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Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food

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Abstract

Pressurized liquid extraction (PLE, ASE) was compared with the Folch procedure (a solid–liquid extraction with chloroform/methanol 2:1, v/v) for the lipid extraction of egg-containing food; the accuracy of PLE for the quantitative determination of oxysterols in whole egg powder was evaluated. Samples of spray-dried whole egg, an Italian vanilla cake (Pandoro) and egg noodles were used. Two different extraction solvents (chloroform/methanol 2:1, v/v, and hexane/isopropanol 3:2, v/v) were tested at different extraction temperatures and pressures (60°C at 15 MPa, 100°C at 15 MPa, 120°C at 20 MPa). No significant differences in the lipid recovery of the egg powder sample using PLE were found. However, PLE of the vanilla cake and egg noodles with the chloroform/methanol mixture was not selective enough and led to the extraction of a non-lipid fraction, including nitrogen-containing compounds. In the same samples, the pressurized hexane/isopropanol mixture gave a better recovery result, comparable to that obtained using the Folch method. Cholesterol oxidation products of the Folch extract and the pressurized liquid extract of spray dried egg powder (obtained with hexane/isopropanol 3:2, v/v, at 60°C and 15 MPa) were determined by gas chromatography. PLE performed under these conditions is suitable to replace the Folch extraction, because the differences between the two methods tested were not statistically significant. Moreover, PLE shows important advantages, since the analysis time was shortened by a factor of 10, the solvent costs were reduced by 80% and the use of chlorinated solvents was avoided.

Keywords: Food analysis; Eggs; Pressurized liquid extraction; Lipids; Oxysterols

1. Introduction

Sample preparation is a laborious, but necessary, step for characterization of the lipid fraction of food or the determination of specific analytes, when using exhaustive and non-selective extraction techniques. Many methods for the purification of food lipids were developed during the last century by well-known scientists, such as Soxhlet [1], Bligh and Dyer [2], and Folch et al. [3,4]. Organic solvents (alone or in combination) are used for these extractions. At that time, the laboratory work load and the amount of solvent consumed were much less than those nowadays; moreover, there was no consciousness of the health risks due to the continued exposure of operators to aromatic hydrocarbons and chlorinated solvents. In the last decades, supercritical carbon dioxide extraction and, recently, pressurized

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liquid extraction (PLE), have been successfully applied to the extraction of lipids. PLE is well known with the acronym of ASE™ (accelerated solvent extraction), which is a registered trade mark [5]. The amount of organic solvents is strongly reduced and the extraction time is shortened by employing high pressure and high temperature during PLE. This technique is used for the analysis of food lipids [6], and the determination of pollutants in environmental samples [7–9] and food [10,11]. Since a large spectrum of solvents can be utilized under these operative conditions, it is necessary to study the quantitative extraction of lipids from food samples of different composition. In addition, heat and high pressure may lead to the formation of artifacts in the lipid extract, such as oxidation products.

Cholesterol oxidation products (COPs, oxysterols) are toxic compounds found in thermally stressed food [12]. They are absorbed by the organism by intake of foods of animal origin and/or are generated in vivo by peroxidation of cholesterol. They have been found in the lipoprotein fraction of lymph and blood and are implicated in the etiology and progression of atherogenesis and cardiovascular disease, as well as in cancer and heart stroke; however, their precise role is not yet understood [13]. They can also be formed as artifacts during sample preparation, such as hot saponification at 80°C for 15 min [14] or direct cold saponification at room temperature for 10–12 h [15]. The GC determination of oxysterols is not yet a routine method due to the laborious clean-up procedure.

The aim of the present investigation was to study the reliability of PLE with respect to lipid extraction of egg-containing food (an Italian vanilla cake, powdered whole egg, egg noodles). Two solvent mixtures (chloroform/methanol 2:1, v/v, and hexane/isopropanol 3:2, v/v) were tested at different pressure and temperature conditions and the results were compared to the method described by Folch et al. [4]. The Folch procedure allows a quantitative extraction of all the lipid classes with very little formation of artifacts and is thus suited for the further determination of the minor components, such as oxysterols. However, it is a tedious and time-consuming method [3]. This work also suggests a new rapid and semi-automated extraction of COPs using PLE. The determination of COPs was performed on the Folch and pressurized liquid (PL) extract with two aims: to test the formation of artifacts due to PLE and to verify the reliability of the latter by comparing the results obtained with both extraction procedures.

2. Materials and methods

2.1. Chemicals and materials

Methanol R.S. and chloroform R.S. were from Carlo Erba (Rodano, Italy), while propan-2-ol R.P. was from Prolabo (Paris, France). *n*-Hexane P.A. was purchased from Merck (Darmstadt, Germany).

The standards used for the identification of oxysterols were supplied by Sigma (St. Louis, MO, USA): 5-cholesten-3β-ol-7-one (7-k), 5-cholestan-5α,6α-epoxy-3β-ol (alpha), 5-cholestan-5β,6β-epoxy-3β-ol (beta), 5-cholesten-3β,19-diol (19-OH) as the internal standard, 5-cholesten-3β,20α-diol (20-OH) and cholestane-3β,5α,6β-triol (tr). The standards 5-cholesten-3β,7α-diol (7-a), and 5-cholesten-3β,7β-diol (7-b) were obtained from Steraloids (Wilton, NH, USA).

Samples of a traditional Italian vanilla cake (Pandoro) and egg noodles were bought from a local food store and finely ground in the laboratory. The spray-dried whole egg powder was obtained from a local factory (Eurovo, S. Maria Fabriago, Italy). The labelled moisture content was 21.5, 11.5 and 4% for the vanilla cake, egg noodles and the spray-dried whole egg powder, respectively.

The samples were homogenized with a T 25 blender (Janke and Kunkel, Staufen, Germany). Paper filters no.1 (Whatman, Maidstone, UK) were used for the Folch extraction. Oxysterols were purified by using aminopropyl (NH_2) cartridges (500 mg/3 ml) from Applied Separations (Allentown, PA, USA). The silylation agent was a mixture prepared from dried pyridine (Merck), hexamethyldisilazane R.P.E. and trimethylchlorosilane R.P.E. (Carlo Erba) in a ratio of 5:2:1 by volume.

2.2. Procedures

2.2.1. PLE

Different amounts of celite were mixed with the

samples: 5 g vanilla cake/5 g celite, 12 g egg noodles/4 g celite and 4 g whole egg powder/4 g celite. Extractions were carried out using a Dionex ASE™ 200 (Dionex, Idstein, Germany). Table 1 summarizes the six different experimental conditions assayed for PLE. All three types of samples were extracted by using two cycles of 10 min each at the reported conditions. Successively, solvents were eliminated from the extract using a vacuum evaporator and the total residue was weighed and dissolved in hexane-isopropanol (4:1, v/v) for further analysis. If an insoluble residue was present (samples of vanilla cake and egg noodles extracted with the chloroform/methanol mixture), this was separated, dried under gentle nitrogen flow, weighed and subjected to elemental analysis (EA).

2.2.2. Folch extraction

Samples were extracted according to Folch et al. [4]. The pulverized samples (egg powder, 3 g; egg noodles, 25 g; vanilla cake, 10 g) were homogenized in a bottle with 200 ml of a chloroform/methanol mixture (1:1, v/v) for 3 min. In order to increase the extraction yield, the bottle was placed at 60°C for 20 min. Subsequently, 100 ml chloroform were added to the bottle. After 3-min homogenization, the content of the bottle was filtered through filter paper. The filtrate, which contained the lipids accompanied by non-lipid substances, was mixed thoroughly with a 1-*M* KCl solution and left overnight at 4°C, in order to allow the separation into two phases. The lower phase, freed from non-lipid substances, was collected and dried with a vacuum evaporator.

2.2.3. COP determination

The pressurized liquid extracts and 250-mg lipid subfractions of the Folch extract were added with a known amount of the internal standard solution (12.5 μg of 19-hydroxy-cholesterol). Cold saponification was performed at room temperature according to Sander et al. [15]. Oxysterols were purified by NH_2 solid-phase extraction (SPE) according to Rose-Sallin et al. [16], silylated according to Sweeley et al. [17] and dried under gentle nitrogen flow. After redissolution in 100 μl hexane, 1 μl was analyzed by capillary gas chromatography.

2.2.4. Statistical analysis

The determination of the experimental Student's *t*-test and the parametric tests were performed by using Unistat Statistical Package (Megalon, Neuchâtel, Switzerland).

2.3. Instrumentation

2.3.1. GC analysis

The gas chromatograph (HRGC 5300, Carlo Erba Instruments, Rodano, Italy) was equipped with a split-splitless injector and a flame ionization detector (FID). A fused-silica capillary column (30 m×0.32 mm I.D., 0.25-μm film thickness) coated with 100% dimethyl-polysiloxane (DB-1, J&W Scientific, Folsom, CA, USA) was used. The samples were injected in the split mode. The oven temperature was programmed from 250 to 325°C at 3°C/min; the injector and detector temperatures were set at 325°C. Helium was used as the carrier gas at a flow of 2 ml/min; the split ratio was 1:15.

2.3.2. Elemental analysis (EA)

The elemental analysis was performed on the insoluble residue obtained with PLE using method D. The residue from egg noodles (150 μg) and vanilla cake (70 μg) was analyzed in triplicate with an EA 1110 CHN-S (CE Instruments, Rodano, MI, Italy), as suggested by Sweeney [18]. The calibration was

Table 1
Experimental conditions assayed for PLE

Method	A	B	C	D	E	F[a]
Extraction solvent	a:b (3:2, v/v)	a:b (3:2, v/v)	a:b (3:2, v/v)	c:d (2:1, v/v)	c:d (2:1, v/v)	c:d (2:1, v/v)
Temperature (°C)	60	100	120	60	100	120
Pressure (MPa)	15	15	20	15	15	20

a, *n*-hexane; b, isopropanol; c, chloroform; d, methanol.
[a] Method F was only applied to the extraction of the whole egg powder.

based on theoretical 10.1% (w/w) nitrogen in pure acetanilide (C_8H_9NO) primary standard (Merck, Darmstadt, Germany).

3. Results

3.1. Total fat content

The amount of lipids extracted by the Folch method were 45, 24.8 and 5% for the egg powder, vanilla cake and egg noodles, respectively. These three samples were chosen for the development of a PLE procedure for sample preparation, because they represent food with high, intermediate and relatively low fat content.

The PLE methods reported in Table 1 were performed in triplicate on all samples. Figs. 1–3 summarize the normalized PLE results with respect to those found in the Folch extraction.

The statistical *t*-test showed that the recovery obtained with hexane/isopropanol (methods A, B and C applied to the egg powder and the vanilla cake) was not significantly different ($P<0.05$) with respect to the Folch procedure. Regarding egg noodles, only method B did not show significant differences. These results show that the pressurized hexane/isopropanol (3:2, v/v) mixture can be an efficient extraction solvent for PLE and can replace chlorinated solvents. The recovery registered with hexane/isopropanol is satisfactory in most cases, even when using gentle conditions, such as 60°C and 15 MPa. Moreover, almost no coextraction of the non-lipid fractions occurred using hexane/isopropanol; in fact, the recovery was similar to that of the Folch extract, except for the egg powder extracted with method B (107%). However, the pressurized chloroform/methanol mixture was particularly critical when PLE was carried out on egg noodles and the vanilla cake (Figs. 1 and 2). In the cake, methods D and E showed three main disadvantages: (a) they were less efficient than methods A, B and C; (b) the

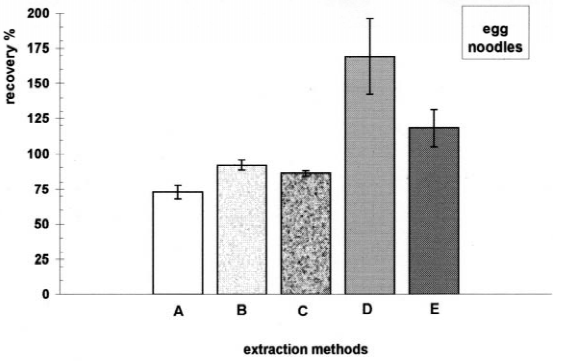

Fig. 2. Normalized lipid contents of egg noodles obtained by different ASE methods, with respect to those determined by the Folch method. The concentrations were calculated from three separate analyses of each sample. RSD are included as a bar.

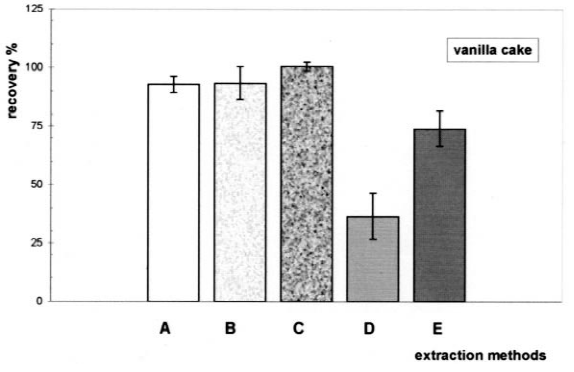

Fig. 1. Normalized lipid contents of vanilla cake obtained by different ASE methods, with respect to those determined by the Folch method. The concentrations were calculated from three separate analyses of each sample. The relative standard deviations (RSD) are included as a bar.

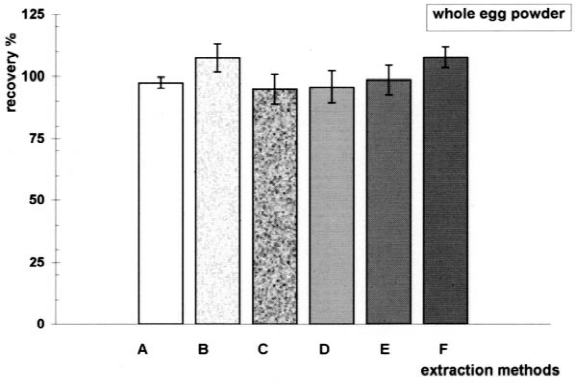

Fig. 3. Normalized lipid contents of spray-dried whole egg powder obtained by different ASE methods, with respect to those determined by the Folch method. The concentrations were calculated from three separate analyses of each sample. RSD are included as a bar.

recovery was affected by the temperature to a great extent; (c) the standard deviation among the three replicates was higher than that obtained by using methods A, B and C. Thus, methods D and E did not represent a robust procedure in the case of samples other than egg powder.

On the other hand, a fraction of the dried pressurized liquid extract of egg noodles and the cake obtained with chloroform/methanol, was not soluble in a mixture of hexane/isopropanol. This shows that non-lipid components were extracted as well. This insoluble residue (not present in the egg powder extract), obtained with methods D and E, ranged between 4±0.4 and 17±2.4% of the sample weight. As reported in Ref. [18], wheat proteins consist of 17.5% nitrogen (N×5.70). Elemental analysis (EA) showed that the nitrogen content of the insoluble residues was less than 17.5% (14±0.3 and 8±1% for the egg noodles and the vanilla cake, respectively). Thus, it can be assumed that not only the nitrogen fraction, but also other components (e.g. carbohydrates) are extracted from the vanilla cake with methods D and E (chloroform/methanol).

3.2. Oxysterol determination

High extraction temperatures were tested because they are currently used for many applications [5–10] or suggested for the determination of the total fat content of food with PLE, when lipid autoxidation is not an issue [19,20].

Since no significant differences among PLE performed with hexane/isopropanol at 60, 100 and 120°C, were found, method A was selected for the extraction of oxysterols from egg powder samples. This method was chosen because chloroform is replaced by non-chlorinated solvents and the extraction temperature (60°C) should limit cholesterol autoxidation. The results of the oxysterol determination using the Folch extraction and method A are reported in Fig. 4. A typical gas chromatographic trace obtained with PLE is shown in Fig. 5. The extractions were run in triplicate and no significant differences were observed between the two methods ($P<0.05$). However, the total amount of oxysterols obtained with the Folch extraction (134 ppm) was 8% higher than that obtained with method A (125

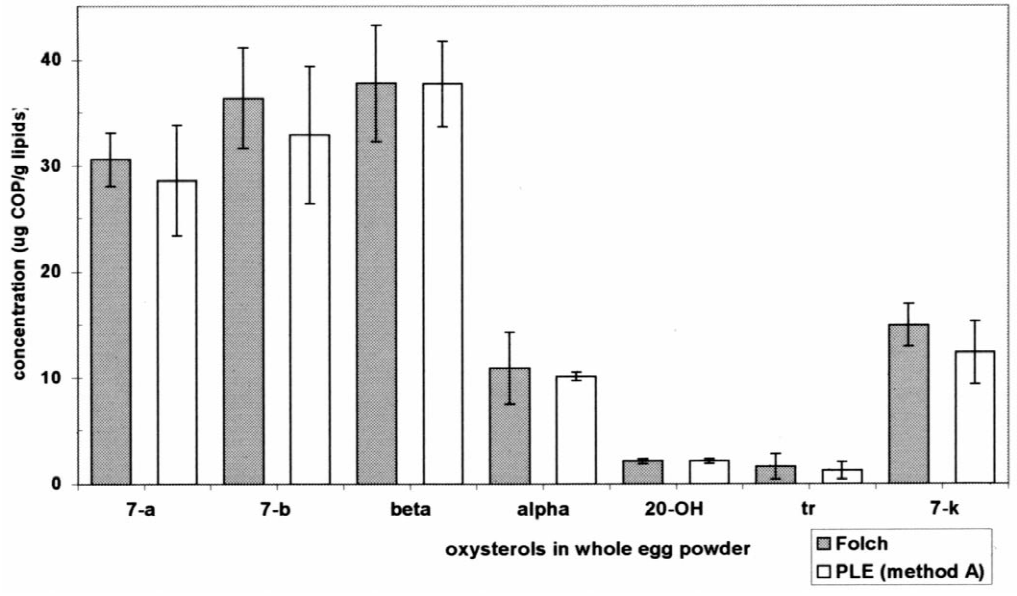

Fig. 4. Comparison between PLE and Folch extraction regarding the recovery of cholesterol oxidation products (μg COPs/g lipids) from whole egg powder. 7-a, 5-cholesten-3β,7α-diol; 7-b, 5-cholesten-3β,7β-diol; 7-k, 5-cholesten-3β-ol-7-one; 19-OH, 19-hydroxy-cholesterol (internal standard); 20-OH, 5-cholesten-3β,20α-diol; alpha, 5-cholestan-5α,6α-epoxy-3β-ol; beta, 5-cholestan-5β,6β-epoxy-3β-ol; chol, cholesterol; tr, cholestane-3β,5α,6β-triol.

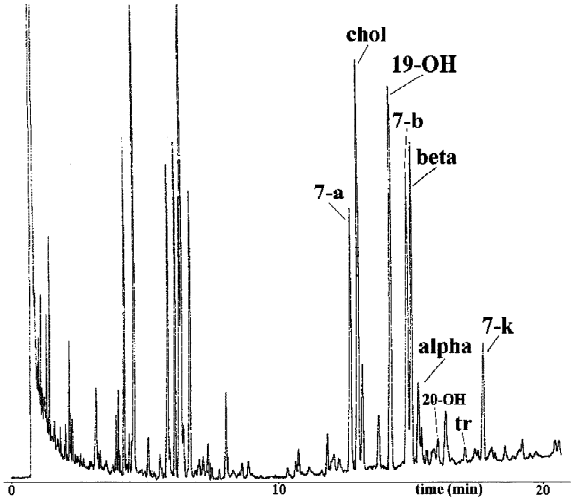

Fig. 5. Gas chromatographic trace of oxysterols of whole dried egg extracted by PLE. Refer to Fig. 4 for abbreviations.

ppm). This could be due to long analysis time and large sample manipulation; in fact, sample manipulation in the presence of oxygen can cause cholesterol autoxidation and can lead to a higher COP concentration in the Folch extract rather than in the PLE extract.

4. Conclusions

Folch extraction can be successfully replaced by PLE for the analysis of the lipid fraction of egg-containing food, provided that a mixture of hexane/isopropanol is used. The major advantages shown by the proposed method are: (a) reduction of the analysis time to about one tenth, (b) reduction of the solvent costs of ~80%, (c) reduction of sample manipulation with the consequent decrease of contamination risk, and (d) the exclusion of chlorinated solvents. The chloroform/methanol mixture (2:1, v/v) is not a suitable solvent at the temperature and pressure conditions that PLE requires.

PLE is also a valid sample preparation method for the determination of oxysterols and can replace the Folch extraction: no artifacts are formed during the sample extraction under the experimental conditions selected in this study.

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