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# DETERMINATION OF STEROLS AND THEIR ESTERS IN FATS BY WAY OF TRANSESTERIFICATION IN DIFFERENT SOLVENTS

**Key Words**: fat, gas chromatography, sterols, steryl esters, transesterification, unsaponificable.

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## ABSTRACT

The interference of triglycerides in the analysis of sterols and their esters in fats can be eliminated by transesterification of the sample dissolved in n-hexane with a solution of sodium hydroxide 2 mol/L. Since the analytes are distributed equally in n-hexane and in methanol, better results can be obtained by accurate measurements of the volumes, and processing the standards as the samples. Triglycerides and steryl esters are transesterified quantitatively by a solution of sodium hydroxide 2 mol/L using diethyl ether as solvent. At 40°C, ten minutes are sufficient to complete the reaction. The sterols are determined in fats using a



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mass spectrometer detector, at concentrations as low as 5 mg/Kg. Using FID, it was possible to analyze sterols present in concentrations higher than 100 mg/Kg.

## INTRODUCTION

The determination of sterols is of great importance in the evaluation of fatty substances. Cholesterol analysis can detect particular qualities of butter which do not contain cholesterol. The determination of sterols is used to detect food adulteration, such as the addition of vegetable fats to butter, the addition of sunflower seed oil to olive oil, etc.[1]. Sterols are present in a variety of foods both as free alcohols and fatty acid esters[2]; in particular, in eggs and in grain, steryl esters represent 36% and 25% of the total sterols, respectively[3,4]. The results of the analyses of the sterols are generally expressed as total free sterols, so it is necessary to carry out ester saponification before the analysis.

The most commonly used method requires a long period of reaction, which combined with the subsequent sample treatment, makes the procedure time consuming and laborious. In fact this method[5] requires saponification of the fat, the extraction of the unsaponified material, its fractionization by means of thin-layer chromatography (TLC), and finally, gas chromatographic analysis. Another method analyzes sterols by means of a HPLC-G.C. procedure[6]; unfortunately, this method requires the use of a sophisticated and expensive apparatus.

It is not possible to directly analyze the sterols present in fats, in as much as both in G. C. and in HPLC, many of the sterols, both free and esterified, do not separate quantitatively from the triglycerides (FIG. 1A). The development of methods to measure particular sterols[7,8], which are added as tracers to the concentrated butter product of the European Economic Community (EEC), led to the set up of an efficient procedure for the transesterification of triglycerides proposed by Christopherson and Glass[9]. The transesterification reaction converts the triglycerides into fatty acid methyl esters; and so, after the reaction, the

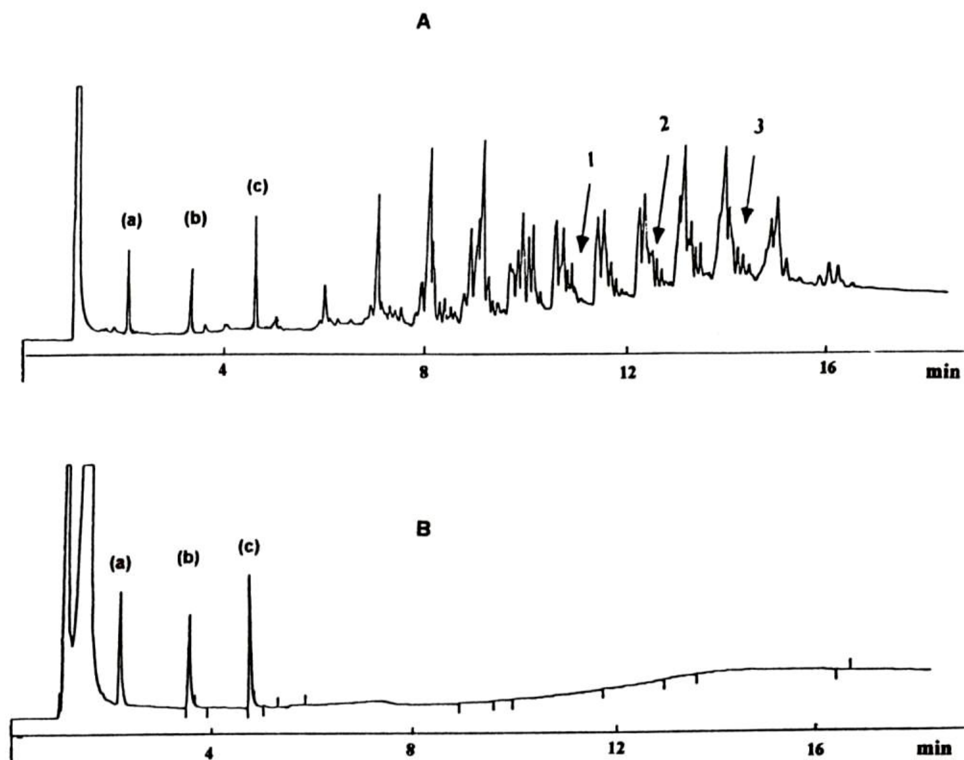


**FIG. 1 Gas chromatogram of butter-oil with the addition of Δ-5 avenasterol (a), cholesterol (b), β-sytosterol (c), cholesteryl laurate (1), Δ-5 avenasteryl palmitate (2) and β- sytosteryl oleate (3) before (A) and after transesterification (B) with sodium hydroxide 2 N in methanol.**

triglycerides no longer constitute an interference with the analysis of the sterols. In addition, since the formation of the methyl esters, under the best gas chromatographic conditions for the eluation of the sterols, finish with practically a "dead" volume, the time of the analysis is greatly reduced (FIG. 1B). The idea was in the possibility to obtain sterols in a free form, starting with the steryl esters, using the same procedure of transesterification used for the triglycerides.

Experimental tests carried out on cholesteryl esters with fatty acids of different lengths have demonstrated that the method of Christopherson and Glass[9], which uses n-hexane as a solvent, partially hydrolyzes the short chain

esters, while it does not hydrolyze at all the fatty acid esters with more than six atoms of carbon. We attribute this behavior to the different structural conformations of steryl esters in respect to triglycerides. Furthermore, since the sodium hydroxide (2 mol/L in methanol solution) is not soluble in n-hexane, it does not change the equilibrium of the reaction towards the product, by the mass action law. With this procedure, therefore, the free and esterified sterols mixed with triglycerides can be determined. Transesterification reactions of the sterols esterified with both short- and long-chain fatty acids, carried out with more polar solvents than n-hexane, such as chloroform or diethyl ether, showed quantitative results. In this way, both the free sterols and the esterified sterols can be analyzed as free sterols.

**Aim of the paper**

The transesterification procedure by Christopherson and Glass[9] uses immiscible solvents, such as n-hexane-methanol, with gas chromatographic analysis of the sole hexane phase. To obtain accurate analytical results, it is necessary to establish how the free sterols and their esters are distributed between the two solvents. We propose to determine the partition coefficient of some of the most interesting sterols and their esters between n-hexane and methanol. Being methanol miscible with diethyl ether, the procedure of transesterification of the steryl esters in diethyl ether leads to a final addition of water to obtain a phase separation. Even in this case, to obtain accurate analytical results, it is necessary to establish how the sterols are distributed between these two phases. We propose, therefore, to determine the partition coefficient of the sterols between diethyl ether and water.

## EXPERIMENTAL

**Chemicals**

All chemicals used were analytical grade: n-hexane (Lab-Scan, Dublin, Ireland); methanol (Lab-Scan, Dublin, Ireland); diethyl ether (Lab-Scan, Dublin,

Ireland); sodium hydroxide (Fluka Chemie, Buchs, Switzerland); hydrochloric acid (Fluka Chemie, Buchs, Switzerland); cholesterol (Larodan, Malmo, Sweden); β-sitosterol (Larodan, Malmo, Sweden); Δ-5 avenasterol (Larodan, Malmo, Sweden); cholesteryl laurate (Larodan, Malmo, Sweden); Δ-5 avenasteryl palmitate (Larodan, Malmo, Sweden); β-sitosteryl oleate (Larodan, Malmo, Sweden); and triolein (Larodan, Malmo, Sweden).

**Instruments**

Autosystem XL GC gas chromatograph, equipped with autosampler, PSS injector and FID detector (Perkin Elmer, Norwalk, CT, U.S.A.); HP electronic integrator, model 3394, (Hewlett-Packard, Palo Alto, CA, U.S.A.); RTX 65-TG, 65% phenyl methyl polysiloxane capillary column, l=30 meters, i.d.=0.25 mm; f.t.=0.1 μ (Restek Corporation, Bellefonte, PA, U.S.A.)

**Gas chromatographic conditions**

Carrier gas: helium; flow rate: 1.5 mL/min; split: 1:80; oven 250°C for 2 min., increasing 5°C/min to a final temperature of 360°C for 10 min; PSS injector: 60°C for 10 sec, increasing 900°C/min to a final temperature of 370°C, for 5 min; FID temperature: 370°C; injection mode: total; split valve open at 1.5 min.

**GC/MS instruments and gas chromatographic conditions**

Finnigan GCQ$^{TM}$ ion trap mass spectrometer (ThermoQuest Corporation, San Jose, CA, U.S.A.); DB-5MS capillary column, l=30m, i.d.=0.25 mm, f.t.=0.25 μ, (J & W Scientific, Folsom, CA, U.S.A.)

Carrier gas: Helium; linear speed: 40 cm/s; injector: split-splitless held at 270°C; oven program: 200°C for 1 min, increasing at 5°C/min to 260°C for 25 min. Injection technique was splitless. Mass spectral data were obtained under the following conditions: ionization electron energy: 70 eV; scan time: 2 sec.;

source temperature: 200°C; transfer line temperature: 275°C; filament emission current: 170 μA. Spectra were acquired in the full scan mode ranged from 350 to 450 uma.

SIM acquisition mode was performed isolating fragments at m/z 414 and m/z 396 for β-sitosterol; m/z 386 and m/z 368 for cholesterol; m/z 400 and 382 for campesterol; m/z 412 and m/z 394 for stigmasterol.

**Transesterification reagent**

A 2 mol/L solution of sodium hydroxide in methanol was prepared by dissolving 8,0 g sodium hydroxide in 100 mL anhydrous methanol (Solution T).

## PROCEDURE

**Determination of the partition coefficient of the free and esterified sterols between n-hexane and methanol, in absence of matrix**

A solution of cholesterol, β-sitosterol, $\Delta^5$ avenasterol and their esters reported in the experimental was prepared in n-hexane, saturated with methanol, approximately 500 mg/L for each compound (Solution 1); Solution 1 was analyzed five times, by gas chromatography; for each compound the average of the areas deduced from the five chromatograms was calculated. 2.00 mL of Solution 1 and 2.00 mL of methanol saturated with n-hexane were placed in a centrifuge tube; the n-hexane phase was agitated in a stirrer for two minutes and then it was centrifuged and analyzed five times, by means of gas chromatography (Solution 2).

For each analysis the average of the areas deduced by the five chromatograms were calculated.

Calculation

$$K = \frac{A2}{A1 - A2}$$

where :

A1 is the average of the areas of the analysis, deduced from the areas of the five chromatograms of Solution 1.

A2 is the average of the areas of the analysis, deduced from the areas of the five chromatograms of Solution 2.

**Determination of the partition coefficient of the free and esterified sterols between n-hexane and methanol, in the presence of matrix**

A solution of triolein in n-hexane, saturated with methanol, 50mg/ml was prepared; from this a solution of cholesterol, β-sitosterol, $\Delta^5$ avenasterol and their esters (reported in the chemicals section) was prepared, approximately 500 mg/L (Solution 1). The procedure was as above, substituting the 2.00 mL of methanol with 2.00 mL of Solution T, saturated with n-hexane.

**Determination of the partition coefficients of the free sterols between the two phases that form by mixing diethyl ether, Solution T and a solution of hydrochloric acid, in the presence of matrix**

A solution of triolein in diethyl ether 50 mg/mL was prepared; from this was prepared a solution of cholesterol, β-sitosterol, $\Delta^5$ avenasterol approximately 500 mg/L (Solution 1). Solution 1 was analyzed five times, by means of gas chromatography. For each analysis, the average of the areas was calculated, deduced by the five chromatograms. 1.00 mL of Solution 1; 1.00 mL of Solution T and 5.00 mL of HCl 0.4 M were placed in a graduated centrifuge tube; the solution was agitated in a stirrer for two minutes and then centrifuged. The volumes of the two phases were measured; Vs and Vi were the upper and lower volumes, respectively. The upper ether phase (Solution 2) was analyzed five times, by means of gas chromatography .

For each analysis the average of the areas was calculated from the five chromatograms.

Calculation

$$K = \frac{A2 \times Vi}{5A1 - A2 \times Vs}$$

where :

A1 is the average of the areas, deduced from the five chromatograms of Solution 1.

A2 is the average of the areas, deduced from the five chromatograms of Solution 2.

Vs is the volume of the ether phase after extraction.

Vi is the volume of the aqueous phase after extraction.

**Analysis of the sterols and steryl esters after transesterification with n-hexane**

In a centrifuge tube about 250 mg of fatty material, accurately weighed, were dissolved in 5.00 mL of n-hexane; 1.00 mL Solution T was added and stirred for two minutes, the tube was centrifuged at 3,000 rpm for two minutes, then 1 μL of the hexane phase was injected into the gas chromatograph.

**Calibration Curve**

A series of standard solutions of sterols and steryl esters in n-hexane were prepared in the range of concentration of 10 mg/L to 600 mg/L; 5.00 mL of each solution were transesterificated, as explained above. The peak areas were plotted against the concentration of the sterols and steryl esters.

**Analysis of the total sterols, as free sterols, after transesterification in diethyl ether**

To a quantity of fatty material, accurately weighed to about 250 mg, in 5.00 mL of diethyl ether was added 1.00 mL of Solution T. The solution was stirred for 1 min. and allowed to sit for 10 min. at 40°C. The solution was neutralized

with 1.00 mL of HCl (2 mol/L), stirred again and then centrifuged for 1 min. at 4000 rpm. Finally, 1 μL of the upper ether phase was injected into the gas chromatograph.

**Calibration Curve**

A series of standard solutions of sterols and their esters were prepared in the range of concentration of 10 and 600 mg/L in diethyl ether; 5.00 mL of each solution were transesterified, as described above.

The peak areas were plotted against the concentration of the sterols and the steryl esters.

## RESULTS AND DISCUSSION

**Transesterification in n-hexane**

The reaction of transesterification in n-hexane is quantitative for triglycerides. Under the same experimental conditions, analytical results obtained with standard solutions of free and esterified sterols with short, medium and long-chain fatty acids demonstrate that only esterified sterols with short-chain fatty acids of up to six atoms of carbon are partially hydrolyzed, while longer chain esters don't hydrolyze at all. The reaction does not alter the concentration of the analytes. The yield of the reaction also does not vary over time. In foods, the concentration of sterols esterified with short-chain fatty acids is negligible; so, this procedure, with some limitations, allows the determination of both free sterols and esterified ones. In FIG. 1A and FIG. 1B the gas chromotograms of genuine butter obtained, with the addition of Δ5-avenasterol, cholesterol, β-sitosterol, cholesteryl laurate, Δ5-avenasteryl palmitate and β-sitosteryl oleate, before and after transesterification in n-hexane, are reported. As can be observed in the gas chromatogram, before transesterification the steryl esters are eluted along with the triglycerides. After the reaction, they are broken down into perfectly isolated fatty acid methyl esters and their respective sterols.

**Transesterification in diethyl ether**

The steryl esters, both short and long chain, are quantitatively hydrolyzed with 2 mol/L sodium hydroxide in diethyl ether. The kinetics of the steryl ester hydrolosis reaction seems to be independent of the length of the fatty acid. At room temperature, about 40 minutes are necessary for a complete reaction; at 40°C, the reaction is quantitative after 10 minutes.

**Evaluation of partition coefficients for the transesterification in n-hexane**

The procedure of transesterification reported by Christopherson and Glass[9] uses n-hexane in methanol, solvents which are immiscible. The values of the partition coefficients, reported in Table 1, indicate that both the sterols and their esters are equally soluble in these two solvents. The matrix does not seem to influence the value of the partition coefficient. In order to avoid a substantial part of the compounds being transferred into the methanol phase, making it particularly difficult to reveal the peaks relative to the less abundant components, it is advisable to utilize a relatively large volume of n-hexane in comparison to methanol. A ratio of 5:1 appears to be sufficient. In any case, even in these experimental conditions, about 17% of the concentration of analytes are transferred into the methanol. Considering that the compounds of interest are equally soluble in both solvents, to obtain satisfying results it is necessary to accurately measure the volumes of the solvents used and treat the samples in the same way. In Table 2 are reported the values of the determinations of a sample of cholesterol-free butteroil, with the addition of Δ-5 avenasterol, cholesterol, β-sitosterol and their esters, as reported above. As can be observed, the error does not exceed 3% of the average value.

**Evaluation of the partition coefficients for the reactions in diethyl ether**

In the aim of evaluating the partition coefficient of the free sterols between diethyl ether and water, we prepared a solution of Δ-5 avenasterol, cholesterol and β-sitosterol in diethyl ether, stirred it with a five times larger volume of

**Table 1. Partition coefficients of free sterols and esterified sterols between n-hexane and methanol.**

| COMPOUNDS | PARTITION COEFFICENT |
|---|---|
| Cholesterol | 1.0 |
| β-sytosterol | 1.1 |
| Δ-5 avenasterol | 1.0 |
| Cholesteryl laurate | 0.9 |
| β-sytosteryl oleate | 1.0 |
| Δ-5 avenasteryl palmyate | 0.9 |

water, and analyzed it via G.C.. We did not observe any difference in concentration in the ether phase after being treated with water. Therefore, under our experimental conditions, it seems that the sterols are insoluble in water. In the analysis of the total sterols, to avoid errors caused by the contraction of the volume of diethyl ether, it is advisable that the sample and the standard solutions undergo the same treatments. In Table 3 are reported the values of the determinations of a sample of cholesterol-free butteroil with the addition of Δ-5 avenasterol, cholesterol, β-sitosterol and their esters as reported above. As can be observed, the error does not exceed 3% of the average value.

**Table 2. Percentage of standard deviation between experimental values and the true value of a standard mixture of sterols and their esters added to a cholesterol free butteroil. Analysis was performed in n-hexane and repeated ten times.**

| COMPOUNDS | TRUE VALUE (mg/Kg) | EXPERIMENTAL VALUE (mg/Kg) | PERCENTAGE DEVIATION (%) |
|---|---|---|---|
| Cholesterol | 4873 | 4756 | 2.4 |
| β-sytosterol | 3151 | 3205 | 1.7 |
| Δ-5 avenasterol | 1213 | 1249 | 3.0 |
| Cholesteryl laurate | 224 | 230 | 2.8 |
| β-sytosteryl oleate | 216 | 210 | 2.8 |
| Δ-5 avenasteryl palmytate | 208 | 214 | 2.9 |

**Sensitivity of the detection**

The concentration of sterols in fats is in the range of 5 and 6000 mg/Kg. Since in the procedures it is advised to dissolve 50 mg of the sample in 1 mL of n-hexane or of diethyl ether, the concentration of the sterols is between 0.25 and 250 mg/L. The sensitivity of the flame ionization detector (FID) does not easily allow the analysis of the analytes in concentrations lower than 5 mg/L, which corresponds to a concentration of 100 mg/Kg in fats. Using the mass spectrometer in the SIM acquisition mode, it is possible to analyze the sterols and their fatty acid esters in concentrations even lower than 0.25 mg/L, which

**Table 3. Percentage of standard deviation between experimental values and the true value of a standard mixture of sterols and their esters added to a cholesterol free butteroil. Analysis was performed in diethyl ether and repeated ten times.**

| COMPOUNDS | TRUE VALUE (mg/Kg) | EXPERIMENTAL VALUE (mg/Kg) | PERCENTAGE DEVIATION (%) |
|---|---|---|---|
| Cholesterol | 4738 | 4831 | 2.0 |
| β-sytosterol | 3005 | 3096 | 3.0 |
| Δ-5 avenasterol | 1483 | 1445 | 2.6 |
| Cholesteryl laurate | 213 | 207 | 2.8 |
| β-sytosteryl oleate | 237 | 231 | 2.5 |
| Δ-5 avenasteryl palmyate | 248 | 242 | 2.4 |

correspond in fats to a concentration of 5 mg/Kg. Using the m/z 414 and 396 ion extracted for β-sitosterol, m/z 386 and 368 for cholesterol, m/z 400 and 382 for campesterol and m/z 412 and 394 for stigmasterol fragments, the result of the mass spectrometer is linear at least between 0.25 and 300 mg/L for each sterol analyzed.

## CONCLUSIONS

The transesterification reaction with potassium methanol in n-hexane is quantitative only for triglycerides; the sterol esters do not react at all. And so in

n-hexane it is possible to determine both the free sterols and their esters. Since the free sterols and their esters distribute between n-hexane and methanol with a partition coefficient (Kd) close to 1, satisfying results are obtained by accurately measuring the volumes of the reagents and using the same procedure for the standard solutions and the sample. In the ether phase, both the triglycerides and the sterol esters are transesterified quantitatively. Temperature has an important role in the kinetics of the reaction; in fact, at 40°C, 10 minutes are sufficient for the reaction to reach the equilibrium. Therefore it is possible to analyze the sterols and their esters as free sterols. The sterols present in fats with a concentration higher than 100 mg/Kg can be detected with FID; sterols with a low concentration such as 5 mg/Kg must be analyzed by means of a mass detector.

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