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Determination of Diglycerides in Virgin Olive Oil, Salami and Cheese by Solid Phase Extraction

Određivanje diglicerida u prirodnom maslinovom ulju, te salami i siru primjenom ekstrakcije na čvrstoj fazi

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Summary

The capillary column coated with a polar stationary phase stable at high temperature was applied to separate diglycerides on the basis of their molecular weight, the unsaturation degree of fatty acids and their position on the glycerol molecule. The solid phase extraction (SPE) with cartridges of amino phase were used to achieve the separation of lipids into polar and non polar fraction, before the GLC analyses. This method was applied to virgin olive oil, salami and cheese samples from which the repeatability was assessed; the recovery was determined in olive oil and salami fat samples.

Sažetak

Ekstrakcijom na čvrstoj fazi uz kolone s aminopropil-silikatnim punjenjem razdvojene su polarna i nepolarna frakcija lipida. Digliceridi iz polarne frakcije razdvojeni su na temelju njihove molekularne mase, stupnja nezasićenosti masnih kiselina te njihova položaja na molekuli glicerola, primjenom plinske kromatografije uz kapilarnu kolonu s polarnom stacionarnom fazom, stabilnom pri visokim temperaturama. Ovaj je postupak primijenjen na prirodnom maslinovom ulju, uzorcima salame i sira za koje je određena ponovljivost; podaci o točnosti metode u postupku određeni su za pojedine digliceride u prirodnom maslinovom ulju i masti iz salame.

Introduction

The determination of diglycerides (DG) and 1,2-DG/ /1,3-DG ratio in foods that contain a high amount of fat is considered to be useful to locate the age of virgin olive oils, *(1–3)*, the ripening stage of fermented foods (salami, cheese) *(4)*, or to make evident possible frauds (partial neutralization of virgin olive oils) *(2)*.

Fresh virgin olive oils usually have small amounts of 1,3-DG and the 1,2/1,3-DG ratio is over 1 *(1)*. A rearrangement takes place while oil ages and the 1,2-DG come to the 1,3 form, which is thermodinamically more stable *(5)*.

On the other hand, 1,3-DG originates mainly from hydrolytic processes which decrease the 1,2/1,3 ratio while at the same time, free organic acidity rises. A partial neutralization of an oil removes free fatty acids, but does not change the 1,2/1,3 ratio that remains at low values. Therefore the 1,2/1,3 ratio can be useful to detect this kind of fraud *(2)*.

The above discussed ratio also changes in fermented foods as ripening goes on: lipolysis takes place as salami and cheese ripe and the monitoring of the 1,2/1,3-DG ratio and the estimation of the total amount of each DG can give useful information about the age of the product, as well as about the kind of microorganisms which are active in the considered step *(4)*.

The usual method for quantitative determination of partial glycerides present in lipid materials is capillary gas chromatography with a previous separation of neutral and polar lipid classes. This lipid separation can be accomplished by preparative TLC, silicic acid or alumina column chromatography, or HPLC isolation. However, there are problems inherent in these procedures such as

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incomplete recoveries of polar lipid classes from TLC *(6)*. The column chromatography requires considerable amount of solvent and gradient procedure *(7)*, while the HPLC equipment is expensive.

In this work a capillary column coated with a polar stationary phase which is stable at high temperatures with the possibility of separating diglycerides on the basis of their molecular weight, the unsaturation degree of fatty acids, and their position on the glycerol molecule, was used. The solid phase extraction with cartridges of amino phase was used to achieve the separation of lipid into polar and non polar fraction.

The advantages of this method are: short time of analyses, small amounts of samples (50 mg), solvent saving (50 mL for sample), and the avoidance of any overloading of GLC column caused by the high amount of triglycerides.

This method was applied to olive oil, salami and cheese samples for which the repeatability was assessed; the recovery was determined in olive oil and salami fat samples.

Materials and Methods

Fat extraction from cheese and salami

Fat was extracted from cheese and salami at low temperature following the procedure described by Caboni *et al. (8)*.

Preparation of pure mixture of diglycerides from food

Extra virgin olive oil and pork fat were subjected to pancreatic lipase action following the reaction conditions reported in the EEC official method *(9)*. After extraction from the reaction mixture with diethyl ether, the DG were purified by preparative TLC using n-hexane/diethyl ether/acetic acid (80:20:2 volume ratio) as elution mixture. 2,7-dichlorofluorescein (sodium salt, 0.2 % in ethanol) was used as visualisation reagent under UV lamp at 254 nm. The bands corresponding to 1,2-DG and 1,3-DG were scraped off and extracted with 50 mL of methanol/acetic acid (98:2 volume ratio) for 30 minutes in a blender. This mixture of DG was used for peak identifications.

Preparation of pure matrix of triglycerides from olive oil and pork fat

The samples of olive oil and pork fat in n-hexane were both prepared in mass concentration of 0.1 g/mL and filtered throught aluminium oxide (neutral) column to purify samples from free fatty acids (FFA), monoglycerides (MG) and diglycerides (DG). The efficiency of purification was controlled using TLC (elution conditions were described in the part of text for preparation of pure mixture of DG). In so obtained pure matrix, we added the standards of DG (1 %, mass ratio) whose recoveries were determined.

Solid phase extraction of diglycerides

The method of Hopia *et al.(10)* was used with minor modifications to clean up DG.

Isolute TM, NH_2 500 mg/3mL cartridges were used through the following steps:

1) cartridge conditioning with 5 mL of n-hexane;

2) loading of sample: 50 mg of fat or oil in 500 µL of n-hexane/diethyl ether (99:1volume ratio);

3) elution with 30 mL of n-hexan/diethyl ether (99:1 volume ratio);

4) elution with 10 mL of methanol/acetic acid (98:2 volume ratio).

The internal standard (cholesterol 0.08 %, of mass ratio) was added to olive oil before the loading of sample. The cholesterol naturally present in cheese and salami was used as internal standard (I.S.). Its amount had

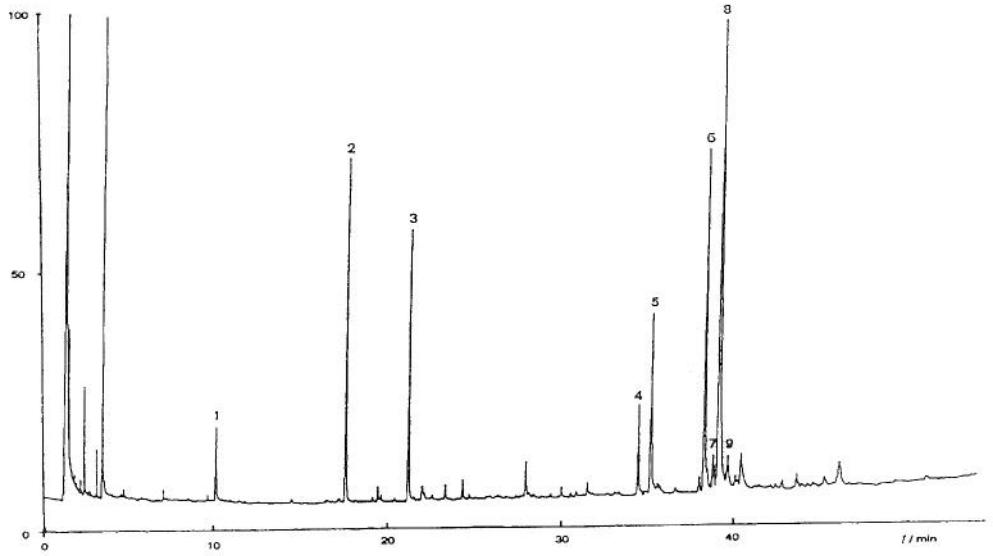

Fig. 1. Gas – cromatographic trace of olive oil patrial glycerides
Slika 1. Kromatogram parcijalnih glicerida maslinovog ulja dobiven plinskom kromarografijom
1 = 1-monooleoyl-glycerol; 2 = cholesterol (IS); 3 = β-sitosterol; 4 = 1-palmitoyl-2-oleoyl-glycerol; 5 = 1-palmitoyl-3-oleoyl-glycerol; 6 = 1,2-dioleoyl-glycerol; 7 = 1-oleoyl-2-linoleyl-glycerol; 8 = 1,3-dioleoyl-glycerol; 9 = 1-oleoyl-3-linoleyl-glycerol.

Table 1. Repeatability of the analytical procedure (areas ratio of peaks)
Tablica 1. Ponovljivost analitičkog postupka (omjer površina pikova)

	Virgin olive oil			Salami fat			Cheese fat		
	Average	S. D.	C.V./ %	Average	S. D.	C.V./%	Average	S. D.	C.V./%
Cholesterol/DG	0.036	0.003	7.7	0.235	0.044	17.4	0.089	0.002	1.8
1,2/1,3-diglycerides	0.48	0.027	5.5	0.66	0.037	5.6	n.d.(*)	n.d.(*)	n.d.(*)

(*) n.d. = not determined

been estimated by GLC using β-sitosterol as I.S. These conditions allowed the elution of MG and DG together with negligible amount of triglycerides.

Capillary gas liquid chromatography

Solvents were removed from the solution of partial glycerides eluted from the SPE cartridge, by nitrogen stream; then silylation was carried out with the *Sweeley* reagent *(11)*, 100 μL/mg of diglycerides. After 20 minutes at room temperature, excess of reactants was evaporated under nitrogen stream; the analytes were dissolved in n-hexane to obtain the mass concentration of 40 μg/mL of DG.

GLC was carried out using a Carlo Erba 5160 Mega capillary gas chromatograph, equipped with a fused silica TAP capillary column (Chrompack), 30 m · 0.25 mm i.d., 0.10 μm film thickness; carrier gas (He) flow rate was 1.8 mL/min, injection was realized in a split mode, with 1/80 split ratio, injector and flame ionization detector (FID) temperature were kept at 350 °C, while column temperature was programmed from 200 to 290 °C at 3 °C/ /min, then from 290 °C to 350 °C at 2 °C/min. The gas-chromatographic traces of partial acylglycerols are presented in Figures 1–3.

Results and Discussion

In this work, a TAP column was used, as it tourned out to be suitable for DG separation both on the basis of their unsaturation degree, and of the 1,2/1,3 distribution, as reported in the literature *(2)*. Peak identification was carried out by comparing their retention times with those of the mixture of 1,2 and 1,3-DG, purified by TLC separation of pancreatic lipase reaction products, and with those of the available standard.

SPE cartridges packed with aminopropyl phase were preferred to silica phase, as they were apparently not influenced by traces of water present in the sample. Water traces represent a problem when fats extracted from salami and cheese are to be analyzed. Previous experiences *(4)* and literature data *(10)* demonstrate that the use of silica column leads to less reproducible data.

Amino groups present on the solid phase formed bonds with polar and ionic molecules, in our case DG, MG, sterols and FFA, while less polar and non polar molecules (triglycerides) easly removed by moderately polar solvents mixtures (n-hexane/diethyl ether 99:1 volume ratio). A more polar mixture was employed for elution of partial glycerides.

Method repeatability was tested by evaluation of the ratio between the area of cholesterol peak and the total area of DG peaks; 1,2-DG peak area / 1,3-DG peak area ratio had been already evaluated. Repeatability trials have been carried out on oil, cheese fat and salami fat samples and each trial was repeated ten times. Cholesterol was added as internal standard to olive oils at a 0.08 % mass ratio.

Table 2. Recoveries of diglycerides standards from olive oil and salami fat
Tablica 2. Točnost metode u postupku za standarde diglicerida u maslinovom ulju i masti salame

Olive oil diglycerides		Average of 5 replicates	S. D.	C.V./%
1,2 PP	Added/(μg/μL)	19.4		
	Found/(μg/μL)	21.9		
	Recovery/%	112.9	1.0	0.9
1,3 PP	Added/(μg/μL)	18.6		
	Found/(μg/μL)	17.1		
	Recovery/%	92.0	0.8	0.9
1,3 PS	Added/(μg/μL)	18.6		
	Found/(μg/μL)	10.4		
	Recovery/%	56.0	0.9	1.7
1,2 SS	Added/(μg/μL)	10.4		
	Found/(μg/μL)	7.9		
	Recovery/%	76.3	2.9	3.9
1,3 SS	Added/(μg/μL)	18.6		
	Found/(μg/μL)	15.5		
	Recovery/%	83.2	2.2	2.7
Salami fat				
1,2 PP	Added/(μg/μL)	28.8		
	Found/(μg/μL)	32.5		
	Recovery/%	112.8	4.9	4.2
1,3 PP	Added/(μg/μL)	28.9		
	Found/(μg/μL)	25.4		
	Recovery/%	87.8	4.1	4.7
1,3 PS	Added/(μg/μL)	30.9		
	Found/(μg/μL)	19.2		
	Recovery/%	63.0	2.8	4.5
1,2 SS	Added/(μg/μL)	29.2		
	Found/(μg/μL)	20.7		
	Recovery/%	70.8	3.2	5.3
1,3 SS	Added/(μg/μL)	29.7		
	Found/(μg/μL)	33.8		
	Recovery/%	113.9	6.4	5.5

PP : dipalmitin
PS : palmitoyl-stearoyl-glycerol
SS : distearin

Data concerning repeatability of the method, reported in Table 1, show that salami samples are affected by more error sources than oil and cheese. Coefficient of variation of cholesterol/DG ratio is in fact 17.4 % for salami samples, while it is lower for oil (7.7 %) and cheese (1.8 %).

The repeatability concerning cheese fat was calculated considering peaks with a retention time higher than cholesterol, even though it is possible that peaks

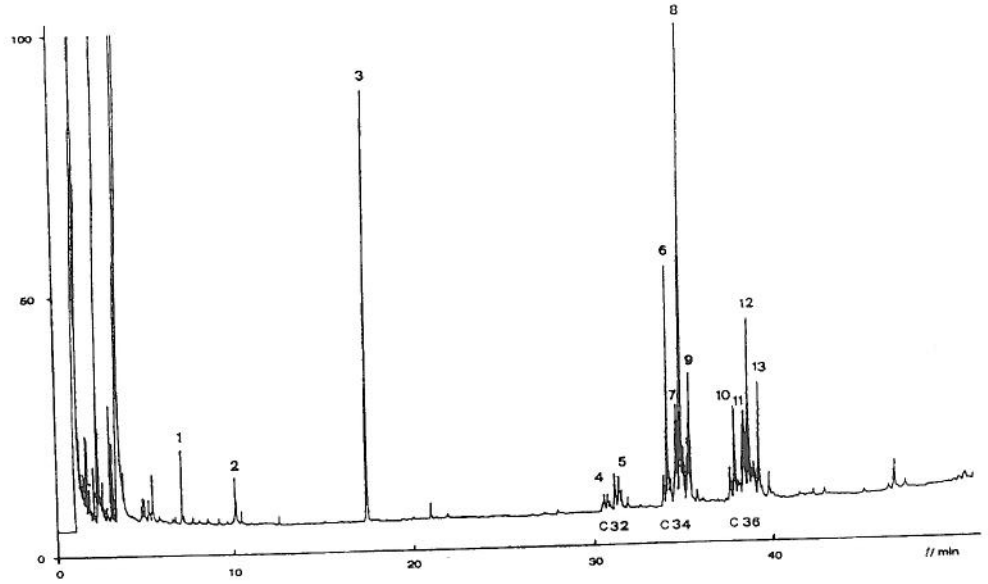

Fig. 2. Gas – cromatographic trace of salami fat patrial glycerides
Slika 3. Kromatogram parcijalnih glicerida masti salame dobiven plinskom kromatografijom

1 = 1-monopalmitoyl-glycerol; 2 = 1-monooleoyl-glycerol + 1-monostearoyl-glicerol; 3 = cholesterol;
4 = 1,2-dipalmitoyl-glycerol; 5 = 1,3-dipalmitoyl-glycerol; 6 = 1-palmitoyl-2-stearoyl-glycerol; 7 = 1-palmytoyl-2-oleoyl-glycerol;
8 = 1-palmitoyl-3-stearoyl-glycerol; 9 = 1-palmitoyl-3-oleoyl-glycerol; 10 = 1,2-distearoyl-glycerol; 11 = 1,2-dioleoyl-glycerol;
12 = 1,3-distearoyl-glycerol; 13 = 1,3-dioleoyl-glycerol.

C32 = diglycerydes with 32 carbon atoms from fatty acids; C32 = digliceridi s 32 atoma ugljika iz masnih kiselina;
C34 = diglycerydes with 34 carbon atoms from fatty acids; C34 = digliceridi s 34 atoma ugljika iz masnih kiselina;
C36 = diglycerydes with 36 carbon atoms from fatty acids; C36 = digliceridi s 36 atoma ugljika iz masnih kiselina;

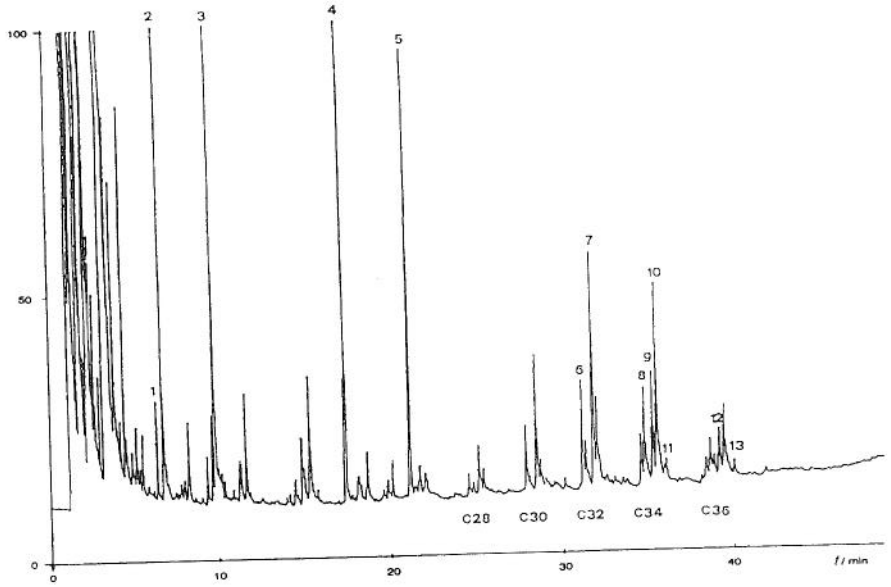

Fig. 3. Gas – cromatographic trace of cheese fat patrial glycerides
Slika 3. Kromatogram parcijalnih glicerida masti sira dobiven plinskom kromatografijom

1 = 2-monopalmitoyl-glycerol; 2 = 1-monopalmitoyl-glycerol; 3 = 1-monooleoyl-glycerol + 1-monostearoyl-glicerol; 4 = cholesterol;
4 = 1,2-dipalmitoyl-glycerol; 5 = β-sitosterol (IS); 6 = 1,2-dipalmitoyl-glycerol; 7 = 1,3-dipalmitoyl-glycerol;
8 = 1-palmitoyl-2-stearoyl-glycerol; 9 = 1-palmitoyl-2-oleoyl-glycerol; 10 = 1-palmitoyl-3-stearoyl-glycerol; 11 = 1-palmitoyl-2-oleoyl-glycerol; 12 = 1,2-dioleoyl-glycerol; 13 = 1,3-dioleoyl-glycerol.

C28 = diglycerydes with 28 carbon atoms from fatty acids; C28 = digliceridi s 28 atoma ugljika iz masnih kiselina;
C30 = diglycerydes with 30 carbon atoms from fatty acids; C30 = digliceridi s 30 atoma ugljika iz masnih kiselina;
C32 = diglycerydes with 32 carbon atoms from fatty acids; C32 = digliceridi s 32 atoma ugljika iz masnih kiselina;
C34 = diglycerydes with 34 carbon atoms from fatty acids; C34 = digliceridi s 34 atoma ugljika iz masnih kiselina;
C36 = diglycerydes with 36 carbon atoms from fatty acids; C36 = digliceridi s 36 atoma ugljika iz masnih kiselina;

with a shorter retention time are constitued by diglycerides of short chain fatty acids (Fig. 3).

Salami fat is not well soluble in n-hexane, which is the solvent used for SPE loading. Nevertheless, no increase in loading solvent polarity can be realized, as polar compounds must be trapped in the SPE cartridge. To obtain an optimum solubility of salami fat in this solvent, the addition of squalane was necessary (10 mg of squalane/50 mg of fat). During SPE, squalane was eluted together with triglycerides.

Variation coefficient of 1,2/1,3-DG ratio is similar for oil and salami; no data are reported for cheese fat concerning this parameter, as the complexity of its DG fraction requires more studies in order to come to a correct identification of the different forms, and this will be the subject of a forthcoming paper. The above discussed difficulties concerning solubility of salami fat may be the most important source of variation.

Recovery data were calculated on matrix from olive oil and pork fat, spiked with a standard mixture. The results are shown in Table 2. The recovery of 1-palmitoyl-3-stearoyl-glycerol is low in both of cases; most of other DG shows a similar behaviour. No recovery trials were carried out on cheese fats because of its complex composition.

The basic character of the adsorbent could catalyze the isomerization of the partial glycerides *(12)*, so further investigations are in progress with the aim of clarifying this aspect and, if necessary, to test other SPE phases in order to identify the most suitable ones.

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