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Journal of Chromatography A, 715 (1995) 333–336

JOURNAL OF CHROMATOGRAPHY A

Determination of cholesterol in milk fat by supercritical fluid chromatography

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First received 6 December 1994; revised manuscript received 18 May 1995; accepted 19 May 1995

Abstract

A rapid, accurate and precise method for the determination of cholesterol in milk fat using supercritical fluid chromatography (SFC) is described. The accuracy and precision of the developed method are confirmed by analyzing the BCR Reference Material (CRM 164) and by recovery studies of spiked sunflower oil. Furthermore, SFC was compared with gas chromatography (GC). In our case, SFC seems to be more accurate than GC.

1. Introduction

It is generally accepted that an elevated level of serum cholesterol is an important risk factor of coronary heart disease. Based on studies on this subject, the American Heart Association recommended to reduce the daily intake of fat, especially of saturated fat and cholesterol [1]. For this reason, the food industry is interested in developing new methods to analyze cholesterol in food samples.

Up till now gas chromatography has been the method of choice for the accurate determination of cholesterol in foodstuffs, because it is fast, cheap and well developed [2,3]. However, the development of supercritical fluid chromatography (SFC) as a promising chromatographic technique [4] and the possibility to couple supercritical fluid extraction and SFC has led to the development of several new methods for the determination of cholesterol and cholesteryl esters by SFC, such as: (i) King [5] analyzed the cholesterol content in a fish-oil capsule by capillary SFC (analysis time, 90 min). However this study did not contain information about the precision of the method. (ii) Ong et al. [6] determined cholesterol in egg yolk and blood serum using capillary SFC (analysis time, 40–45 min). In this paper the conditions for the analysis of cholesterol in egg yolk were given, but no information about sample preparation and precision. (iii) Nomura et al. [7] published a very interesting paper which exactly described the determination of cholesterol and cholesteryl esters in human serum on an ODS-silica gel column, where the analysis took less than 20 min.

In the present work a rapid, accurate and precise method is described for the determination of cholesterol in milk fat by SFC using a simplified sample preparation method. Addition-

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0021-9673/95/$09.50
SSDI 0021-9673(95)00602-8

ally, a comparison between SFC and gas chromatography (GC) is carried out.

2. Experimental

A Lee Scientific supercritical fluid chromatograph (Model 602) from Dionex (Salt Lake City, UT, USA), equipped with a flame-ionization detector (FID), a 20 m × 50 μm I.D. capillary column SB Phenyl 5 with 0.25 μm film thickness (Dionex) and a 15 cm × 15 μm fused-silica restrictor, is used with a timed-split injection. As carrier gas, carbon dioxide (UN 1013 SFC Grade, Scott Specially Gases, USA) is used.

A Hewlett-Packard gas chromatograph (HP 5890A), equipped with an FID, is used with split injection. The column employed is a fused-silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness), coated with HP-5 (Hewlett-Packard, 19091 J-433). Hydrogen is used as carrier gas.

As reference material anhydrous milk fat (CRM 164) from the Community Bureau of Reference in Brussels, Belgium, is used. For this reference material a cholesterol value of 273 mg/100 g (± 39 mg/100 g) is indicated. Cholestane, 98% pure (Aldrich, USA) was used as internal standard.

2.1. Sample preparation procedure

The modified sample preparation is based on the method described by Fenton and Sim [3] for the analysis of cholesterol in egg yolk samples. Modifications were made to minimize the sample volume and the preparation time. The sample preparation was performed as follows. A weighted amount (0.15 g) of milk fat was placed into a screw-cap tube (100 × 20 mm) and 1.0 ml of the internal standard solution (1 mg cholestane/1 ml of hexane) was added. Then, 10 ml of alcoholic KOH solution [9.4 ml 95% (v/v) ethanol plus 0.6 ml 33% (w/v) KOH] was added. The saponification was carried out in a water bath of 70°C during 30 min with occasional shaking. Subsequently, the sample was cooled to room temperature and 5 ml of deionized water was added. Extraction of the unsaponificable constituents was performed with 10 ml of hexane with permanent shaking for 1 min. After separation of the layers, the upper hexane layer was removed using a pipette and evaporated at 35°C. The residue was diluted with 0.5 ml hexane and then analyzed by SFC and GC.

2.2. Supercritical fluid chromatography (SFC)

To analyze cholesterol in milk fat the following method was used: The density of carbon dioxide was held at 0.2 g/ml for 2 min, then programmed at 0.012 (g/ml)/min to 0.45 g/ml. The oven temperature was held at 130°C and the detector temperature (FID) was at 350°C. The total analysis time was 25 min. Fig. 1 shows a typical chromatogram of anhydrous milk fat (CRM 164) using SFC (peaks: 1 = cholestane, 2 = cholesterol).

2.3. Gas chromatography (GC)

Hydrogen is delivered to the column at a head pressure of 0.7 bar and at a flow-rate of 30 ml/min. The injector and the detector temperatures were held at 300°C. The oven temperature was held at 260°C for 1 min, then programmed at a rate of 4°C/min to 300°C. The injection volume was 1 μl and the total analysis time was 12

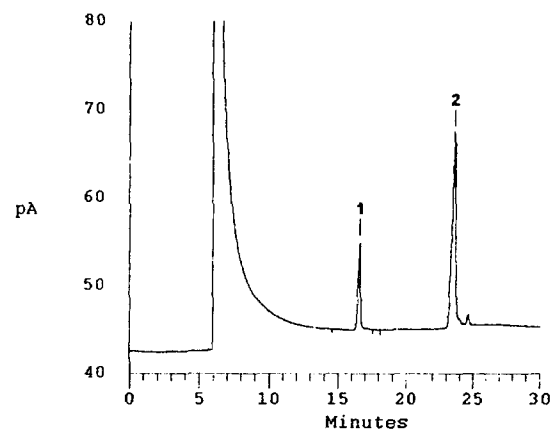

Fig. 1. Supercritical fluid chromatogram of anhydrous milk fat (CRM 164 reference material). Peak: 1 = cholestane, 2 = cholesterol.

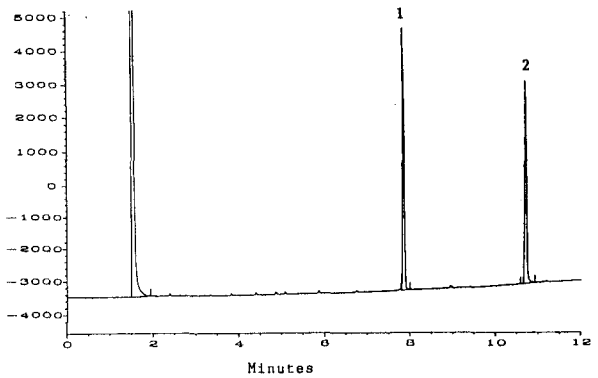

Fig. 2. Gas chromatogram of anhydrous milk fat (CRM 164 reference material). Peaks: 1 = cholestane, 2 = cholesterol.

min. Fig. 2 shows a typical chromatogram of anhydrous milk fat (CRM 164) using GC (peaks: 1 = cholestane, 2 = cholesterol).

3. Results and discussion

The relative response factor (F) was determined by plotting the mass ratios of cholesterol (CH) over the range 0.2–2.0 mg cholesterol/ml hexane and the internal standard (I.S.) at a level of 1 mg cholestane/ml of hexane versus the ratios of their peak areas (Fig. 3).

Using regression analysis the relative response factor (F) and the coefficient of variation (C.V.) were calculated for SFC and GC. In Table 1 the results of the experiments are shown. The cholesterol content (C_{MF}) in any milk fat sample (MF) can be determined by using the following equation:

$$C_{MF}(mg/g) = F \cdot \left(\frac{mg_{I.S.}}{g_{MF}}\right) \cdot \left(\frac{area_{CH}}{area_{I.S.}}\right)$$

where F is the relative response factor, $mg_{I.S.}$ the added amount of the internal standard, $area_{CH}$ and $area_{I.S.}$ are the peak areas of the chromatograms of cholesterol and of the internal standard, respectively.

In order to determine the accuracy and precision of the developed methods two procedures were carried out. First, ten samples of the reference material (CRM 164) were prepared according to the described preparation method. Then, the cholesterol content was analyzed by SFC and GC. Table 2 shows the mean cholesterol content (C_{CRM164}) and the coefficient of variation (C.V.) of these experiments.

For the reference material a cholesterol content of 2.73 mg/g (± 14.3%) was found. SFC seems to be more exact in the determination of

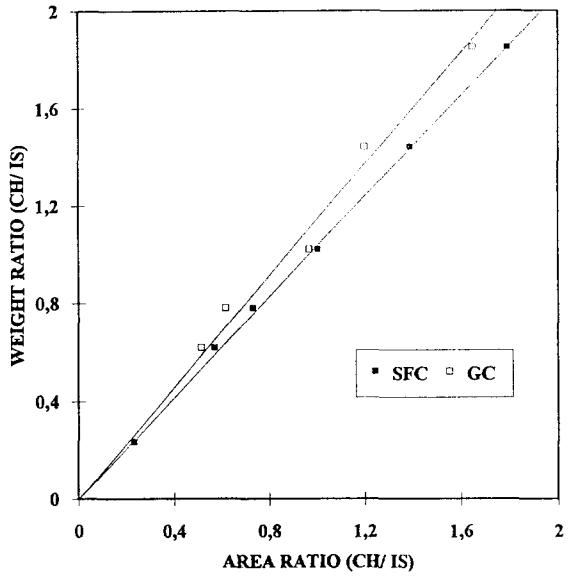

Fig. 3. Mass ratios of cholesterol (CH) and cholestane (I.S.) versus their corresponding area ratios for the determination of the relative response factor (F).

Table 1
Experimental results for the determination of the relative response factors

Method	F	C.V (%)
SFC	1.04	0.04
GC	1.15	6.2

Table 2
Mean cholesterol content and coefficient of variation of the reference material using SFC and GC

Method	C_{CRM164} (mg/g)	C.V. (%)
SFC	2.70	0.002
GC	3.02	0.001

cholesterol in milk fat than GC using our sample preparation method.

Ulberth and Reich [2], analyzing nine samples of the same reference material (CRM 164) by GC, have reported an overall mean value of 2.66 mg/g (C.V. 1.2%), which did not differ significantly from the indicated value; however, the sample preparation was different from our sample preparation. In the same paper, it was demonstrated that an accurate method for the analysis of cholesterol in anhydrous milk fat is also suitable for the analysis of cholesterol in foodstuffs such as sausages, mayonnaise, noodles or cheese.

Secondly, cholesterol was added to a cholesterol-free sunflower oil at a level of 3.10 mg/g. Three samples of spiked sunflower oil were prepared and analyzed by SFC and GC. The mean experimental values of the cholesterol content (C_{SO}), the coefficient of variation and the recovery are shown in Table 3.

According to our recovery studies, there is no difference between the use of SFC and GC in analyzing spiked sunflower oil at a level of 3.1 mg/g.

Finally, information about the long-term reliability of the developed SFC method is given. We have analyzed the cholesterol content in ca. 300 milk fat samples over a period of six months. We have compared the results of our experiments analyzing five samples of the reference material at the beginning and the end of a 6-months period, and we did not find any significant changes in the cholesterol content ($P > 0.05$, Analysis of Variance [8]). We found it very useful to repeat the first analysis after every non-run period to get best results.

Table 3
Mean cholesterol content of spiked sunflower oil analyzed by SFC and GC

Method	C_{SO} (mg/g)	V.C. (%)	Recovery (%)
SFC	3.08	0.29	99.4
GC	3.13	0.71	100

4. Conclusions

A supercritical fluid chromatographic method for the determination of cholesterol in milk fat has been developed with carbon dioxide as carrier gas. The method uses sample preparation, saponification and extraction, is relatively rapid (25 min), very accurate and precise (C.V. < 1%). In our case, SFC seems to be more accurate than GC for the analysis of cholesterol in milk fat, but GC is faster and cheaper.

Acknowledgement

The authors would like to thank Prof. M. García Vargas (University of Cádiz) for his critical comments during the preparation of this paper.

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