The Extraction of Cholesterol from Solid and Liquid Matrices Using Supercritical CO_2

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Abstract. The design and construction of a relatively simple supercritical CO_2 system capable of extracting compounds from solid and liquid matrices are described. The system consists of a conventional liquid chromatography pump, a cooler, a stainless steel extraction chamber, and a modified collection system. A preliminary evaluation of the extractor is carried out here by using it to extract cholesterol from egg yolk and blood serum. The extracts were analyzed by supercritical fluid chromatography, and the results compared with those obtained by Soxhlet extraction.

Key words: *supercritical fluid extraction, supercritical fluid chromatography, cholesterol, extraction chamber, liquid and solid matrices*

INTRODUCTION

Supercritical fluid extraction (SFE) and chromatographic separation of a large variety of compounds have been enjoying widespread popularity in the past few years (1,2). The relatively mild conditions that supercritical fluids such as CO_2 (critical temperature, T_{cr}, of 31°C) offer in the extraction of these compounds have made SFE and supercritical fluid chromatography (SFC) important techniques in the extraction and analysis of thermally labile compounds. Coupled with this, CO_2 is also relatively cheap, easily available, nontoxic, nonflammable, and conveniently removed after extraction — all very desirable properties for an extraction solvent. Several reviews on SFE have previously been published (3–5).

In this paper, we describe the assembly of a simple supercritical CO_2 extraction system from commercially available components. We also describe the design of an extraction chamber that can be used for either solid or liquid samples. A preliminary evaluation of the system used for the extraction of cholesterol from egg yolk (solid sample) and blood serum (liquid sample) is presented.

EXPERIMENTAL

Hardware for SFE system. A conventional HPLC solvent pump (model RR/066 supplied by HPLC Technology, Macclesfield, Cheshire, U.K.) was used as the fluid compression and delivery system. (It should be mentioned that as long as there is a way to cool the pump head, any HPLC pump could be used for an SFE system.) To cool the CO_2 being delivered by the pump, a 30:70 ethylene glycol and water mixture, which was chilled using a Frigomix cooler (Braun, Melsungen, FRG), was circulated through polypropylene tubing that was wrapped around the pump head. By ensuring good insulation around the tubing over this area, it was possible to achieve a temperature of about −10°C at the pump head. We found that this temperature could be maintained satisfactorily by having a circulator (for example, Thermomix from Braun) in the cooler. Centrifugal pumps such as those used in aquariums (for example, RENA C-40, supplied by RENA SA, Annecy, France, with pumping capacities of 10 L min^{-1}) were used to circulate the cold ethylene glycol and water mixture to and from the pump head and in other parts of the system. A diagram of the supercritical CO_2 extraction system is depicted in Figure 1.

One-quarter-inch (o.d.) (6 cm × 4 mm i.d.) 316 stainless steel tubes were used as the extraction chambers. With a slight modification, the solid sample extractor can be converted easily to the liquid sample extractor (Figure 2). Such a design offers great flexibility and versatility. Furthermore, variable amounts of the respective matrices can be extracted by using different lengths of the tubing. Stainless steel frits were placed at both ends of the extraction tube to prevent clogging during extraction. Stainless steel Swagelok fittings were used for most un-

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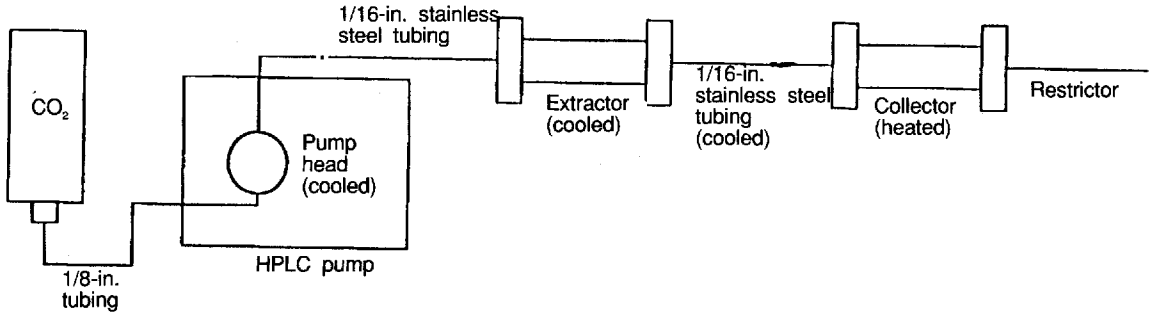

Figure 1. *Diagram of supercritical CO_2 extraction system.*

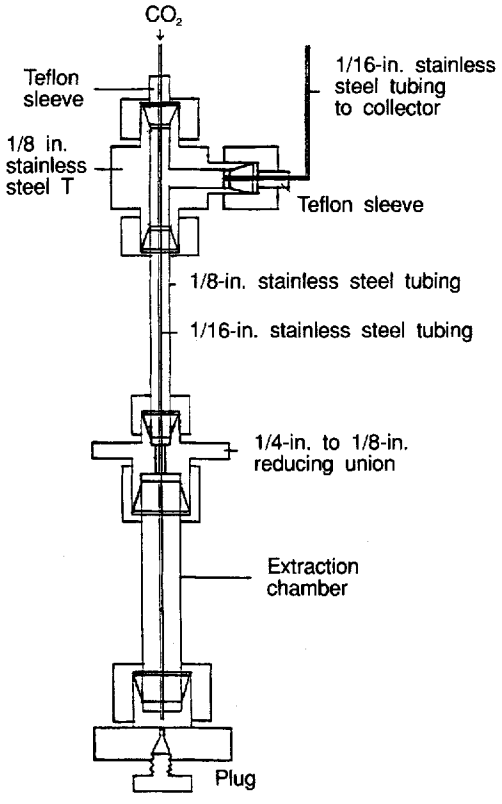

Figure 2. *Diagram of liquid sample extractor.*

ions. However, to facilitate disconnection of the extraction tube assembly (for cleaning, removal, and introduction of sample to be extracted, etc.), it was convenient to use high-pressure Fingertight II fittings (Upchurch Scientific, Oak Harbor, Washington, USA). All connecting tubings were made of stainless steel. The oven of a Hewlett-Packard series 5790 gas chromatograph (Palo Alto, California, USA) was used to maintain the temperature of the extractor.

Collector. A 1/4-in.-o.d. (15 cm × 4 mm i.d.) stainless steel tube, similar to that used for the extraction chamber, was used as the collector. Swagelok stainless steel ferrules and nuts were used for all other fittings.

Restrictor. Tapered fused-silica glass capillary restrictors (0.073-mm i.d., calibrated flow rate 18 mL min^{-1}) were used to maintain the high pressure within the system.

Chemicals. Carbon dioxide (purity 100%, supplied by BOC, London, U.K.) was used in the extraction system. HPLC-grade hexane (J.T. Baker, Phillipsburg, New Jersey, USA) was used to prepare standard solutions of cholesterol and cholesteryl chloroacetate. The latter compounds were of the purest grade available (Aldrich Chemical Co., Milwaukee, Wisconsin, USA).

Procedures. *Delivery of CO_2.* It was necessary to place the cylinder upside down to deliver liquid CO_2 satisfactorily to the pump. In this regard, it was convenient to use the 3-kg CO_2 bottles, which can be more easily lifted and upended.

Extraction of cholesterol. To illustrate the utility of the two types of extractors and evaluate their performance, we selected blood serum and hard-boiled egg yolk from which to extract cholesterol. The egg yolk was simply "packed" into the extraction chamber and mixed with prewashed sand and glass wool. The sand and glass wool were added to improve the dispersion of the egg yolk so that a more efficient extraction could be carried out. As for the liquid matrix, horse blood serum was used. The serum, spiked with cholesterol as well as the internal standard, was simply introduced into the extractor by a syringe. In order to assess the recovery of cholesterol, cholesteryl chloroacetate was used as an internal standard. The reasons for this choice are that both peaks are well separated chromatographically and the total analysis time for these two components was reasonably short (6). The structures of cholesterol and cholesteryl chloroacetate are shown in Figure 3.

Chromatography. Chromatograms were obtained on a Carlo Erba SFC 3000 (Carlo Erba Strumentazione, Milan, Italy) that was equipped with a hydrogen flame ionization detector. The following pressure program was used: initial pressure — 14 MPa (held for 0 min, rate of increase 0.1 MPa min^{-1}); final pressure — 20 MPa. The temperature of the oven (column) was maintained at 85°C. The 10 m × 0.1 mm i.d. column used was coated with SE-52 (J&W Scientific, Folsom, California, USA).

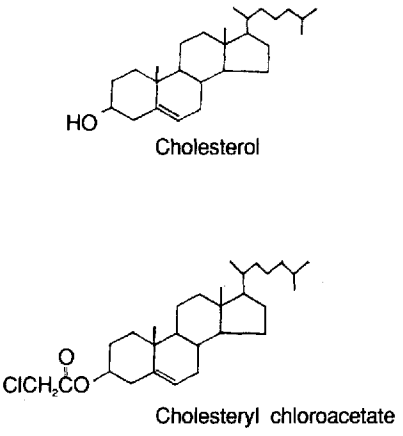

Figure 3. *Structures of cholesterol and cholesteryl chloroacetate.*

Injections were made with an air-actuated VICI Valco injection valve (Schenkon, Switzerland) equipped with a 1-μL loop. The injection port, split outlet, and detector temperatures were maintained at 40°C, 250°C, and 320°C, respectively.

RESULTS AND DISCUSSION

Cholesterol is an appropriate test material in our system because of the inherent difficulties of its extraction and subsequent analysis by conventional chromatographic techniques. The extraction of this compound from biological materials usually involves several tedious steps, including hydrolysis with alcoholic potassium (or sodium) hydroxide solution, followed by liquid–liquid partition, and then HPLC or GC analysis. The main problem with HPLC analysis is that the weak UV absorption exhibited by cholesterol precludes the use of a UV detector, and the refractive index detector generally is not suitably sensitive. On the other hand, if gas chromatographic analysis is desired, a derivatization step is needed; this adds to the total analysis time. Moreover, high-temperature conditions are required. The measurement of cholesterol in blood serum by the Abell–Kendall colorimetric method (7) also suffers from many problems (8). Therefore, we set out to develop an alternative procedure involving SFE and SFC.

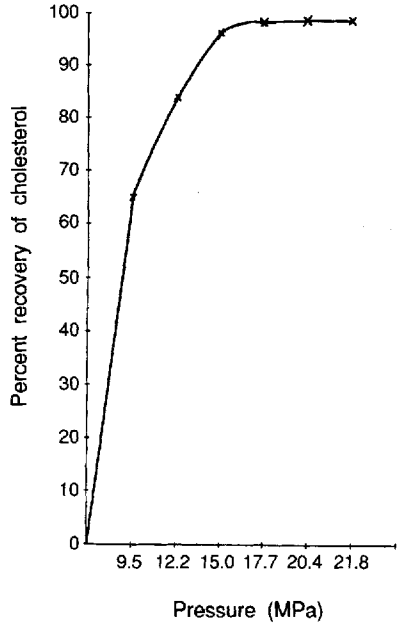

Figure 4. *Percentage recovery of cholesterol from spiked glass wool with respect to pressure of extraction medium. x = Supercritical CO_2.*

The SFE system described is very easy to use. A simple on/off toggle valve at the cylinder was used to supply CO_2 to the LC pump head. For an extraction run, the latter was cooled to −10°C prior to switching on the CO_2 flow. Initially, the problem of clogged restrictors was encountered when the extract was collected from the extraction chamber directly into hexane. There was no attempt to collect the extract into warm hexane, although that might have solved the problem because of the high volatility of the solvent. We tried heating the restrictor, but because the submerged tip was effectively excluded from the heating, no improvement was observed. Wright et al. have extracted polycyclic aromatic hydrocarbons using a heated restrictor such as this (2). Specifically, the heated restrictor was enclosed in a sealed flask cooled in liquid nitrogen. To avoid the special handling procedures for liquid nitrogen as well as a more elaborate design of the collector under this arrangement, an alternative way of collecting the cholesterol extract was devised.

The collector was placed between the extractor and the restrictor, which was maintained at an elevated temperature. This setup differs from the more familiar arrangement in which the restrictor is connected to the outlet of the extractor. Using the latter arrangement, restrictor clogging was a common occurrence, and thus it was not possible to satisfactorily extract cholesterol. No such problems were encountered with the modified arrangement. Furthermore, with this modification, it was no longer necessary to heat the restrictor. As long as the collector was heated to a temperature (100°C) slightly greater than the oven temperature, negligible losses of extracted cholesterol were observed. Various extraction conditions initially were evaluated by considering the extraction of cholesterol from spiked glass wool. A discussion of these conditions follows.

Temperature. It has been recommended that SFE be carried out at a temperature at least 10°C greater than the T_{cr} of the fluid (2). For this work, therefore, the temperature of the extraction assembly was maintained at about 45°C. Higher temperatures were not used for fear of decomposition of the thermally labile components, especially the blood serum, in the respective matrices.

Pressure. As expected, with increasing pressure, the efficiency of extraction improved. The relevant data are shown in Figure 4. The minimum pressure required for 98% recovery of cholesterol within 30 min was found to be 17.7 MPa. A pressure of 17.7 MPa was subsequently adopted as the working pressure for extractions.

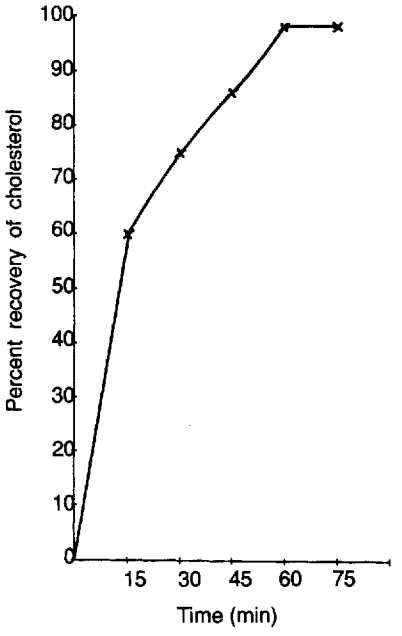

Figure 5. *Quantity of cholesterol recovered (as a percentage of total amount) from egg yolk with respect to duration of extraction. x = Supercritical CO_2.*

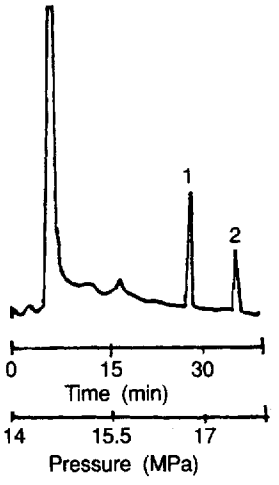

Figure 6. *Typical supercritical fluid chromatogram of (1) cholesterol and (2) cholesteryl chloroacetate extracted from egg yolk.* k′ *(cholesterol) = 4.48;* k′ *(cholesteryl chloroacetate) = 5.84. Chromatographic conditions: isothermal at 85°C; pressure programmed from 14 MPa to 20 MPa in 60 min.*

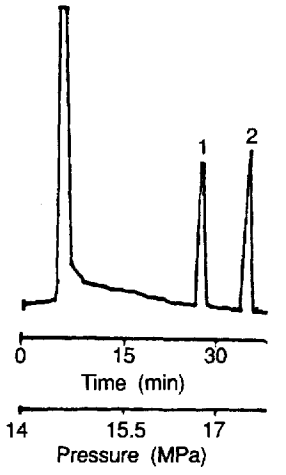

Figure 7. *Typical supercritical fluid chromatogram of (1) cholesterol and (2) cholesteryl chloroacetate extracted from spiked blood serum.* k′ *(cholesterol) = 4.47;* k′ *(cholesteryl chloroacetate) = 5.82. Chromatographic conditions: same as Figure 6.*

Extraction time. Figure 5 shows that the quantity of cholesterol extracted from egg yolk was proportional to the duration of extraction and leveled off after about 60 min. It should be noted that the longer time (i.e., 60 min, compared to 30 min for the glass wool–spiked sample) was required to allow for complete extraction of both bound and unbound cholesterol. The extraction time compares very favorably with the Soxhlet extraction, which required 7 h. Moreover, in our view, the SFE's ease of operation is preferable to the widely used liquid–liquid partition technique (after sample saponification) for cholesterol.

Figures 6 and 7 show typical chromatograms of cholesterol (and the internal standard) extracted by SFE from egg yolk and spiked blood serum, respectively.

As Table I indicates, excellent recoveries of cholesterol and cholesteryl chloroacetate from both spiked glass wool and blood serum were obtained. Table II contains, as a means of comparison, the amount of cholesterol extracted from egg yolk by the

Table I. *Extraction efficiencies. SFE of cholesterol and cholesteryl chloroacetate from spiked glass wool and spiked blood serum.*

	Percent Recoveries	
	Cholesterol	Cholesteryl Chloroacetate
Glass wool	98.0	98.0
Blood serum	98.0	98.2

Conditions: pressure — 17.7 MPa for 30 min; oven temperature — 45°C.

Table II. *Quantity of cholesterol extracted from egg yolk.*

Method of Extraction	Cholesterol (mg/100 g egg yolk)[a]
Supercritical CO_2	1450 ± 2
Soxhlet[b]	1380 ± 2

[a]Average values of duplicate extractions.
[b]Extraction with hexane for 7 h.

different extraction methods used in this work; the values listed reflect the expected quantity of the compound present in eggs. The results also bear out, as mentioned above, an important advantage of supercritical CO_2 extraction; that is, less time is required for near-complete recovery of the cholesterol from the sample.

A commercial SFC instrument was used for the analysis of the two compounds considered in this work. This instrument was used because the homemade system lacked a pressure programmer, which precluded the optimization of the analytical conditions; nevertheless, we have successfully used it to analyze steroids (9).

CONCLUSIONS

We have successfully demonstrated the use of supercritical CO_2 for the extraction and analysis of cholesterol from food samples and blood serum. This is the first report of a simple design in which the extractor and collector are interchangeable. A further advantage is that the extraction chamber can be used for both solid and liquid samples.

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