no enhancement of extraction was observed. Other researchers found similar results [15,17]. They attributed this finding to the quickly equilibrium reaching and independency of DLLME to the extraction time.

Overall, the optimized experimental conditions found here were as follows: 0.8 ml ethanol and 35 μl CCl_4 as disperser solvent and extraction solvent, respectively, pH value of 8.5, and 0 min of extraction time.

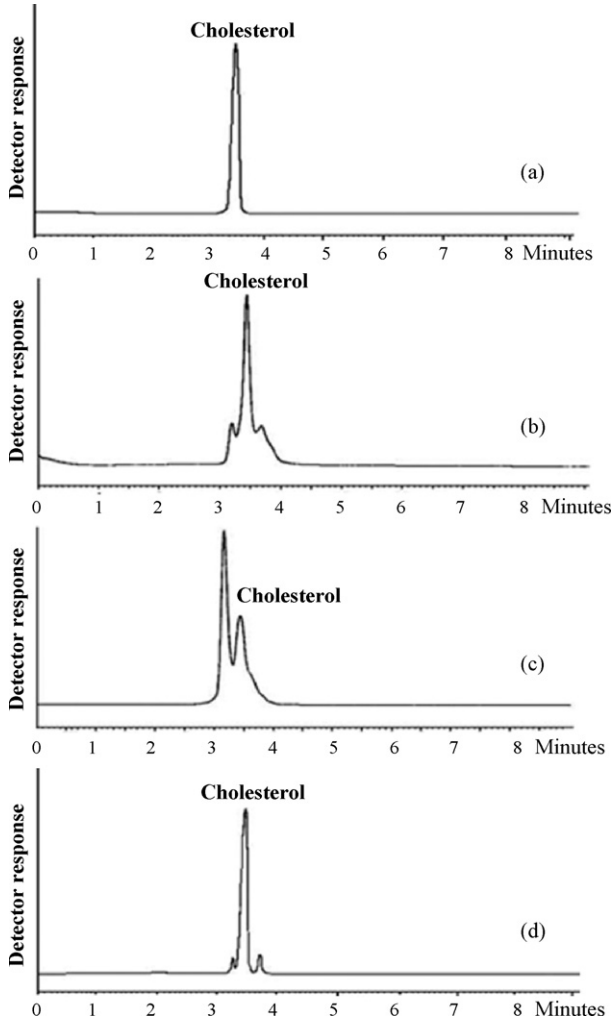

Fig. 6. LC–UV chromatograms obtained from the analysis of real samples spiked with cholesterol at concentration level 1 $\mu g\,l^{-1}$: (a) standard solution of cholesterol; (b) cow milk; (c) egg yolk; and (d) extra virgin olive oil. Chromatographic conditions: shim-pack CLC-ODS-C8 column; acetonitrile and ethanol (50:50, v/v) as mobile phase at a flow rate of 1 ml min^{-1}; detection at 210 nm.

3.6. Method validation

Under the above-optimized experimental conditions, the proposed method was validated by linearity, precision, recovery, LOD and LOQ. The calibration plot was found to be linear in the range of 0.03–10 $\mu g\,l^{-1}$, with a correlation coefficient (r^2) of 0.9996 ($n = 11$). For each concentration level, three replicate extractions were performed. The limits of detection (LOD, S/N = 3) and quantification (LOQ, S/N = 10) were 0.01 and 0.03 $\mu g\,l^{-1}$, respectively. As can be seen, the proposed method has low LOD and LOQ and can be used for trace analysis of cholesterol in food samples.

The intra-day and inter-day precisions and accuracies of the assay were evaluated by analyses of quality control samples at three concentration levels on the same day and the five consecutive days. The relative standard deviations (R.S.D.s) and accuracies were in the range of 1.00–2.97% and 95.0–105.0%, respectively. The estimated results are shown in Table 2.

Fig. 6a–d show the chromatograms of (a) standard solution of cholesterol in ethanol; (b) spiked cow milk sample; (c) spiked egg yolk sample; and (d) spiked extra virgin olive oil sample. As can be seen, no significant interference peaks were found at the retention position of cholesterol.

Table 3 indicates the limit of detection (LOD), linear range (LR), R.S.D., extraction time, recovery (%) and extraction solvent volume, using electrophoresis [18], reverse micelle [19], solid phase extraction–gas chromatography–flame ionization detection (SPE–GC–FID) [20], molecularly imprinted polymers–gas chromatography–flame ionization detection (MIP–GC–FID) [1], liquid chromatography–fluorescence detection (HPLC-fluorimetric) [3], and dispersive liquid–liquid microextraction–high performance liquid chromatography–UV detection (DLLME–HPLC–UV) methods for the determination of cholesterol in food samples. The proposed method provides similar quantification extraction efficiency, with advantages of being faster, using smaller volume of organic solvents, no saponification, and lower limit of detection.

4. Conclusions

A fast, simple, and sensitive DLLME combined with isocratic reverse phase high performance liquid chromatography (RP-HPLC) and UV detection was developed and optimized for direct determination of cholesterol in food samples with a chromatographic run time less than 10.0 min. As well as the consumption of the toxic organic solvents (at μl level) were minimized without affecting the method sensitivity. The results show that the proposed method can be used for the determination of cholesterol in food samples.

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