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Prefractionation of aroma extracts from fat-containing food by high-performance size-exclusion chromatography

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Abstract

A method for the prefractionation of aroma extracts of fat-containing food using high-performance size-exclusion chromatography is presented. The aim was to obtain a fraction of volatile compounds with a residual triglyceride content as low as possible, in order to allow its direct analysis by gas chromatography. Two different mobile phases, diethyl ether and dichloromethane, were tested and the elution volumes of triglycerides and a large variety of aroma compounds were measured. The quality of separation between triglycerides and volatile compounds as a function of column load was studied. The method was successfully applied to the analysis of goat cheese volatiles.

Keywords: Food analysis; Aroma extracts; Cheese; Sample preparation; Triglycerides; Alcohols; Hydrocarbons; Organic acids

1. Introduction

Size-exclusion chromatography (SEC) is typically a separation method for high-molecular-mass compounds, i.e., synthetic polymers or biopolymers. The separation is achieved by partial or total exclusion of the solutes from the pores of the stationary phase. However, solute–stationary phase interactions may occur and may affect the retention dramatically, depending on the mobile phase used. For proper SEC, these interactions should be reduced to a minimum.

The molecular mass region within which solutes of different size can be separated depends on the mean pore diameter. Despite the fact that styrene–divinylbenzene copolymers with a small mean pore diameter (i.e., 50 or 100 Å) have been available for many years, high-performance SEC of small molecules is not a very widespread technique. Two main areas of application in organic media can be distinguished. First, the technique has been applied to the separation of individual compounds, e.g., mono- and sesquiterpenes [1] and coffee components [2]. Second, SEC has been used as a sample clean-up method for fat-containing organic extracts. Related publications concern the determination of pesticides [3,4], pollutants [5] and pharmaceuticals [6] in various matrices.

Aroma analysis of food rich in fat is usually done by headspace or vacuum distillation techniques. Headspace methods are limited to major

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compounds of good volatility. Minor or less volatile compounds can hardly be determined. Vacuum distillation is laborious and time consuming and considerable losses of heavy aroma compounds may also occur.

These difficulties led us to consider the possibilities of using SEC to clean up organic aroma extracts rich in fat. The aim was to obtain a fraction of volatile compounds exempt from fat or at least sufficiently skimmed to be directly analysable by gas chromatography (GC). This approach is original and different in two respects from the above-cited applications of SEC for sample clean-up. First, it is not one specific compound family but a great variety of compounds of different chemical classes that must be recovered. Second, the volatility of aroma compounds implies the use of a highly volatile mobile phase in order to allow subsequential fraction concentration.

2. Experimental

2.1. Chemicals

Diethyl ether was of HPLC grade (Fisons, Loughborough, UK). Dichloromethane (SDS, Peypin, France) was freshly distilled. Prior to use, all solvents were filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA).

The standards were obtained from Sigma (St. Quentin Fallavier, France) or from our laboratory collection of volatile compounds.

2.2. Equipment

The styrene–divinylbenzene SEC column [PLgel 50 Å (5 μm), 300 × 7.5 mm I.D.] was obtained from Polymer Laboratories (Church Stretton, Shropshire, UK). The flow-rate was set to 1.0 ml/min. Detection of standard compounds was performed using a Waters R 401 refractometer. All other liquid chromatographic experimental conditions were as previously published [7]. Triglyceride quantification was performed by GC on a Hewlett-Packard Model 5890 Series II instrument equipped with an on-column injector and an electronic pressure control (EPC) module. The column was a DB-1 (15 m × 0.25 mm I.D., 0.1 μm film thickness) (J&W Scientific, Folsom, CA, USA). The carrier gas was hydrogen at 50 cm/s (40°C). In order to obtain a constant mass flow, the column head pressure was programmed from 47 kPa (40°C) to 118 kPa (350°C). Compound identification was carried out on a Hewlett-Packard Model 5890 Series II gas chromatograph coupled to a Nermag R 10-10 quadrupole mass spectrometer.

2.3. Computations

Elution volumes were calculated from the mean of three injections. Correction for flow-rate variations was performed as described previously [7].

2.4. Sample extraction

A 100-g amount of goat cheese was homogenized in 600 ml of water, stirred for 1 h at 40°C and centrifuged at 13 000 *g* at 4°C. Supernatant solid fat was eliminated and aqueous extraction and centrifugation were repeated on the pellet. The liquid phases of both centrifugations were mixed, the pH was adjusted to 2 with dilute HCl (0.1 *M*) and the mixture was extracted with dichloromethane and the extract was concentrated to 1.2 ml (Kuderna–Danish apparatus). The contents of triglycerides and volatile compounds in the concentrated extract were about 200 and 3 μg/ml, respectively.

3. Results and discussion

3.1. Elution of standard compounds

To our knowledge, no study of the chromatographic behaviour of aroma compounds in high-performance SEC has been undertaken previously, except for the work of Komae and Hayashi [1,8] on terpenes. We therefore measured the elution volumes of volatile reference compounds and triglycerides using two different highly volatile mobile phases, diethyl ether and dichlorome-

thane. The values obtained are given in Table 1. Figs. 1 and 2 show elution volumes (V_e) as a function of molecular mass. In Fig. 1 it can be seen that elution volumes are a quasi-linear function of the logarithm of molecular mass within homologous series. This confirms that the column porosity is well adapted to the molecular sizes, even for solutes as small as pentane. On the other hand, large mass discrepancies exist between solutes with comparable elution volumes but belonging to different chemical classes, e.g., butanol, $V_e = 8.64$ ml, and tributyrine, $V_e = 8.61$ ml. Volatile compounds and triglycerides overlap to a large extent and no separation is obtained with diethyl ether. We attribute this phenomenon to solute–stationary phase interactions which, in the case of certain solutes such as triglycerides, largely overcome exclusion.

A change of the mobile phase to a stronger eluting solvent should reduce these undesirable interactions. Robinson et al. [9] has shown that the elution strength of dichloromethane is higher than that of diethyl ether on Amberlite XAD-2, another type of styrene–divinylbenzene copolymer. Table 1 and Fig. 2 suggest that this is also the case for the column used in this study. The elution volumes of all the compounds tested decreased. However, the importance of this effect depends largely on the solutes. Whereas for pentanal it is negligible ($\Delta V_e = 0.08$ ml), the decrease is dramatic for 2-pentanone ($\Delta V_e = 2.08$ ml) and tributyrin ($\Delta V_e = 1.96$ ml).

Three more observations can be made from Fig. 2. First, some part of the specific solute–stationary phase interaction still remains, as shown by the dispersion of elution volumes for volatile compounds of comparable mass. Second, triglycerides and volatile compounds are clearly separated, the latter being eluted at $V_e > 6.5$ ml. Elimination of fat from aroma extracts by fractionation on this column with dichloromethane as a mobile phase should therefore be possible, at least in principle. Third, volatile compounds are eluted within a relatively narrow zone. SEC is certainly not the method of choice when prefractionation of aroma extracts is needed in order to "thin out" gas chromatograms. What may be a disadvantage in these cases turns out to be an advantage here: flavour solutes of different polarity, molecular mass and volatility can be collected in a narrow fraction.

3.2. Separation as a function of column load

In the second part of this work, we studied the separation efficiency when analysing fat-containing aroma extracts, where more important amounts of triglycerides have to be injected. We chose goat cheese as a model. Various amounts of a dichloromethane extract obtained as described previously were injected. The amount of triglycerides injected ranged from 0.1 to 7 mg. A typical size exclusion chromatogram is shown in Fig. 3. Fractions were collected every 30 s (0.5 ml) and analysed by GC for their triglyceride content. Fig. 3 shows a triglyceride peak of good shape. In fact, owing to peak tailing, some minor but non-negligible part of the triglycerides is present in fractions collected later than 6.5 ml. This is illustrated by the gas chromatograms presented in Fig. 4a, b and c, showing profiles of fractions corresponding to the front, the apex and the tail of this peak, respectively. Characteristic split peaks, from a retention time of ca. 1000 s on, represent groups of triglyceride isomers with an equal carbon number, whereas earlier peaks indicate the presence of volatile compounds.

The quality of the separation obtained between volatile compounds and triglycerides can be expressed as the amount of triglycerides concomitantly eluted with the former. Fig. 5 shows this amount as a function of total column load. At 0.1 mg of triglycerides injected, 1 μg is recovered in the volatiles-containing fraction. At 10 mg, nearly half of this injected amount contaminates the fraction of volatile compounds. It is noteworthy that for an injected amount of triglycerides <1 mg, at least 99% of the triglycerides are eluted in fractions (before the elution volume of 6.5 ml) which do not contain any volatile component. Grob and Kälin [10] found that tailing of the triglyceride peak can be reduced by a factor of ten on by-passing the injection valve shortly after injection. Using this method, we were not able to find any significant

Table 1
Elution volumes of reference compounds

Compound	Molecular mass	Diethyl ether		Dichloromethane	
		V_e (ml)	R.S.D. (%)	V_e (ml)	R.S.D. (%)
Hydrocarbons					
Pentane	72	8.78	0.18	8.77	0.54
Hexane	86	8.60	0.05		
Heptane	100	8.53	0.13	8.61	0.67
Octane	114			8.30	0.48
Decane	142	8.25	0.08	8.08	0.46
Dodecane	170	8.09	0.06	7.85	0.10
Hexadecane	226	7.81	0.02		
Eicosane	282	7.58	0.02	7.23	0.18
Tetreicosane	338			7.00	0.02
Octeicosane	394			6.83	0.05
Hexatriacontane	506	6.93	0.07		
Cyclohexane	84	9.58	0.26	9.15	0.04
1-Decene	140			8.17	0.02
γ-Terpinene	136			8.10	0.17
Myrcene	136			7.99	0.03
Phellandrene	136			8.11	0.12
Copaene	204			7.65	0.06
Caryophyllene	204			8.00	0.03
Benzene	78			8.67	0.06
p-Cymene	136			8.05	0.03
Styrene	104			8.51	0.01
Naphthalene	128			8.72	0.11
Phenanthrene	178			8.79	0.07
1,2-Dihydro-1,1,6-trimethylnaphthalene	172			8.00	0.07
Alcohols					
Methanol	32	9.18	0.19	8.88	0.14
Ethanol	46	8.71	0.05		
1-Propanol	60	8.71	0.05	8.28	0.06
1-Butanol	74	8.64	0.18	8.15	0.10
1-Pentanol	88	8.57	0.06	8.04	0.05
1-Hexanol	102	8.48	0.08	7.93	0.15
1-Octanol	130	8.31	0.10		
1-Decanol	158	8.13	0.06	7.55	0.37
1-Dodecanol	186	7.99	0.06	7.39	0.09
2-Methyl-1-butanol	88	8.52	0.02	8.08	0.06
3-Methyl-1-butanol	88	8.48	0.09	8.02	0.02
2-Methyl-2-butanol	88	8.49	0.14	7.95	0.02
3-Methyl-2-butanol	88	8.48	0.10	8.01	0.13
2-Pentanol	88	8.48	0.12	7.97	0.03
Menthol	156			7.82	0.05
Cedrol	222			7.82	0.09
3-Penten-2-ol	86			8.40	0.04
1-Penten-3-ol	86	8.53	0.16	8.42	0.18
α-Terpineol	154	8.59	0.28	7.19	0.06
Pinocarveol	152			7.55	0.12
Geraniol	154			7.13	0.08
Linalool	154			7.03	0.12

Table 1 (*continued*)

Compound	Molecular mass	Diethyl ether		Dichloromethane	
		V_e (ml)	R.S.D. (%)	V_e (ml)	R.S.D. (%)
cis-Nerolidol	224			7.28	0.03
2-Phenyl-ethanol	122			7.84	0.09
p-Cymen-7-ol	150			7.66	0.02
Phenols					
Phenol	94			9.44	0.10
2-Methylphenol	108			9.01	0.03
3-Methylphenol	108			9.09	0.02
4-Methylphenol	108			9.06	0.14
4-Ethylphenol	122			8.79	0.12
5-Isopropyl-2-methylphenol	150			8.34	0.01
4-Vinylphenol	120			9.31	0.05
2-Methoxyphenol	124			8.11	0.00
2-Methoxy-4-vinylphenol	150			7.87	0.44
2-Methoxy-4-allylphenol	164			7.67	0.04
2-Methoxy-4-(1-propenyl)phenol	164			7.79	0.01
o-Cresol	110			8.09	0.03
Aldehydes					
Pentanal	86	7.84	0.28	7.76	0.30
2-Pentenal	84	8.71	0.08	7.56	0.32
Geranial	152			7.21	0.01
Benzaldehyde	106			7.99	0.08
4-Hydroxybenzaldehyde	122			7.99	0.08
Ketones					
2-Pentanone	86	9.69	0.39	7.61	0.02
2-Decanone	156			7.25	0.02
Camphor	152			7.53	0.01
Carvone	138			7.45	0.23
1-Penten-3-one	84	9.70	0.35		
Indanone	132			7.77	0.02
Furaneol	128			7.75	0.10
Acids					
Formic acid	46			9.76	0.30
Acetic acid	60			8.30	0.05
Propanoic acid	74			8.22	0.05
Butanoic acid	88			8.13	0.07
Hexanoic acid	116			7.87	0.17
Tetradecanoic acid	228			7.02	0.19
Octadecanoic acid	284			6.95	0.09
Esters					
Methyl acetate	74			7.53	0.12
Methyl tetradecanoate	242			6.65	0.19
Ethyl formate	74			8.40	0.03
Ethyl acetate	88			8.40	0.07
Ethyl octanoate	172			7.23	0.19
Pentyl pentanoate	172			7.23	0.03

(*Continued on p. 376*)

Table 1 (*continued*)

Compound	Molecular mass	Diethyl ether		Dichloromethane	
		V_e (ml)	R.S.D. (%)	V_e (ml)	R.S.D. (%)
3-Methylbutyl pentanoate	172			7.23	0.10
Pentyl 3-methylbutanoate	172			7.24	0.25
Pentyl 2-methylbutanoate	172			7.29	0.19
3-Methylbutyl 2-methylbutanoate	172			7.28	0.09
3-Methylbutyl 3-methylbutanoate	172			7.28	1.05
Ethyl *trans*-2-octenoate	170			7.22	0.13
Bornyl acetate	196			6.65	0.11
Methyl vanillate	182			7.93	0.08
2-Phenylethyl acetate	164			7.36	0.02
Diethyl succinate	174			7.62	0.06
Ethyl lactate	118			7.39	0.48
Tributyrine	302	8.61	0.40	6.65	0.07
Tricaproine	386			6.52	0.10
Tricapryline	470			6.39	0.13
Trilaurine	639			6.17	0.12
Tristearine	891	6.69	0.14	5.93	0.11
Lactones					
γ-Butyrolactone	86			7.53	0.23
γ-Decalactone	170			7.02	0.02
δ-Decalactone	170			7.03	0.19
Heterocyclic compounds					
2-Methyltetrahydrofuran	86	10.06	0.14	8.10	0.03
2-Methylfuran	82	9.34	0.14	8.51	0.10
Menthofuran	150			7.97	0.11
3,4-Dihydro-2*H*-pyran	84	8.74	0.16	8.33	0.07
Safrole	162			7.93	0.04
Pyrazine	80			8.17	0.02
Methylpyrazine	94			7.97	0.06
Ethylpyrazine	108			7.89	0.06
2,3-Dimethylpyrazine	108			7.85	0.05
2,5-Dimethylpyrazine	108			7.81	0.06
Tetramethylpyrazine	136			7.67	0.05
Methoxypyrazine	110			8.12	0.04
2-Isobutyl-3-methoxypyrazine	152			7.77	0.02

decrease with our specific equipment (Rheodyne Model 7010).

3.3. Optimization for the analysis of volatile compounds

As stated before, the aim of the fractionation was to obtain a sample that is directly analysable under normal GC conditions (i.e., on stationary phases that do not allow sufficiently high temperatures for the elution of triglycerides). The maximum tolerable amount of triglycerides remaining in the volatile fraction after fractionation depends on the mode of injection in GC (on-column or split–splitless), the required precision of the method and the part of the fraction that is effectively injected (the entire fraction for on-line GC experiments [11] or an aliquot part for conventional syringe injection). In our case, we assume that the injection of 0.5 μg of triglycerides per 1 μl injected into the GC system is acceptable. In order to maximize the sample

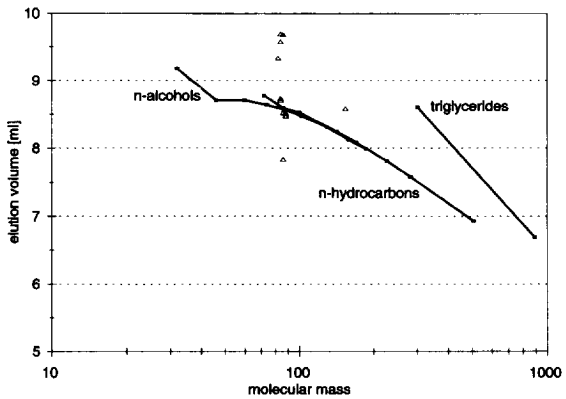

Fig. 1. Elution volume of reference compounds as a function of molecular mass. Mobile phase: diethyl ether. Compounds belonging to the same homologous series are connected by lines.

throughput and minimize the number of injections required, we used the following procedure: first, ten injections at 5 mg of triglycerides per injection, which slightly overloads the column; and second, recombination of the fraction from 6.5 to 10 ml of all injections, and refractionation in one injection. Owing to the small amount of triglycerides present in the recombined fractions, the final step yields a fraction of volatile compounds with an undetectable residual triglyceride content (Fig. 6). The concentration factor obtained is ca. 2000. For comparison purposes, it should be stressed that a more conventional

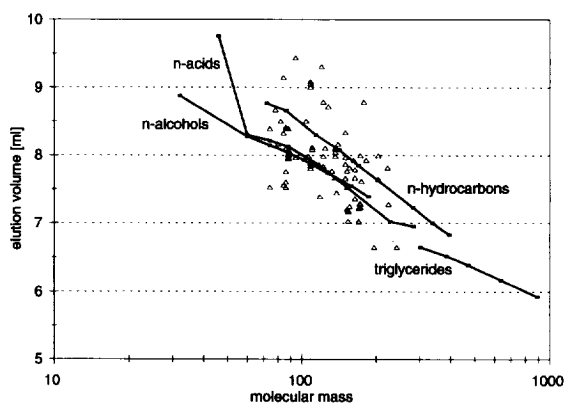

Fig. 2. Elution volume of reference compounds as a function of molecular mass. Mobile phase: dichloromethane. Compounds belonging to the same homologous series are connected by lines.

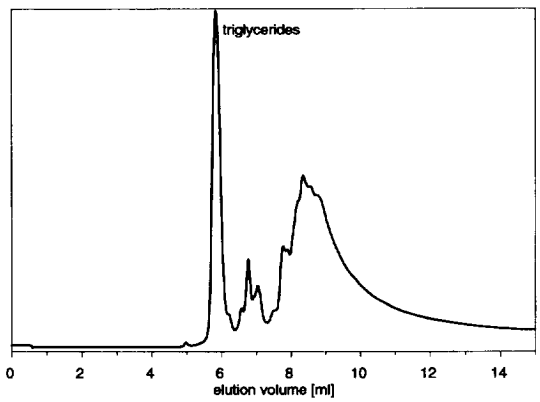

Fig. 3. Size-exclusion chromatogram of a crude goat cheese extract. Detection at 225 nm. Total column load: 5 mg.

extraction method, dichloromethane extraction of an aqueous vacuum distillate of 100 g of cheese, yielded an extract that contained 160 μg of triglycerides after concentration to 100 μl. This was three times more than our objective for the SEC aromatic fractions.

3.4. Analysis of goat cheese volatiles

Fractionation of the goat cheese extract carried out according to this scheme allowed us to collect a fraction of volatile compounds which was further chemically separated into acidic and neutral subfractions. Both subfractions were analysed by GC–MS. A total of 80 compounds could be identified, including 23 acids, 19 alcohols, 13 ketones, 13 lactones and 7 aldehydes. The qualitative and quantitative composition of the aromatic fractions thus determined was found to be comparable to that of the extracts obtained via the more conventional method described above [12]. However, the main interest in the SEC method described here resides in the limited number of injections necessary and in the reduced final volume of the fractions of interest. This last point is particularly noteworthy in terms of final concentration, prior to GC analysis, which appeared significantly quicker, and gave rise to less degradation of thermolabile products and to smaller losses of the most volatile compounds.

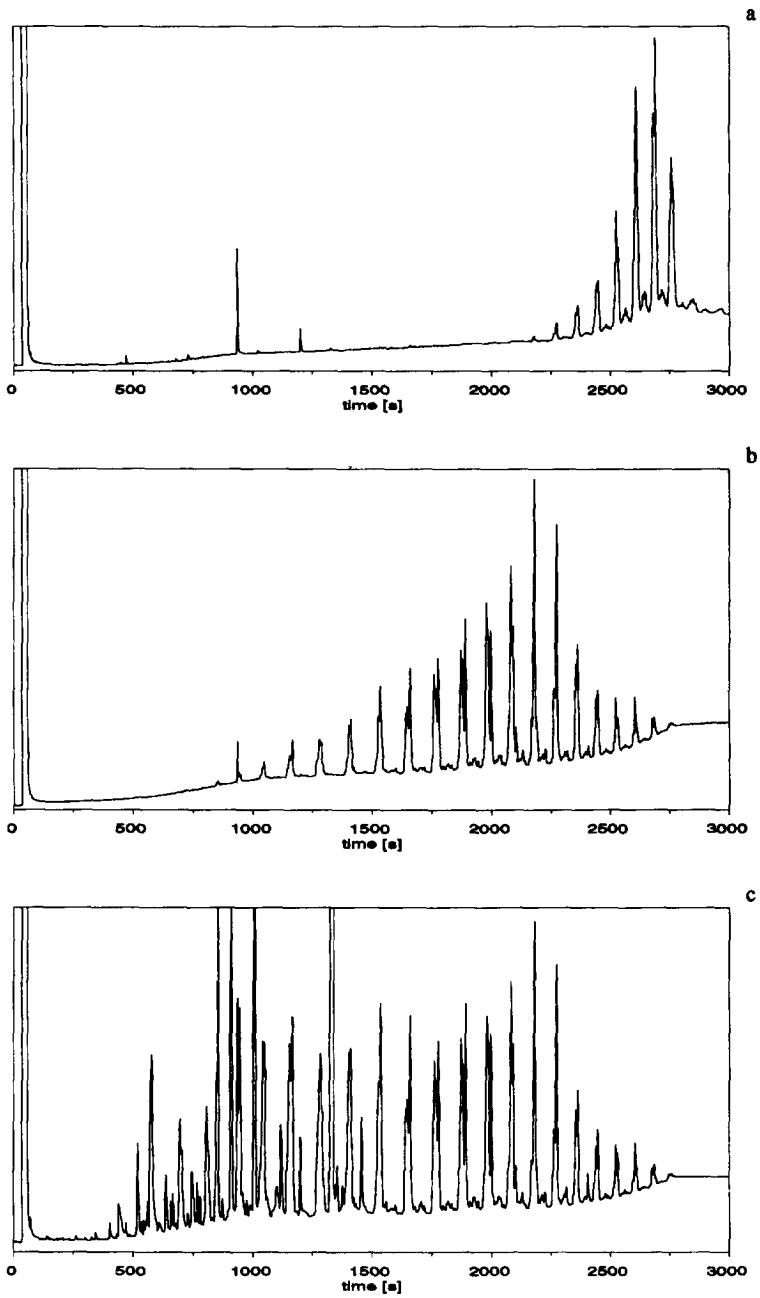

Fig. 4. Gas chromatograms of SEC fractions (total column load: 1 mg) of a goat cheese extract. Temperature programme: from 40 to 200°C at 10°C/min, then to 350°C at 5°C/min. (a) Fraction from 5 to 5.5 ml; (b) from 6 to 6.5 ml; (c) from 6.5 to 7 ml.

4. Conclusions

It has been shown that SEC is a simple and effective method for elimination of triglycerides in aroma extracts of fat-containing food. We believe this technique is an interesting alternative to the more classical, but often time-consuming, clean-up methods for such samples. The unique

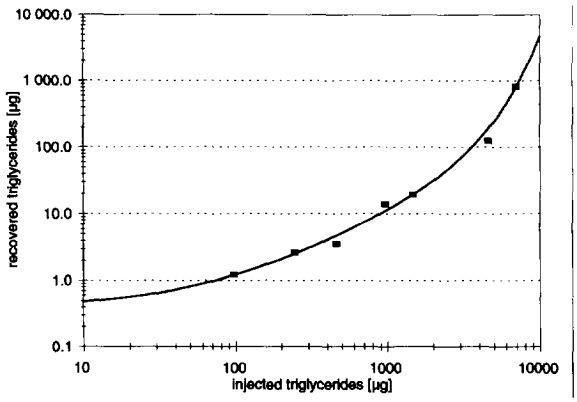

Fig. 5. Residual triglyceride content in SEC fractions from 6.5 to 10 ml as a function of the total injected amount.

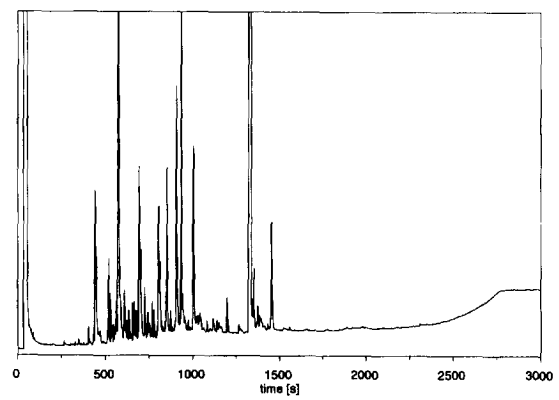

Fig. 6. Gas chromatogram of the volatile goat cheese fraction obtained after one injection of ten recombined fractions corresponding to Fig. 4c. GC conditions as in Fig. 4.

feature of SEC, elution of all solutes within a defined time, makes this method rapid and ensures quantitative recovery of the compounds of interest. On-line coupling of SEC with GC may further increase the attractiveness of the proposed method.

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