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# Combined silver-ion and reversed-phase high-performance liquid chromatography for the separation and identification of $C_{20}$ metabolites of conjugated linoleic acid isomers in rat liver lipids

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**Abstract**

Reversed-phase high-performance liquid chromatography (RP-HPLC) and silver-ion high-performance liquid chromatography (Ag-HPLC) were successively combined for the separation of the longer-chain metabolites of conjugated linoleic acids (CLAs). Commercial silver nitrate-impregnated columns were used with an eluting solvent composed of a mixture of hexane–acetonitrile. Fatty acid methyl esters (FAMEs) from liver lipids of rats fed CLA were analysed. This method allowed separation both of the non-conjugated FAME, as C16:1, C18:2, C18:3, C20:4 and C22:5, but also the conjugated fatty acids like CLA, 8,12,14-20:3, 5,8,12,14-20:4 and 5,8,11,13-20:4. The presence of 8,11,13-20:3 is reported for the first time. This method is of interest for the isolation and identification of the $C_{20}$ conjugated metabolites that cannot be resolved by gas chromatography. Furthermore, it allows the isolation of FAME for further characterisation by GC–mass spectrometry (MS). 

*Keywords:* Silver ion chromatography; Linoleic acid

## 1. Introduction

The number of studies on conjugated linoleic acids (CLAs) has increased considerably in recent years as it has been shown that they could have a beneficial effect on carcinogenesis [1–4], atherosclerosis [5–7] and body fat deposition [8]. It has also been demonstrated that CLAS could be metabolised by desaturation and elongation to form C20:3 and the C20:4 conjugated acids [9–11], as is the case for other *trans* polyunsaturated fatty acids [12]. Sébédio et al. [9] described three structures for such metabolites: 8,12,14-20:3, 5,8,12,14-20:4 and 5,8,11,13-20:4, and recently identified the two C20:4 isomers as being 5-*cis*,8-*cis*,12-*trans*,14-*cis*-20:4 and 5-*cis*,8-*cis*,11-*cis*,13-*trans*-20:4 [13].

Ag–high-performance liquid chromatography (HPLC) [14] has been developed and used for the separation and identification of *cis*/*trans* fatty acid isomers [15,16]. Recently, separations of CLAs were described [17–20]. Our aim was therefore to isolate the different geometric isomers of C20:3 and C20:4 that are difficult to resolve by GC or to isolate by Ag–TLC [13], and to complete the identification of all conjugated $C_{20}$ components present in the liver

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lipids of rats fed CLAs. We have developed a method combining Ag–HPLC with a pre-fractionation by RP-HPLC, in order to separate the C20:3 and C20:4 conjugated fatty acids according to their double bond positions.

## 2. Experimental

### 2.1. Chemicals and biological samples

Hexane and acetonitrile were of UV grade (SDS, Peypin, France). The mixture of CLAs and the chemical reagents were obtained from Sigma (L'Isle d'Abeau, France).

The methyl esters of total lipids of rat liver were obtained as previously described [9]. Briefly, nine rats were force-fed with 180 mg of CLA (administered in triacylglycerol form) for one week. The liver lipids were extracted using the method of Folch et al. [21], and converted into fatty acid methyl esters as described by Carreau and Dubacq [22].

### 2.2. High-performance liquid chromatography

A ternary Varian 9010 pump (Varian, Les Ulis, France), equipped with a Valco compressed air injector fitted with a 25- or 100-μl loop was used for the analysis.

The first column was a Nucleosil $C_{18}$ column, 5 μm, 250×10 mm I.D. (Shandon, Eragny, France). Acetonitrile alone was used as the mobile phase at a flow-rate of 4 ml/min. A Waters model 401 refractometer was used as the detector. The fractions were collected manually.

The second separation involved the use of a Chromspher Lipids column, impregnated with silver (5 μm, 250×4.6 mm I.D.) and purchased from Chrompack (Middelbourg, The Netherlands). The isocratic mobile phase were composed of hexane with a mixture of hexane–acetonitrile (100:2, v/v), the percentage of each solvent being determined by the FAME fraction required. The flow-rate was 1 ml/min. The column was maintained at 10°C using a refrigerated circulating bath (Julabo, Seelbach, Germany). The different FAME fractions were detected

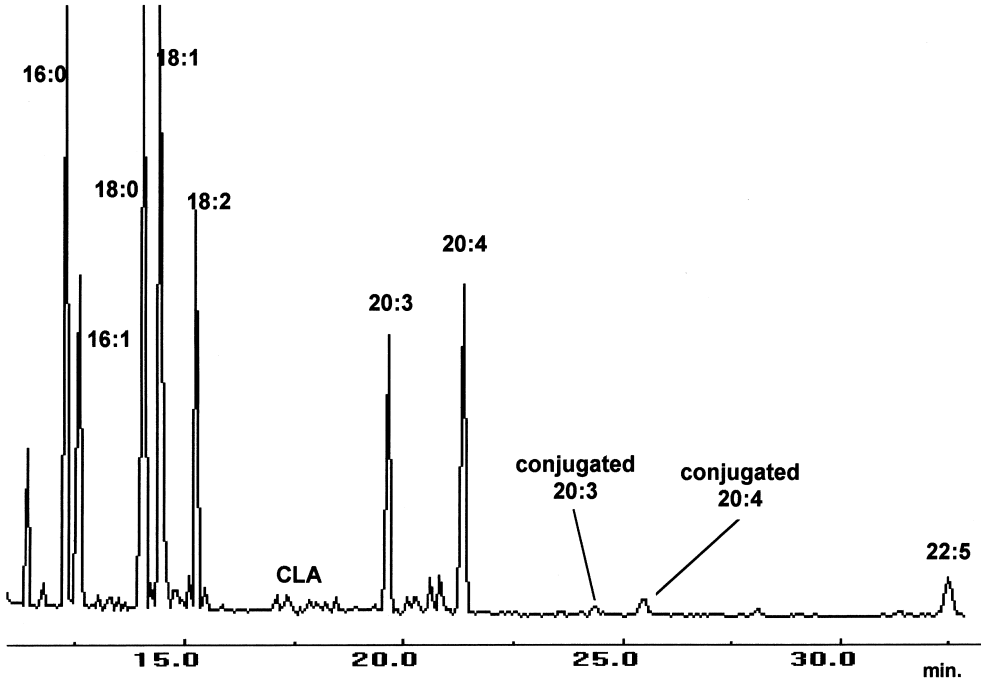


Fig. 1. Part of the gas chromatographic analysis of FAMEs of the total liver lipids of rats.

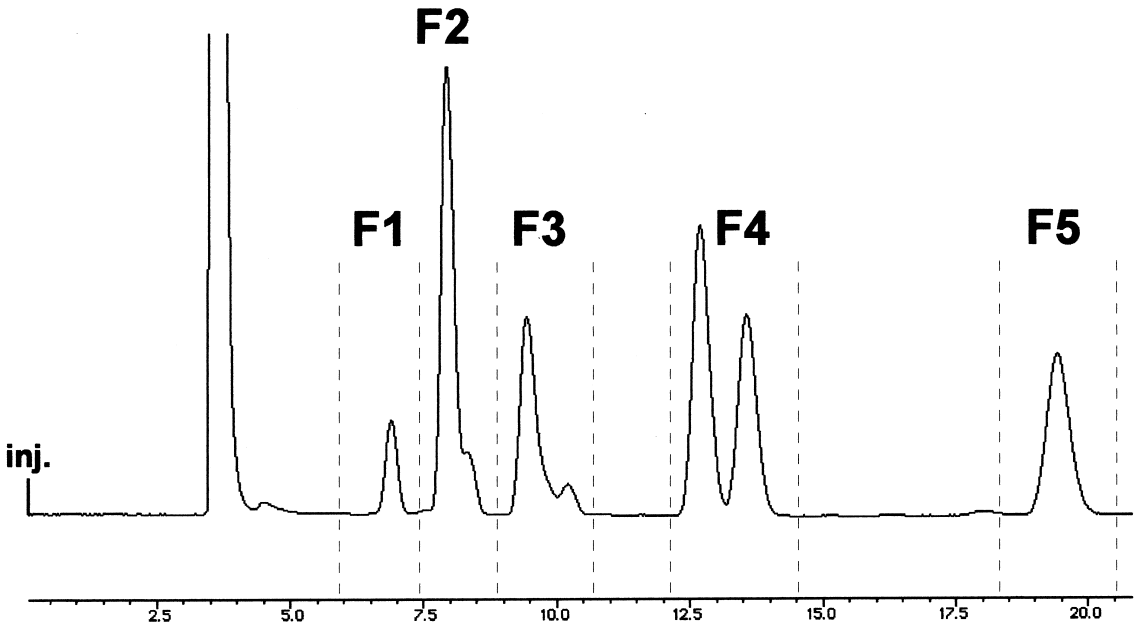


Fig. 2. Reversed-phase HPLC of FAMEs of liver lipids. Flow-rate, 4 ml/min, acetonitrile, refractive index (RI) detection. F1: 20:5, 22:6; F2: 18:3, 20:4, 22:5; F3: 16:1, 18:2, 20:3, 22:4; F4: 16:0, 18:1, 20:2; F5: 18:0.

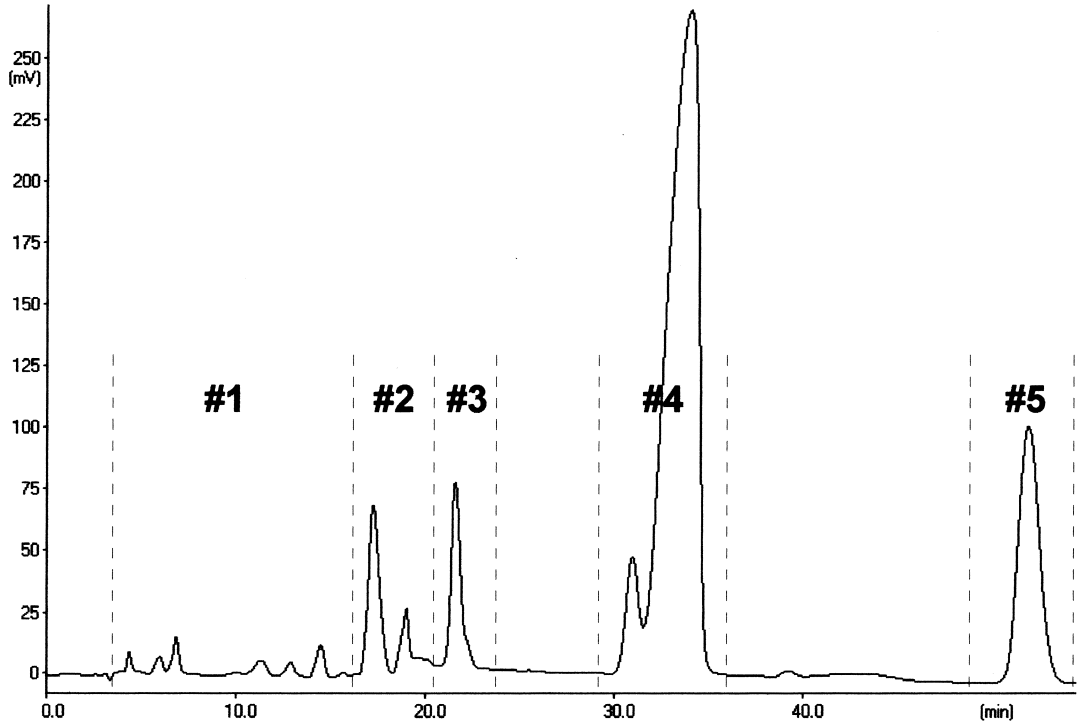


Fig. 3. Fractionation of fraction F2 by Ag–HPLC. Flow-rate, 1 ml/min, hexane–acetonitrile (99.75:0.25, v/v), UV detection. ''≠n'' between two interrupted ticks indicates the fractions collected.

Table 1
Fatty acid composition (%) of fraction F2 and of the different fractions collected (fr. no.) after Ag–HPLC

| | Total | fr. no. 2 | fr. no. 3 | fr. no. 4 | fr. no. 5 |
|---|---|---|---|---|---|
| Σ 18:3 | 4.2 | 59.6 | – | – | – |
| 4, 7, 10, 13-20:4 | 6.9 | – | 4.8 | 8.6 | – |
| 20:4 *n*−6 | 71.2 | – | 12.9 | 91.4 | – |
| 5, 8, 11, 13-20:4 | 5.7 | 40.4 | – | – | – |
| 5, 8, 12, 14-20:4 | | – | 82.3 | – | – |
| 22:5 | 12.1 | – | – | – | 100 |

Table 2
Fatty acid composition (%) of fraction F3 and of the different fractions collected (fr. no.) after Ag–HPLC

| | Total | fr. no. 1 | fr. no. 2 | fr. no. 3 | fr. no. 4 | fr. no. 5 |
|---|---|---|---|---|---|---|
| Σ 16:1 | 38.2 | 90.1 | – | – | – | – |
| iso 18:2 | 5.1 | – | 12.7 | 73.7 | 21.7 | – |
| 18:2 *n*−6 | 20.3 | – | 87.3 | 11.6 | 1.8 | – |
| Σ CLA | 4.1 | 9.9 | – | – | – | – |
| Σ 20:3 | 30.1 | – | – | – | – | 97.1 |
| 8, 11, 13-20:3 | 1.1 | – | | 14.7 | – | – |
| 8, 12, 14-20:3 | | – | – | – | 76.5 | – |
| 22:4 | 1.1 | – | – | – | – | 2.9 |

with a UV detector at 210 nm (Varian 9050, Les Ulis, France) and collected manually.

### 2.3. Gas chromatography

All of the FAME fractions were analysed by GC using a Hewlett-Packard (Palo Alto, CA, USA) 5890 series II, fitted with a split–splitless injector and a flame ionisation detector maintained at 250°C. The column was a BPX70 (50 m×0.25 mm I.D.; film thickness, 0.25 μm) (SGE, Melbourne, Australia) with helium as the carrier gas. The oven temperature was programmed from 60 to 185°C at 20°/min. Quantitative analyses were performed using a Chromjet integrator coupled to a computer equipped with Labnet software (Spectra-Physics, Les Ulis, France).

### 2.4. Gas chromatography–mass spectrometry

The fractions collected after Ag–HPLC were converted into 2-alkenyl-4,4-dimethyloxazoline derivatives (DMOX) according to Yurawecz et al. [23]. Briefly, 100 μl of 2-amino-2-methyl-1-propanol were added to the FAME in a screw cap tube, purged with $N_2$ and sealed using a PTFE cap. The reaction

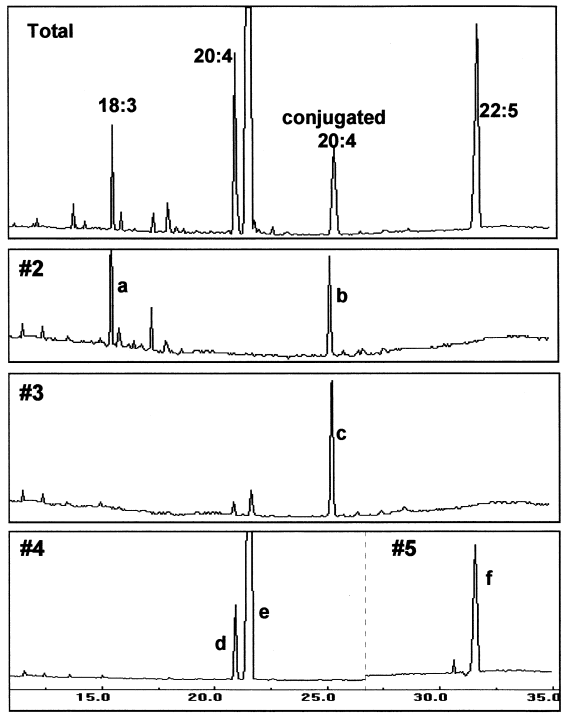


Fig. 4. Gas chromatography of FAMEs of the total fraction F2 and the Ag–HPLC collected fractions. (a) C18:3, (b) 5,8,11,13-20:4, (c) 5,8,12,14-20:4, (d) 4,7,10,13-20:4, (e) 5,8,11,14-20:4 and (f) 4,7,10,13,16-22:5.

mixture was maintained at 170°C for 8 h. The DMOX derivatives were analysed by GC–MS using a HP 5890 coupled to a HP 5970 mass spectrometer. The column was a BPX70, as described previously. The oven temperature was programmed from 60 to 200°C at 20°/min. The injector (splitless mode) was maintained at 250°C. The carrier gas velocity was 35 cm $s^{-1}$ at 200°C. The electron impact mass spectra were recorder at 70 eV between 100 and 400 amu.

## 3. Results and discussion

GC analyses of the total liver fatty acid methyl esters (Fig. 1) showed the presence of CLAs and other $C_{20}$ conjugated metabolites, as described by Sébédio et al. [9]. In spite of the low concentrations of the C20:3 and C20:4 metabolites, 0.1 and 0.7% of the total lipids, respectively, three metabolites have already been identified, 8,12,14-20:3, 5-*cis*,8-*cis*,12-*trans*,14-*cis*-20:4 and 5-*cis*,8-*cis*,11,*cis*-13-*trans*-20:4. However, identification of the second C20:3 conjugated fatty acid was not achieved as further fractionation steps would be required to purify it.

Consequently, the total FAME was fractionated by RP-HPLC [24], as shown in Fig. 2. Five fractions were collected. Fraction F2 contained all of the 18:3 (4%), C20:4 and its isomers (78%), C22:5 (12%) and 5.7% of the conjugated C20:4 isomers (Table 1). Fraction F3 was composed of 16:1 (38%), 18:2 (25%), CLAs (4%), 20:3 (29%), 22:4 (1%) and 1% of conjugated C20:3 isomers (Table 2). The direct identification of the conjugated $C_{20}$ DMOX derivatives by GC–MS was very difficult due to the high concentration of the other fatty acids.

Fig. 3 shows the fractionation of the fraction F2 by Ag–HPLC, using an isocratic solvent (hexane–acetonitrile, 99.75:0.25, v/v). No variation of re-

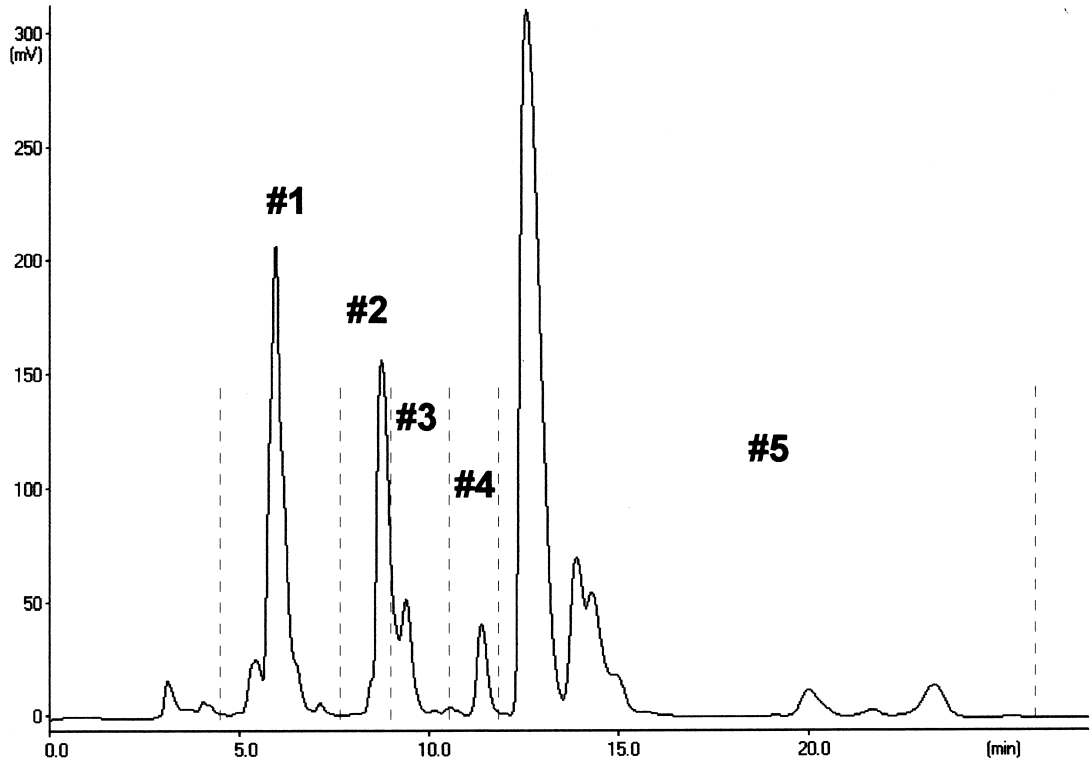


Fig. 5. Fractionation of fraction F3 by Ag–HPLC. Flow-rate, 1 ml/min; hexane–acetonitrile (99.71:0.29, v/v); UV detection. ''≠n'' between two interrupted ticks indicates the fractions collected.

tention time was detected. Repeated injections (300 μg/100 μl) were made to obtained a sufficient quantity of product for further analysis. Each collected fraction was analysed by GC and GC–MS. Fig. 4 shows the GC trace of the total fraction F2 and the detailed composition of each of the fractions collected. In fraction 1, no peaks were detected by GC–MS. In fractions 2 and 3, we found 5,8,11,13-20:4 (40%) and 5,8,12,14-20:4 (82%), respectively. Fraction 2 also contained different isomers of 18:3, but no conjugated 18:3 could be detected. 5,8,11,14-20:4 (arachidonic acid), 4,7,10,13-20:4 (C20:4$n-7$) and 4,7,10,13,16-22:5 (C22:5$n-6$) were present in the two last fractions (i.e. fractions 4 and 5).

Fig. 5 shows the separation of fraction F3 by Ag–HPLC. The isocratic solvent was composed of hexane–acetonitrile (99.71:0.29, v/v). Five fractions were collected and analysed by GC and GC–MS. The GC separations are illustrated in Fig. 6. Fraction 1 contained 16:1 ($n-7$ and $n-9$) and the CLAs (8c,10t; 9c11t; 10t12c; 11c,13t-18:2 and some minor cc, tc, tt conjugated isomers). All of the 20:3 ($n-9$, $n-6$, $n-3$ and Δ5,8,11) isomers and 22:4$n-6$ were found in fraction 5. The 18:2 and its geometric isomers were in fractions 2 and 3. The 8,12,14-20:3, already identified by Sébédio et al. [9], was in fraction 4. Lastly, the collected fraction 3 contained part of 18:2 and another peak with a similar GC retention time as that of the conjugated 20:3 previously identified. Fig. 7 represents the mass spectrum of the DMOX derivatives of this peak. The spectrum gave two intense ions at $m/z$ 113 and 126, characteristic of the DMOX derivatives and a prominent molecular ion at $m/z$ 359, which indicated that this peak was a C20:3 isomer. Three mass intervals of 12 amu (instead of 14) occurred between 182 ($C_7$) and 194 ($C_8$), between 222 ($C_{10}$) and 234 ($C_{11}$), and between 248 ($C_{12}$) and 260 ($C_{13}$). This indicates the presence of three ethylenic bonds in the Δ8, Δ11 and Δ13 positions. This component is therefore 8,11,13-20:3, which could be formed from 9,11-18:2 by desaturation and elongation.

In conclusion, the combination of the two HPLC procedures allowed us to isolate the positional and geometric isomers of conjugated C20:3 and C20:4. The two methods are complementary. With fractionation RP-HPLC alone, GC–MS analyses of the fractions was very difficult due to the small amounts of conjugated $C_{20}$ isomers isolated and no separation was possible between the two isomers of C20:3 or C20:4. The total liver lipid sample contained too many fatty acids for direct analysis by Ag–HPLC alone and the $C_{20}$ isomers tended to co-elute with other FAMEs (depending on the composition of the mobile phase), which made analysis by GC–MS difficult. With a combination of Ag–HPLC and RP-HPLC, however, the isolation and identification of the conjugated C20:3 and C20:4 isomers can be achieved.

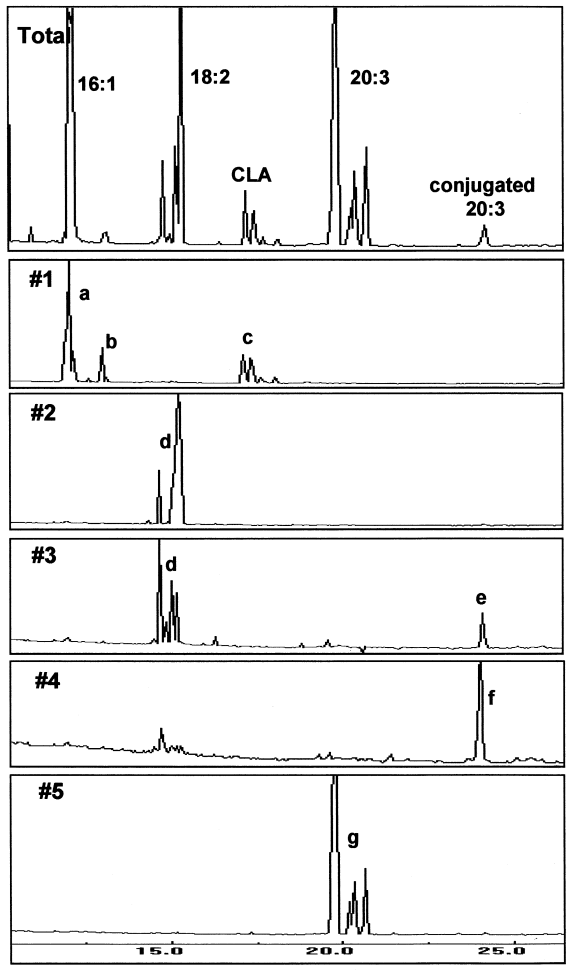


Fig. 6. Gas chromatography of FAMEs of the total fraction F3 and the Ag–HPLC collected fractions. (a) 9−16:1+10−16:1, (b) 9−17:1, (c) CLA, (d) 18:2 (9,12+isomers), (e): 8,11,13-20:3, (f): 8,12,14-20:3 and (g) 20:3.

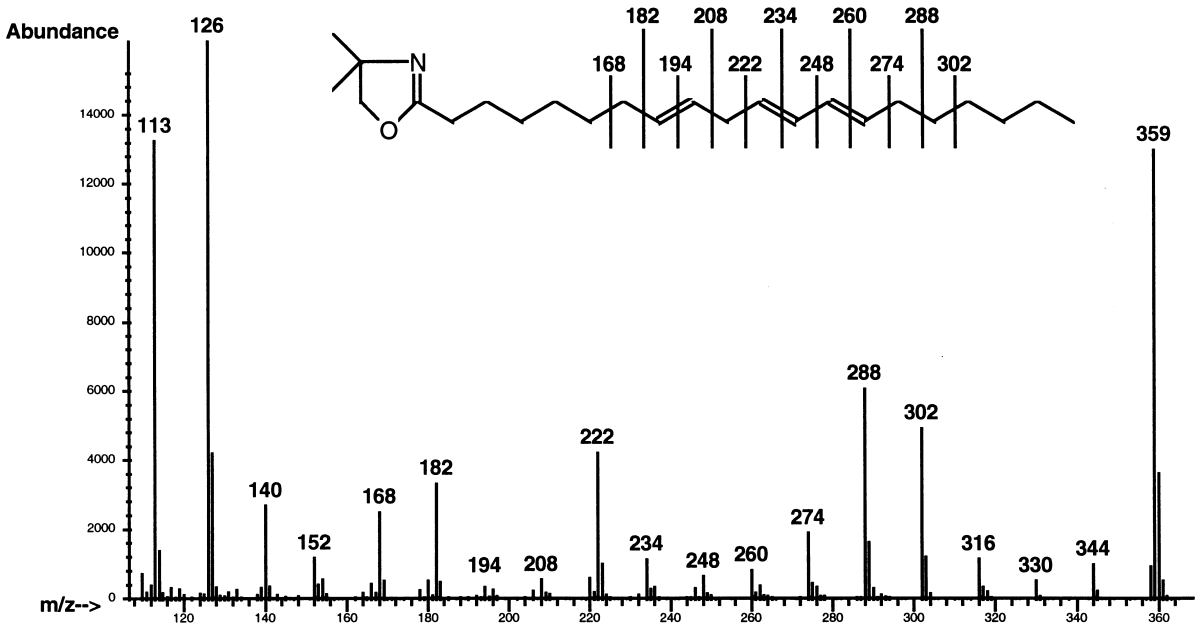


Fig. 7. GS–MS of the 2-alkenyl-4,4-dimethyloxazoline derivative of 5,11,13-20:3.

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