Determination of the triglyceride composition of avocado oil by high-performance liquid chromatography using a light-scattering detector

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ABSTRACT

The triglyceride composition of avocado oil was determined by high-performance liquid chromatography using a light-scattering detector. Two avocado varieties, Fuerte and Hass, were analysed, and the qualitative composition of each was found to be similar, though quantitative differences were detected. The triglyceride composition was predicted using a system of equations based on the relationship between log k' and the molecular variables equivalent carbon number, chain length and number of double bonds for each of the fatty acids in the glycerides. A total of 24 molecular species of triglycerides were identified. The chromatographic system used successfully separated the critical pairs OOO–LOS, PaPaO–LnPP and PaOO–LOP (O = olein; L = linolein; S = stearin; Pa = palmitolein; Ln = linolenin; P = palmitin). Detector response was found to have a linear relationship with the amount of sample injected over the injection range 10–70 μg.

INTRODUCTION

The avocado (*Persea americana*) is a subtropical fruit of the family *Lauraceae* which is becoming increasingly common in Spain. The lipid content is very high and may attain 25% of the edible portion of the fruit. The saponifiable fraction of the lipid material in avocados is highly unsaturated [1], which makes it not only a highly esteemed food and food ingredient, but also a valuable raw material for the cosmetics industry [2].

It is normal practice to follow the avocado maturation process by monitoring the lipid content, because lipid levels are related to the ripening stages in each cultivar [3,4]. Some workers have proposed the more refined method of fatty acid profile analysis carried out by either gas chromatography (GC) or high-performance liquid chromatography (HPLC), which has the advantage of being able to monitor fruit maturity stages as well as to differentiate individual cultivars [5]. Applying the higher power of new analytical instrumentation to this same line of

refinement, it is appropriate to examine the potential benefits of analysing the glycerides themselves.

The triglyceride (TG) composition of mesocarp lipids has been investigated by separating molecular species by thin-layer chromatography (TLC) and GC [6], silver nitrate TLC and pancreatic lipolysis [7], or HPLC [8]. Non-aqueous reversed-phase chromatography separates TGs on the basis of the number of carbon atoms and the number of double bonds [9]. Such factors as the type of stationary phase, composition of the mobile phase and column temperature have been studied with a view to enhancing the resolution of critical pairs of TGs [10–13]. The light-scattering detector [14–16] affords an advantage over refractive index detectors in that elution gradients can be used, and over ultraviolet detectors in that no baseline drift occurs and there are no limitations on the use of mobile phase solvents.

Some workers have reported that the mass detector is subject to a non-linear relationship between reponse and the amount of solute injected [14,17], *i.e.*, the response (A) is proportional to the amount (*m*) injected raised to a power ($A = am^x$). The exponent, *x*, is closely linked to the nebulizer shape (pressure and temperature conditions in the evaporator).

The identification of chromatogram peaks is difficult because of the rarity of mixed TGs in a pure state. To indentify the HPLC-separated TG molecular species, the relationships between the retention time and the partition number [18,19], the equivalent carbon number (ECN) [12,20,21], the theoretical carbon number [10], or with the equivalent chain length [22] have been studied. All are similar concepts with differences in the manner in which they relate the carbon number and the number of double bonds in the fatty acids in the glycerides. Takahashi and co-workers [23,24] suggested a matrix model relating the retention time with the chain length and number of double bonds for each fatty acid in the glyceride, giving a more accurate prediction of the TG molecular species from the retention time than if only the total carbon number and number of double bonds were used, without distinguishing the positions of the fatty acids.

The object of this paper is to report the determination of the glyceride composition of the lipid fraction extracted from the mesocarp of the two major avocado cultivars in Spain, Fuerte and Hass, performed using an HPLC technique with a light-scattering detector. The application of mathematical modelling to the retention behaviour of TGs is used to estimate the TG composition.

EXPERIMENTAL

Sample preparation

Samples of avocado pears (varieties Hass and Fuerte) from Granada (Mediterranean coastal zone, Spain) were analysed.

The avocado pear samples were freeze-dried and the oil was obtained by Sxohlet extraction with chloroform–methanol (2:1). The oil sample (1 g) was extracted with ten volumes of *n*-hexane (Panreac, Madrid, Spain) containing 0.1 mg/ml butyl-hydroxytoluol (Fluka, Buchs, Switzerland) as an antioxidant. The mixture was homogenized and poured into a separation funnel with a solution of ethanol (Panreac) and distilled water (80:20, v/v), in the proportion 3:2 (v/v). The mixture was shaken vigorously and then allowed to stand until separation of the upper, organic fraction from the lower, aqueous alcoholic fraction was complete. The organic fraction was filtered through 1PS filter paper (Whatman, Maidstone, UK), exposed to a stream of nitrogen for 1 min, and evaporated to dryness under low pressure at 30°C. The TG residue was redissolved in HPLC-grade chloroform (2 ml; Ferosa, Madrid, Spain) and passed through a filter with a pore size of 0.2 μm (Millipore, Milford, MA, USA). Portions of the resulting solution were used for HPLC analysis.

HPLC analysis

The HPLC equipment consisted of two pumps (Models 510 and 6000A, Waters Chromatography Division, Milfrod, MA, USA), a system controller (Model 720, Waters Chromatography Division), an injector (Model 7125, Rheodyne, Cotati, CA, USA), two 25 cm × 4.6 mm I.D. stainless-steel columns containing a bonded phase composed of Sperisorb ODS-2 (Phase Separations, Queensferry, UK and Symta, Madrid, Spain), with a particle size of 3 μm, connected in series. The column effluent was passed through a mass detector (Model 750/14, ACS, Macclesfield, UK). The mass detector oven temperature was 60°C and the inlet gas pressure

(from an air compressor) was 20 p.s.i.g.; the detector was connected to an integration system (System Gold, Beckman, San Raman, CA, USA).

The column was placed in a hot air oven (Kariba Instruments, Cardiff, UK) to keep the analysis temperature constant at 30°C. The mobile phase consisted of an elution gradient from 35 to 70% (v/v) HPLC–grade acetone (Panreac) in HPLC–grade acetonitrile (Ferosa) in two stages: a linear increase in acetone at 4.6 μl/min for the first 45 min, followed by a linear increase in acetone at 2.4 μl/min from that point to 75 min. From 75 to 120 min elution was isocratic. The flow-rate was 0.8 ml/min and the pressure 2200 p.s.i.g.

The TGs were quantified based on the percentage peak area in the HPLC chromatogram.

To calculate response factors in relation to the internal standard, trilinolein (LLL) (99% pure, 10 mg/ml, Sigma Chemical, St. Louis, MO, USA), chloroform solutions containing 10 mg/ml of the simple TGs (99% pure, Sigma Chemical) tricaproin (CoCoCo), tricaprylin (ClClCl), tricaprin (CaCaCa), trilinolein (LLL), trilinolenin (LnLnLn), trimyristin (MMM), tripalmitolein (PaPaPa), tripalmitin (PPP), triolein (OOO) and tristearin (SSS) and the mixed TGs 1,2-dimyristoyl-3-palmitoyl glycerol (MMP), 1,2-dioleoyl-3-palmitoyl glycerol (OOP), 1,2-dipalmitoyl-3-oleoyl glycerol (PPO), 1,2-dioleoyl-3-stearoyl glycerol (OOS), 1,2-distearoyl-3-oleoyl glycerol (SSO) and 1,2-stearoyl-3-myristoyl glycerol (SSM) were analysed.

Five replications of the HPLC analysis were performed for one of the avocado oil samples to determine the reproducibility of the HPLC method. All analyses were performed in triplicate.

Calculation of the random composition

The random composition of the TGs in the avocado oil was calculated from the percentage mole fractions of the main fatty acids in the total TG fraction, taking the three positions on the glycerol molecule to be equivalent [25].

The fatty acid composition of the total TG fraction was obtained from data for avocado oil (varieties Fuerte and Hass) from Granada from previously reported data [2]. The percentage mole fraction values for the main fatty acids ranged from 0.50 to 19.22%.

Calculation of the ECN

The ECN for each individual TG was calculated using the formula given by Herslöf et al. [20]:

$$ECN = CN - (a'ND) \qquad (1)$$

where CN is the total number of carbon atoms and ND the total number of double bonds in the fatty acids attached to the glycerol molecule. The value of the constant a' was calculated by multiple linear regression analysis of the experimental values of the dependent variable, log k', on the independent variables CN and ND for the TGs available in pure form [log $k' = q' + b'(CN + c')ND$], where a' is the quotient of the coefficient c' on the coefficient b'.

Calculation of the equations used in the identification system

Linear and multiple regression were aplied to relate the ECN to log k'; log k' to the chain length (CL) and the number of double bonds (DB) in each of the three fatty acids in the TG molecule, taking the three possible positions to be equivalent; and log k' for mixed TGs to log k' for simple TGs [26]. The equations were calculated from experimental data for the available pure mixed and simple TGs.

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC profiles for the TGs in the avocado oil from the two varieties (Fuerte and Hass) studied. The Fuerte variety gave 19 peaks, whereas the Hass variety gave 24. Reproducibility of the retention times of the peaks was good, with coefficients of variation of about 1% in all instances.

The molecular species that corresponded to each of the peaks on the chromatogram was determined by applying a system that had proved effective in an earlier experiment [27]. The first step was to compare the ECN for each peak with the ECNs for the possible TGs established from the fatty acid composition of the avocado oil.

The three stereospecific positions on the glycerol molecule were assumed to be equivalent, inasmuch as the position isomers were not distinguishable with the HPLC system used. Based on the eight main fatty acids, the total possible number of molecular species was calculated to be 120. However, the maximum number of molecular species de-
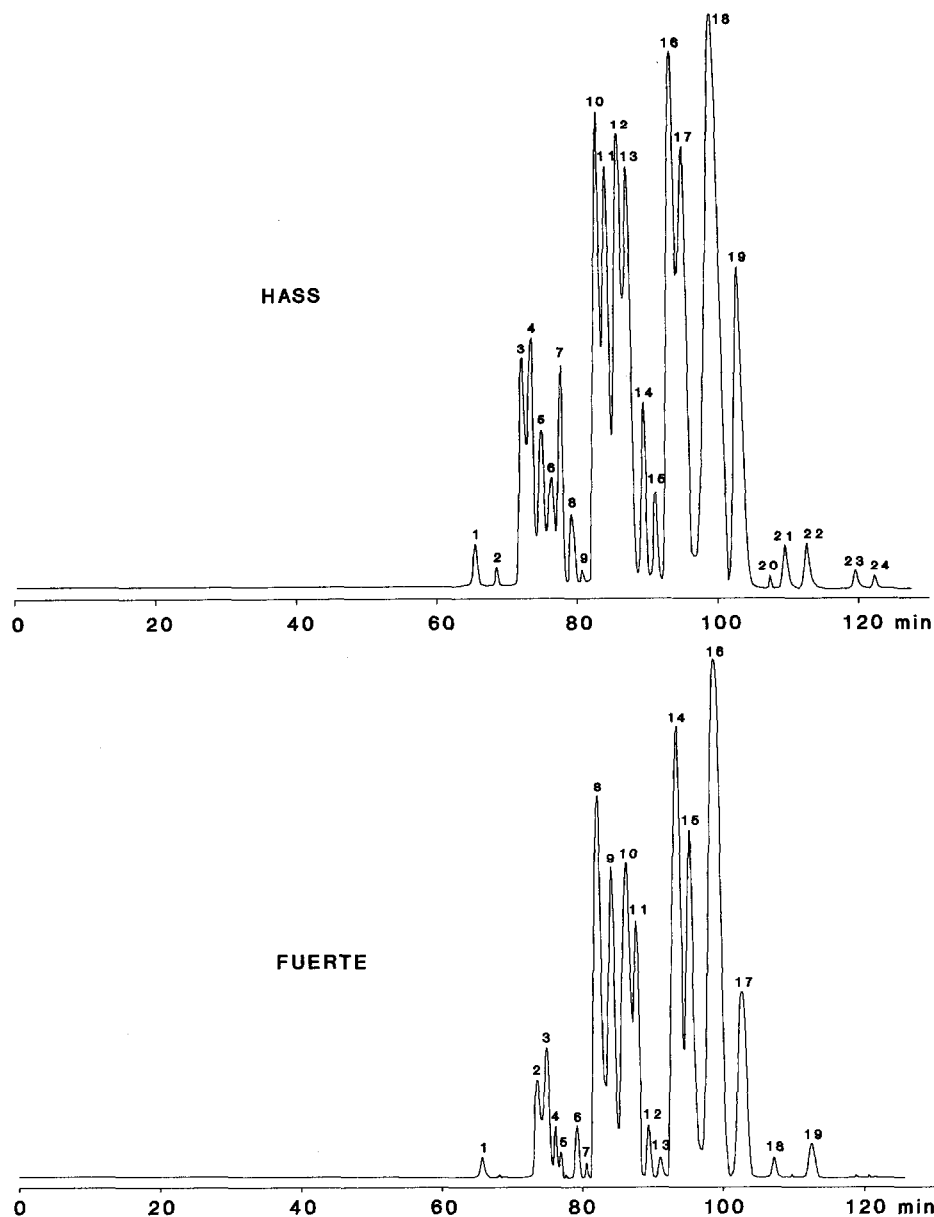
Fig. 1. HPLC analysis of avocado TGs. See Table I for identification of peak numbers.

scribed previously was 33 (28); Lozano [8] has described 31 possible molecular species.

The usefulness of random composition in predicting the molecular species of TGs present in a fat has been shown previously [21,29]. As a result, the most probable molecular species were considered herein to be those with random composition values

higher than 0.01%, which brought the number of possible TGs down to 41.

The ECN was at first calculated using eqn. 1. The value of the constant a' was calcualted to be 1.87 from the equation $\log k' = -0.7665 + 0.0426CN - 0.0797ND$ [standard error (S.E.) = 0.0553]. The TGs eluted, as expected, in ascending order of

ECN, except for certain series of critical pairs such as those with a partition number of 48, namely, OOO, OOP, OPP and PPP, for which the ECN values were 48.39, 48.26, 48.13 and 48.00, respectively, which eluted in descending order of ECN. The reason for this failure to conform to the generally accepted pattern of behaviour for TGs in non-aqueous, reversed-phase HPLC was attributed to improper estimation of the ECN by eqn. 1. The relationship between ECN and the molecular variables CN and ND may possibly be more complex than that described by the equation.

To elucidate this question, stepwise linear regression was applied, taking CN and ND and transforms thereof, as the independent variables.

$$ECN = CN(1 - 0.0062CN) -$$
$$1.1234ND(1 - 0.0256ND) \quad (2)$$

On the basis of the new ECN values calculated using eqn. 2, all the TGs eluted in ascending order of ECN.

Table I gives the ECN values for each of the peaks in the chromatogram for the total TG fraction from the sample of avocado oil from the Fuerte variety calculated using eqn. 3.

$$\log k' = -1.372 + 0.0797ECN \text{ (S.E. } = 0.0152) (3)$$

Table I also gives the distribution of the 41 preselected possible TGs among the 19 peaks on the chromatogram according to their ECN calculated using eqn. 2. Theoretically, there was more than one TG for each of the peaks; and, additionally, certain molecular species could correspond to more than one peak.

In the next step the equation proposed by Takahashi et al. [26] was used to estimate the value of log k' for mixed TGs from the log k' values for the simple TGs composed of the appropriate fatty acids according to the composition of the mixed TGs (eqn. 4). The equation was applied to the possible TGs preselected in the preceding step.

$$\log k'_{ABC} = 1/3\log k'_{AAA} + 1/3\log k'_{BBB} +$$
$$+ 1/3\log k'_{CCC} \text{ (S.E. } = 0.0071) \quad (4)$$

The experimental data for the mixed TGs MMP, OOP, OPP, OOS, MSS and OSS and the corresponding simple TGs confirmed the applicability of this equation to the chromatographic system used.

Table I shows that application of this equation reduced the number of possible TGs for each peak approximately by half and in some instances limited the possibility to a single TG. Thus, in step 1, six TGs (OOO, LOS, PaOS, OOP, LPS and MMP)

TABLE I

IDENTIFICATION OF TGs IN AVOCADO OIL (VARIETY FUERTE)

Peak No.	ECN (eqn. 3)	Possible TGs (eqn. 2)	Possible TGs (eqn. 4)
1	30.51	LLPa, LnOPa, LnPaP, LPaM, LnLP, LPaPa, PaPaPa	LLPa, LnOPa, LnLP, LPaPa
2	31.10	LLO, LnOO, LPaO, LLP, LnOP	LLO, LnOO, LPaOP
3	31.19	LLO, LnOO, LPaO, LLP, LnOP, PaPaO, LOM, LnPP	LLO, LPaP, LLP, PaPaO
4	31.32	LPaO, LLP, LnOP, PaPaO, LOM, LnPP, PaPaP	LPaO, LLP, LnOP, PaPaO, LOM
5	31.42	PaPaO, LOM, LnOP, PaPaP, LnPP, LLP, LPaO	LOM, LnPP, PaPaP, LnOP, LLP
6	31.45	PaPaO, LOM. LnOP, PaPaP, LnPP, LLP, LPaO	LOM, LnOP, PaPaP, LnPP, LLP
7	31.51	LOM, PaPaO, LnPP, PaPaP	LOM, PaPaP
8	31.91	LOO, LnOS, LLS, PaOO, LOP, LPaS, LnPS	LOO, PaOO
9	31.99	LOO, LnOS, LLS, PaOO, LOP, LPaS, LnPS	PaOO
10	32.11	PaOO, LOP, LPaS, LnPS, PaOP, LPP, OOM	LOP, OOM
11	32.19	PaOO, LOP, LPaS. LnPS, OOM, PaOP, LPP, PaPaS	LOP, OOM, PaOP, LPP
12	32.35	OOM, PaOP, LPP, PaPaS	PaOP, LPP
13	32.45	OOM, PaOP, LPP, PaPaS, PaPP, OMP	PaOP, LPP, PaPaS, PaPP, OMP
14	32.61	PaPP, OMP, MPP, OOO, LOS, PaOS, LPS	LPP, PaPaS, PaPP, OMP, OOO
15	32.66	PaPP, OMP, MPP, OOO, LOS, PaOS, LPS	LPP, PaPaS, OOO, PaPP, OMP
16	32.88	OOO, LOS, PaOS, OOP, LPS, MMP	OOP
17	33.17	PaPS, OPP	OPP
18	33.50	PaPS, OPP, PPP	PaPS, OPP
19	33.73	PPP, OOS	OOS

were possible for peak 16 (log k' = 1.249), which had an ECN of 32.88. After application of eqn. 4, log k' for these TGs took on the values 1.237, 1.273, 1.278, 1.252. 1.284 and 1.177, respectively, hence OOP became the only possible TG for this peak.

The next step was to apply the equation put forward by Takahashi and co-workers [23,24] relating log k' for a TG to the chain length (L) and the number of double bonds (D) for the fatty acids making up the TG molecule (eqn. 5).

$$\log k' = -0.8072 + 0.0521L1 + 0.0246L2 + 0.0549L3 - 0.1479D1 - 0.08335D2 - 0.0186D3$$
$$(\text{S.E.} = 0.0499) \qquad (5)$$

Chain length for the acid at position 3 ($L3$) was calculated from the log k' values for each peak, using the order of fatty acids in the TGs established in Table II. Checking which of the TGs preselected using the above equation met the chain length requirement for the fatty acid at position 3 (Table II) yielded a new selection of molecular species.

Table II shows that most of the peaks on the chromatogram had only a single corresponding TG. The identification of peaks 16, 17 and 19 was confirmed by comparing the retention times with

TABLE II

TGs IN AVOCADO OIL (VARIETY FUERTE)

Peak no.	ECN (eqn. 3)	Possible TGs (eqn. 5)[a]
1	30.51	LLPa
2	31.10	LLO[a], LPaO
3	31.19	LLO, LPaO[a]
4	31.32	PaPaO, LLP[a]
5	31.42	PaPaO[a], LLP, LnPP
6	31.45	LnPP[a], PaPaP
7	31.51	PaPaP
8	31.91	LOO
9	31.99	PaOO
10	32.11	LOP
11	32.19	PaOP
12	32.35	LPP
13	32.45	PaPP
14	32.61	OOO[a], LOS
15	32.66	OOO, LOS[a]
16	32.88	OOP
17	33.17	OPP
18	33.50	PaPS
19	33.73	OOS

[a] TGs with greater possibility of corresponding to the particular peak.

those for the mixed TGs available in the form of pure TGs, i.e. OOP, OPP and OOS.

The possible TGs for peaks 14 and 15 were the same, OOO and LOS, for both peaks. The ECN was the same (32.809) for both these TGs, hence it was difficult, in principle, to determine the order of elution. However, based on the retention time for the simple TG OOO, which was available in pure form, the TG OOO corresponded to peak 14. Therefore, it would appear that for TGs with the same ECN, the first to elute was the TG with the greatest number of unsaturated fatty acids.

According to eqn. 5, the TGs LnPP and PaPaP were the possible TGs for peak 6. LnPP was the most likely choice, because PaPaP was the only possible TG for peak 7 and hence should not be considered for peak 6. In addition, the ECN for LnPP (31.389) was lower than that for PaPaP (31.584) and thus eluted first. Consequently, the TG LnPP was disregarded for peak 5, which was thereby limited to the TGs PaPaO and LLP, which were also the most likely candidates for peak 4. The ECNs for these two TGs were 31.389 and 31.202, respectively, hence PaPaO probably corresponded to peak 5 and LLP to peak 4.

The two possible TGs for chromatographic peaks 2 and 3 were LLO and LPaO. Which of the two should elute first can be predicted from the ECN values. As the ECNs for these TGs were 31.023 for LLO and 31.202 for LPaO, the order of elution should be first LLO, then LPaO. Therefore, the likely TGs were LLO for peak 2 and LPaO for peak 3.

Based on the foregoing, it would appear that the relationship between ECN and CN or ND is not as simple as has been presumed up to now. This was to be expected, inasmuch as analytical methods developed in recent years have proved capable of resolving TGs with the same conventional ECN value.

TGs with equal ECNs eluted in ascending order of number of saturated fatty acids in the molecule. This was confirmed for the critical pairs OOO–LOS, LPaO–LLP, PaPaO–LnPP and PaOO–LOP (Table III). The behaviour of TGs in non-aqueous reversed-phase HPLC is therefore dependent not only on the CN and ND, but also on the number of unsaturated acids in the molecule.

The chromatographic peaks corresponding to the TG fraction of avocado oil from the Hass variety

TABLE III

TGs IN AVOCADO OIL (VARIETY HASS)

Peak No.	ECN	TGs
1	30.55	LLPa
2	30.60	LPaPa
3	31.16	LLO
4	31.21	LPaO
5	31.32	PaPaO
6	31.40	LLP
7	31.45	LnOP
8	31.52	LnPP
9	31.67	PaPaP
10	31.90	LOO
11	32.00	PaOO
12	32.10	LOP
13	32.19	PaOP
14	32.34	LPP
15	32.44	PaPP
16	32.60	OOO
17	32.63	LOS
18	32.85	OOP
19	33.14	OPP
20	33.39	PaPS
21	33.49	PPP
22	33.72	OOS
23	33.99	OPS
24	34.08	PPS

were identified using the same procedure described above. The TGs estimated were the same as those for the Fuerte variety, except that there were five additional TGs in the Hass variety, which corresponded to five additional chromatographic peaks.

Table III gives the TG composition of the avocado oil from the Hass variety. Peaks 2, 7, 21, 23 and 24, correspoding to the TGs LPaPa, LnOP, PPP, OPS and PPS, differentiated the oils from the two avocado varieties.

These results showed good agreement with the results reported by Lozano [8] and Gaydou et al. [4]. Although these workers succeeded in separating ten chromatographic peaks and estimating thirteen TGs, in the present experiment nineteen TGs were separated and estimated for the Fuerte variety and 24 for the Hass variety.

The enhanced resolution achieved with the analytical method employed here is reflected in peaks 2–7, for which Lozano [8] separated only two chromatographic peaks and described three TGs (LLO,

LLP and LOPa), compared with the six peaks and TGs (LLO, LPaO, LLP, PaPaO, LnPP and PaPaP) separated here.

The resolution of peaks 8–12, which has been described previously by other workers, improved considerably in this experiment.

The differences between the TGs in the two avocado varieties considered were probably more quantitative than qualitative, because the chromatograms for the Fuerte variety displayed small traces that could not be quantified. The retention times for these trace peaks were the same as those for peaks 2, 7, 21, 23 and 24 in the Hass variety.

Quantitative analysis

As explained earlier, the response of the light-scattering detector is not dependent on the structure of the component detected but is closely related to the size of solute droplets. Therefore, response factor values should be 1 or close to 1. For TGs, the response should be independent of the number of carbon atoms and the number of unsaturated bonds.

To confirm this for the detection system used, the response factors were first calculated for each of the available pure TGs, simple or mixed, with respect to LLL, which eluted at an intermediate point on the chromatogram for the pure TGs and, moreover, was not detected in the avocado oil. The values so obtained all ranged from 0.976 to 1.043.

Next, the relationship between peak area and amount of sample injected was examined for sample amounts (pure TGs) ranging from 10 to 70 μg under the conditions already described for the system, to establish whether the relationship was linear over a smaller range than that studied by Perrin et al. [17] and Stolyhwo et al. [14]. Such a linear relationship is an esential condition if quantitative analysis is to be carried out using an integrator.

The linearity of the relationship was analysed for all the available pure TGs, simple or mixed; linear relationships were detected in all instances with coefficients of determination ranging from 0.990 to 0.999 and regression coefficients ranging from 23.91 to 25.35. The relationship between the log of the chromatographic peak area and the log of the amount of sample injected was also examined; the analysis yielded a regression coefficient of 1.001 $\pm$ 0.003, which was the value of the exponent in the
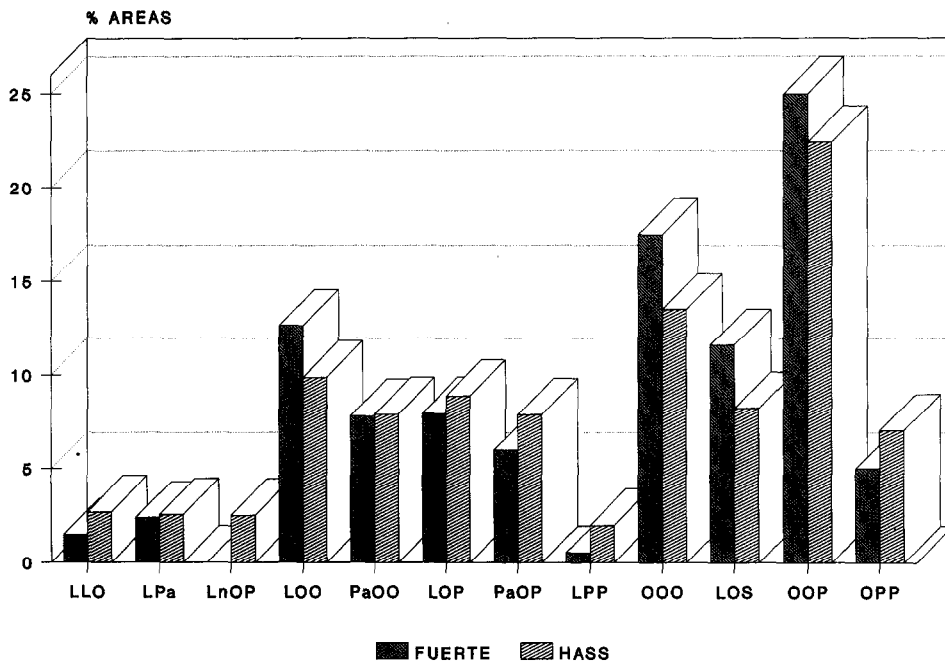
Fig. 2. Composition (%) of the major TGs in avocado oil (varieties Hass and Fuerte).
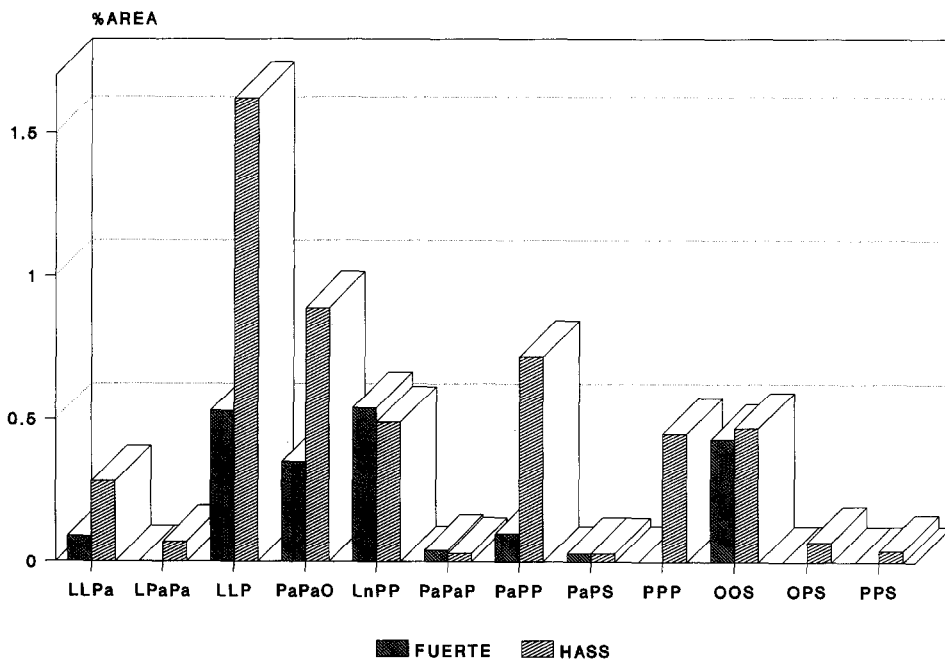

Fig. 3. Composition (%) of the minor TGs in avocado oil (varieties Hass and Fuerte).

equation $A = am^x$ mentioned previously. Thus the response can be assumed to be linear over a small interval of injection amounts, and quantfication of the components using an integrator is therefore possible.

Fig. 2 shows the percentage share of each of the main TGs in the oils of the two avocado varieties considered (Fuerte and Hass).

Fig. 2 shows that the TGs with the highest percentage shares, OOO, LOS, OOP and LOO, were present in higher amounts in the Fuerte variety than in the Hass variety. The rest of the major TGs were similar in the two varieties or lower in the Fuerte variety, whereas the shares of LnOP and PaPP in the Fuerte variety were each less than 0.5%; they were 2 and 7%, respectively, in the Hass variety. OOP was the major TG in both varieties and was present in a proportion of 22.41% in the Hass variety and 24.99% in the Fuerte variety.

Fig. 3 shows the percentages shares of the minor TGs present in the avocado oils from the Fuerte and Hass varieties. In all instances the shares were higher in the Hass variety than in the Fuerte variety, except for the TGs PaPaP and PaPS, which, with values of 0.03 and 0.04%, respectively, were similar in both varieties. In contrast, the shares of the TGs LPaPa, LnOP, PPP, OPS and PPS, which were not present in measurable amounts in the Fuerte variety, ranged between 0.04% for PPS and 2.5% for LnOP in the Hass variety.

Lozano [8] published data on the centesimal composition of major TGs, and the reported values were similar to the values reported here. By way of example, the percentage share values for the major TGs OOO, OOP and OPP reported by Lozano [8] were 18.6, 24.5 and 6.2, respectively, whereas the results recorded in this study were 14.5, 24.9 and 5.0 respectively. For the TGs LOP and PaOO, which Lozano [8] was unable to resolve chromatographically, the reported combined value was 16.2% [8], approximately equal to the sum of the percentages recorded in the present analysis (7.97% for LOP and 7.83% for PaOO), in which both these TGs were resolved.

The differences between the two varieties may have been due either to differing degrees of ripening of the fruit or to varietal differences, two factors which have been considered for various avocado varieties by other workers [4,30].

ABBREVIATIONS

Ca	Caprin
Cl	caprylin
Co	caproin
L	linolein
Ln	linolenin
M	mysristin
O	olein
P	palmitin
Pa	palmitolein
S	stearin

ACKNOWLEDGEMENTS

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