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Formation of stigmasta-3,5-diene in vegetable oils

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The formation of stigmasta-3,5-diene (STIG) in vegetable oils from beta-sitosterol was investigated. Previously, analytical methods for STIG determination were developed and verified. For virgin olive oil and crude vegetable oils, the usual oil production processes (pressure, centrifuging and solvent extraction) and long term storage did not produce measurable amounts of STIG, except in the case of crude olive pomace oils where small quantities arose as a result of the high temperature applied to the solid residues during the drying operation. The influence on STIG generation of variables affecting the refining processes was studied. Although minor amounts of STIG appeared after only heating the oil, this compound was produced mainly during the bleaching earth treatment. The decoloration temperature and the bleaching earth activity were the most important variables involved in STIG formation. After deodorising, carried out under normal conditions, the refined olive oils retained measurable amounts of STIG. The refining of other vegetable oils with high beta-sitosterol content (such as sunflower, rapeseed and soya oils) also rendered considerable amounts of STIG. These results support the method based on STIG determination for detecting low percentages of refined vegetable oils in virgin olive oils and crude seed oils.

INTRODUCTION

In a review article, Kochhar (1983) reports the results of Niewiadomski and Sawicki indicating that the refining process of vegetable oils produces steroidal hydrocarbons which can be used to establish whether an edible oil is crude or refined. One of these, stigmasta-3,5-diene (STIG), is found as a reaction by-product in an experiment on autoxidation of beta-sitosterol at high temperature (Yanishlieva *et al.*, 1980). Later, Lanzón *et al.* (1989) identified STIG in refined olive oils as a dehydration product of beta-sitosterol, not finding this hydrocarbon in virgin olive oils. Consequently, these authors propose STIG determination as a method to detect refined olive oils in virgin olive oils, and describe an analytical procedure including oil saponification, fractionating the unsaponifiable matter on a silica gel column, and gas chromatographic analysis. Recently, Fascioli *et al.* (1992) using a modified analytical method (substituting the fractionation on column by a TLC separation) analysed 20 commercial olive oil samples and claimed that the method was useless for the detection of refined oils in virgin olive oils, since they state that STIG is found in four samples of oxidized virgin olive oils.

The purpose of this work has been to investigate the formation of stigmasta-3,5-diene during the different processes of producing and refining vegetable oils and to know the influence in the operating parameters on STIG generation, in order to confirm the usefulness of the method for refined oil detection. A simple analytical procedure for refined oils has been developed, and laboratory and industrial experiments have been performed.

MATERIALS AND METHODS

Apparatus

A glass chromatographic column (1·5 cm i.d. × 50 cm length) was filled with a slurry of 15 g of silica gel 60,

70–230 mesh, (Merck, Darmstadt, Germany, Ref. 7734) in 40 ml of hexane, with the help of hexane portions.

A UV-spectrophotometer, Uvikon 810 (Kontron, Milan, Italy) was used with a quartz cell of 1-cm path length. For oil oxidation, a Rancimat 679 (Metrohm, Hersau, Switzerland) was used. A gas chromatograph, HP-5890 (Hewlett-Packard, Avondale, USA), equipped with a flame ionization detector and a split injector for capillary columns was used.

Methods

Storage experiments

Oils from three olive varieties (Marteña, Picudo y Picual), each in two ripeness conditions, and each type processed by press and centrifuge, were bottled in glass and PVC 1-litre bottles and then stored under constant illumination (1,000 lux) for 12 months, maintaining a temperature of 28 ± 1°C.

Sterol dehydration

Sterols, obtained from 5 g of olive oil following the EEC method (EEC Commission Regulation 2568/91, 1991), were treated with 1 ml of sulphuric acid–water 1 : 1 at 60°C for 10 min. When cold, the mixture was extracted with two 5-ml portions of hexane. In the evaporated extracts, the hydrocarbons were estimated by gas chromatography.

Laboratory decoloration

Mixtures of oil (50 g) and the bleaching agent were stirred and heated in a thermostatic oil bath (± 1°C). After cooling, the mixtures were filtered through filter paper and the oils analysed for hydrocarbons. The decoloration intensity was evaluated comparing the oil colours by the naked eye.

Laboratory deodorizing

This was performed in a glass apparatus by passing steam through the oil (1 litre) heated at 260°C under a reduced pressure of 0·13 kPa for 2 h.

Industrial deodorizing

The processes took place in a discontinuous system on 10-t batches of olive oil, passing steam (25 kg/t) or nitrogen (5 m^3/t) for 5 h at 1·33 kPa pressure.

Analytical methods

Measurements of UV-absorption at 270 nm, acidity and trans-fatty acid isomers were performed following the EEC methods (EEC Commission Regulation 2568/91 and 1429/92, 1991 and 1992).

Standard method for stigmasta-3,5-diene determination (applied to virgin and crude oil analysis)

A mixture of 20 g of oil, 1 ml of a standard solution of cholestadiene (20 μg/ml, SIGMA, St Louis, MO, USA) and 75 ml of 10% alcoholic potash was heated to gentle boiling for 30 min. The heating was interrupted, 100 ml of water added and the solution transferred to a decanting funnel with the aid of 100 ml of hexane. The mixture was shaken vigorously for 30 s and left to separate. The lower aqueous phase was transferred to a second funnel and extracted again with 100 ml of hexane. The combined hexane extracts were washed with several 100-ml volumes of ethanol–water, 1 : 1, until neutral pH. The solution was passed through anhydrous sodium sulphate and evaporated in a rotating evaporator at 30°C to dryness. With the aid of two 1-ml portions of hexane, the residue was taken to a silica gel column and chromatographic elution started with hexane at a flow rate of 1 ml/min. The first 30-ml fraction was discarded and the following 40-ml fraction was collected and evaporated at 30°C under reduced pressure. The residue was immediately dissolved in 0·2 ml of hexane and the solution kept in the refrigerator. Chromatographic analysis was done on a fused silica capillary column (0·25 mm i.d. by 25 m length) coated with 5%-phenyl methyl silicone phase (0·25 μm thickness) using helium as gas carrier at 120 kPa pressure and a split ratio of 20 : 1. The oven programming temperature was 235°C for 6 min, then rising at 2°C/min up to 285°C, remaining there for 10 min. The injector and detector temperatures were 300 and 320°C respectively.

Simple method for stigmasta-3,5-diene determination (applied to oils with high content of this hydrocarbon, that is, refined oils and their mixtures with virgin oils)

The oil sample (1 ± 0·01 g) was mixed with 1 ml of a standard solution of cholestadiene (see standard method) and the solution taken to the chromatographic silica gel column with the aid of the two 1-ml portions of hexane. The fractionation was started with hexane at a flow rate of 1 ml/min. The first 30-ml fraction was discarded and the following 40-ml fraction was collected and evaporated at 30°C under reduced pressure. The residue was dissolved in 0·2 ml of hexane and kept in the refrigerator until the GC analysis, which was performed as indicated in the standard method.

Samples

Virgin olive oil samples were collected directly from oil mills, except two very old oils taken from storage tanks. The origin of the samples was as follows: about 250 oils produced from crops 87/88 and 88/89 in various Spanish regions (Badajoz, Cáceres, Castellón, Córdoba, Granada, Huelva, Jaen, Lérida, Málaga, Seville, Tarragona, Teruel and Toledo), including oils obtained by press and centrifuge with and without the help of talc, and oils of very different quality (edible and lampant); Greek edible oils (11 samples), crop 91/92, from Heraklion, Ierapetra and Hania regions; Tunisian lampant oils (four samples), crop 91/92, from Tunis and Sfax regions; finally, two samples of lampant oils, crops 78/79 and 79/80, stored in iron tanks at

ambient temperature (ranging from 4 to 40°C) for 9 and 8 years respectively.

Crude olive pomace oils (20 samples) were collected from five extraction factories. In addition, four samples were obtained by hexane Soxhlet extraction of two olive pomaces before and after the drying process occurring in the extraction plant.

Normal and high oleic varieties of crude sunflower oils, each obtained by pressure and solvent extraction, were taken from oil factories.

Refined olive oils (50 samples) were gathered from several refineries using chemical and physical refining processes. Refined olive pomace oils (20 samples) and refined seed oils (12 samples of sunflower, rapeseed and soya oils) were also collected from several refining factories.

All samples were stored at 4°C and kept away from the light until analysis

RESULTS AND DISCUSSION

Assessment of the analytical methods

To determine STIG in virgin olive and crude seed oils, a modification of the method described by Lanzón *et al.* (1989), based on a reduction of the saponification time and the use of capillary gas chromatography, was adopted. Using this procedure, a detection limit lower than 0·01 mg/kg was achieved, whereas by the Fascioli method, only STIG amounts greater than 0·1 mg/kg could be detected.

In oils with high STIG content, the sample weight had to be reduced from 20 to 1 g to achieve an appropriate STIG–internal standard ratio. As STIG remains in the oil as a free compound, the saponification step was omitted and a small oil sample (1 g or less) was taken directly to the fractionating column. This makes a very simple analytical method that was applied to refined oils.

Standard and simple methods showed satisfactory repeatability and equivalence (Table 1).

Virgin olive oils

In all the virgin olive oils analysed, representative of the majority of Spanish olive oil types, the STIG concentration was lower than 0·01 mg/kg (Fig. 1). This indicates that STIG formation is negligible during the oil production processes (by press or centrifuge, with or without talc addition), regardless of olive variety, olive growing conditions or climatic variables.

To assess the effect of storage on STIG content, 26 virgin olive oil samples, including oils obtained from olives of different varieties and ripeness by pressure and centrifugation processes, were exposed to constant illumination for 12 months. At the end of this period, K270 absorption increased from 0·11 to 0·32 approximately but the STIG content remained negligible. Moreover, two virgin oils stored in iron tanks at atmospheric temperature (ranging from 4 to 40°C) for 9 and 8 years showed an acidity of 12·9% and 10·5% and K270 of 0·60 and 0·58 respectively, but no STIG was detected. These results demonstrate that no STIG is produced throughout long storage, even under poor conservation conditions causing oxidation and hydrolysis.

No STIG was formed from virgin oil submitted to oxidation at 98°C in a Rancimat apparatus for 31 h during which time the oil became very rancid. These results are in agreement with the non-detection of STIG after the oxidation of solid beta-sitosterol exposed at atmospheric conditions for 6 months (Yanishlieva *et al.* (1980). On the other hand, STIG was detected when oxidation was performed at 150°C for 1 h, due to a temperature effect that is discussed below. These facts suggest that the STIG found by Fascioli *et al.* (1992) in four reputedly virgin oils stored in poor conditions (which are not described) must be due to the non-genuineness of the oils or to unusual treatments.

Filtrations of virgin oils at 50°C through filter paper and diatomaceous earth did not produce STIG.

Testing of the industrial bottling process in several factories showed no STIG changes in two of them and small increases (0·06, 0·08 and 0·14 mg/kg) in another three. Study of these latter cases demonstrates that the

Table 1. Repeatability of stigmasta-3,5-diene (STIG) determination. Data in mg/kg. Samples A, B and C are mixtures of virgin and refined olive oils 99·75 : 0·25, 99 : 1 and 90 : 10, respectively. Sample D is a refined olive oil.

Sample weight (g)	Assay					$\bar{x}$	RSD%	r(95%)
	1	2	3	4	5			
Standard method								
A (20)	0·026	0·022	0·022	0·022	0·021	0·022	8·6	0·005
B (20)	0·067	0·069	0·060	0·060	0·064	0·064	6·3	0·011
C (20)	0·568	0·588	0·566	0·577	0·582	0·576	1·6	0·026
D (1)	15·5	14·6	14·7	15·6	15·8	15·24	3·6	1·53
Simple method								
D (1)	14·2	14·8	15·3	14·6	15·2	14·82	3·0	1·25

$\bar{x}$ = mean
RSD = relative standard deviation
r(95%) = repeatability value at a significance level α = 5%;
= $t_{0.05,\ \nu=4}$ (s); (t = value of Student's distribution; s = standard deviation).

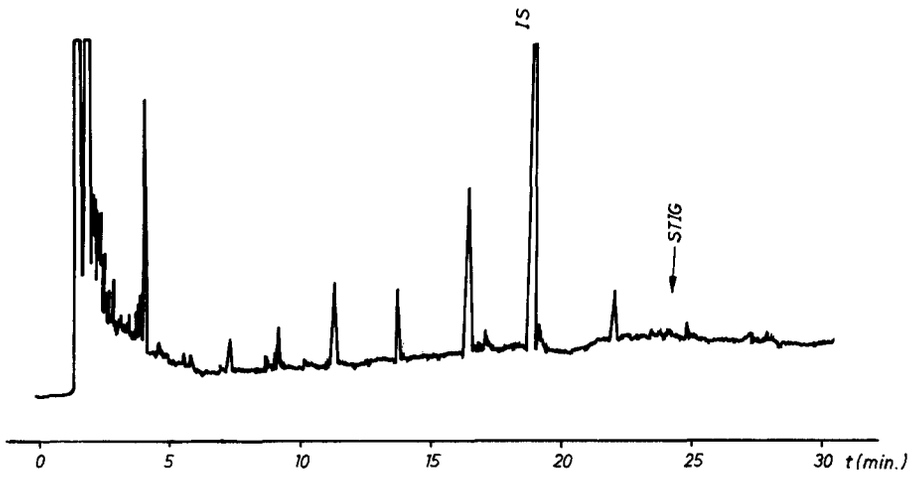

Fig. 1. Gas chromatogram of the steroidal hydrocarbon fraction obtained from a virgin olive oil using the standard analytical method.

appearance of STIG was due to contamination with refined oils, either in bulk transport from the mill or during handling in the factory (residues in storage tanks, filters and pipes).

Crude olive pomace oils

In the extraction plants, the humid solid residues from olive milling are dried in a flow of hot air before proceeding to the hexane extraction. The Soxhlet extraction of solid residues before and after the drying process produced oils with negligible STIG content in the former case and with levels near 0·35 mg/kg in the latter, indicating that high temperatures favour STIG formation. In fact, the STIG values found in crude olive pomace oils obtained in several factories were in the 0·3–0·9 mg/kg range.

Crude sunflower oils

Normal and high oleic sunflower oils, obtained by pressure and hexane extraction, showed a STIG content lower than 0·01 mg/kg (Fig. 2), corroborating that operations carried out at temperatures below 70°C (approximate hexane boiling point) do not yield STIG.

Sterol degradation

Sulphuric acid treatment of the sterols isolated from an olive oil (85% of beta-sitosterol) produced a main degradation product (95%) with the same chromatographic retention time and mass spectrum as the stigmasta-3,5-diene detected in refined olive oils (Lanzón *et al.*, 1989). this confirms that this hydrocarbon arises from beta-sitosterol by dehydration.

Refining processes

The wide range of STIG concentrations (2–100 mg/kg) found in commercial refined olive oils prompted investigation of the effect on STIG generation of the various refining steps, by means of laboratory and industrial experiments and using the simple analytical method for STIG evaluation (Fig. 3).

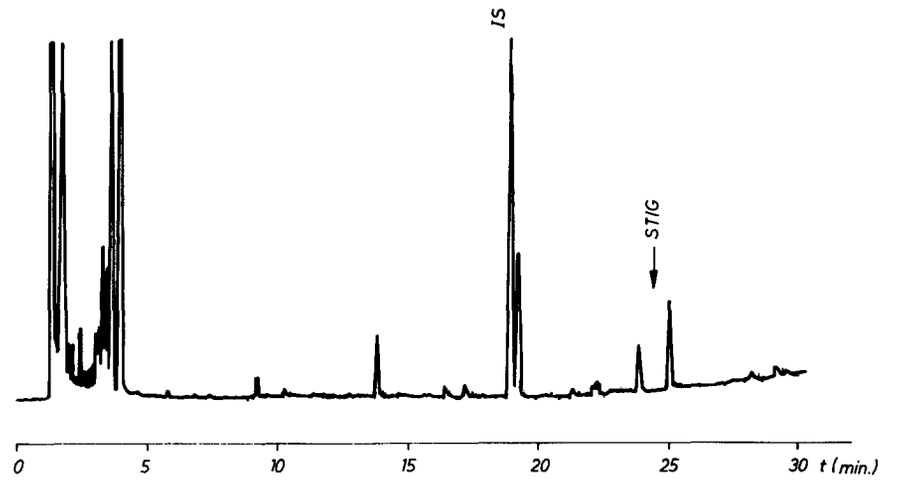

Fig. 2. Gas chromatogram of the steroidal hydrocarbon fraction obtained from a crude high oleic sunflower oil using the standard analytical method.

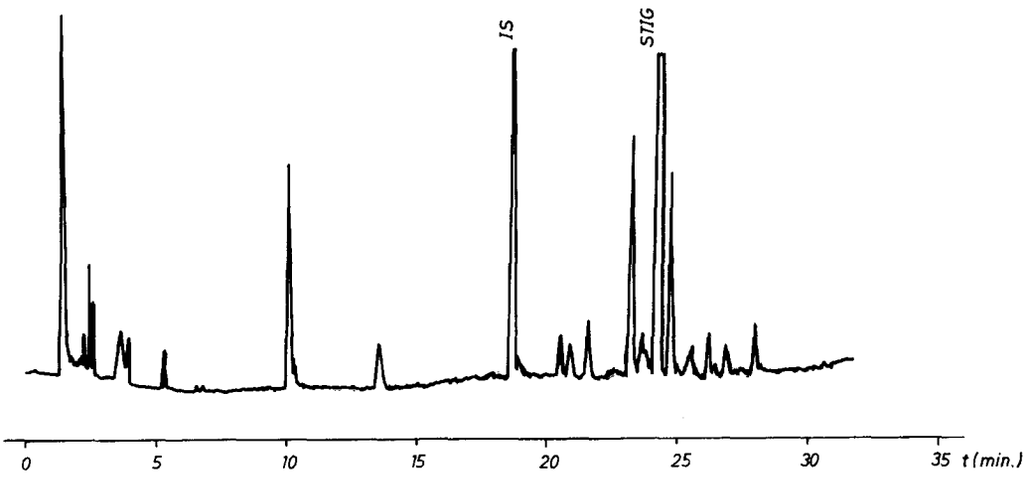

Fig. 3. Gas chromatogram of the steroidal hydrocarbon fraction obtained from a refined olive oil using the simple analytical method.

Table 2. Effect of bleaching agent type on stigmasta-3,5-diene formation and K270 absorption, (virgin olive oil heated with 2% of bleaching agent at 80°C for 15 min).

Decoloration intensity	Bleaching agent	STIG (mg)	K270
	None	<0·010	0·19
+	Activated charcoal	0·012	0·20
+	Trisyl	0·028	0·21
++	Benesa	0·066	0·27
+++	Gador-C	0·189	0·31
++++	Filtrol-105	0·339	0·41
++++	Optima-D	1·085	0·42
+++++	Tonsyl	7·563	0·44

Effect of decoloration

To study the influence of decoloration, the type and rate of bleaching agent, temperature and time were tested with laboratory experiments performed on a virgin olive oil with less than 0·01 mg/kg of STIG and 0·19 of K270. The K270 was chosen with regard to its connection with the refining intensity.

Neutralization with sodium hydroxide (at 70°C for 10 min) produced minimal quantities of STIG (0·013 mg/kg), a decrease of K270 (0·15) and a small loss of colour.

Treatment with bleaching agents, such as activated charcoal and activated bleaching earths, yielded amounts of STIG related to the decoloration intensity (Table 2). The highest STIG increases were due to those earths producing the most effective decoloration. The different STIG levels found in oils showing similar UV absorption and the wide range of STIG concentrations suggest that the intensity of treatment can be better assessed by STIG content than by K270.

With regard to the bleaching earth concentration, the formation of STIG increased in a nearly linear relationship for each earth, the effect being greater with increasing bleaching power of the earth (Fig. 4).

The effect of temperature was minimal at ambient temperature but considerable above 80°C, mainly in the case of the most active earth (Table 3). For both assayed earths (Tonsyl and Gador-C), linear relationships (correlation coefficients 0·9964 and 0·9977) with very similar slopes (19·6 and 20·3) were found between log [STIG] and temperature (Fig. 5), indicating a very strong temperature effect greater for the more active earth. In contrast, only a slight effect was produced on increasing the decoloration time (Table 4).

Table 3. Effect of decoloration temperature on stigmasta-3,5-diene formation, (virgin olive oil heated with 1% of bleaching earth for 15 min).

Gador-C		Tonsyl	
Temperature (°C)	Stig (mg/kg)	Temperature (°C)	STIG (mg/kg)
33	<0·010	33	0·011
60	0·016	60	0·211
80	0·117	80	1·48
100	1·71	100	23·7

Effect of deodorizing

Heating of a virgin olive oil for 15 min under atmospheric pressure at temperatures of 100°C and 150°C did not originate STIG but heating at 175°C and 200°C rendered 0·025 and 0·076 mg/kg STIG respectively. This temperature effect was confirmed by laboratory steam deodorizing at 260°C under 0·13 kPa pressure, where 1·53 mg/kg STIG was found at the end of a 2-h period. STIG formation by the effect of temperature alone explained the appearance of STIG in the oxida-

Table 4. Effect of decoloration time on stigmasta-3,5-diene formation, (virgin olive oil heated with 1% of bleaching earth at 80°C).

Gador-C		Tonsyl	
Time (min)	STIG (mg/kg)	Time (min)	STIG (mg/kg)
15	0·117	15	1·48
30	0·193	30	1·77

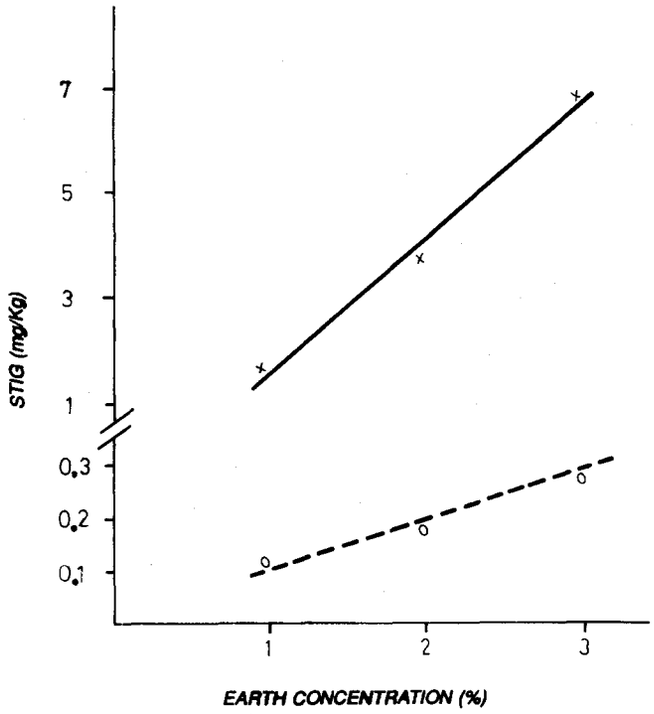

Fig. 4. Variation of the stigmasta-3,5-diene concentration according to the bleaching earth rate in an olive oil. Decoloration at 80°C during 15 min, (o, Gador-C; x, Tonsyl).

tion of beta-sitosterol only when a high temperature (150°C) was applied (Yanishlieva *et al.*, 1980).

To verify the industrial process, two batches of a bleached olive oil containing 50 mg/kg of STIG were submitted to deodorizing in an industrial plant at 245°C for 5 h using steam and nitrogen as stripping gases. STIG fell to 34 and 24 mg/kg respectively, suggesting STIG removal by distillation. In order to determine the extent of this elimination, a bleached oil

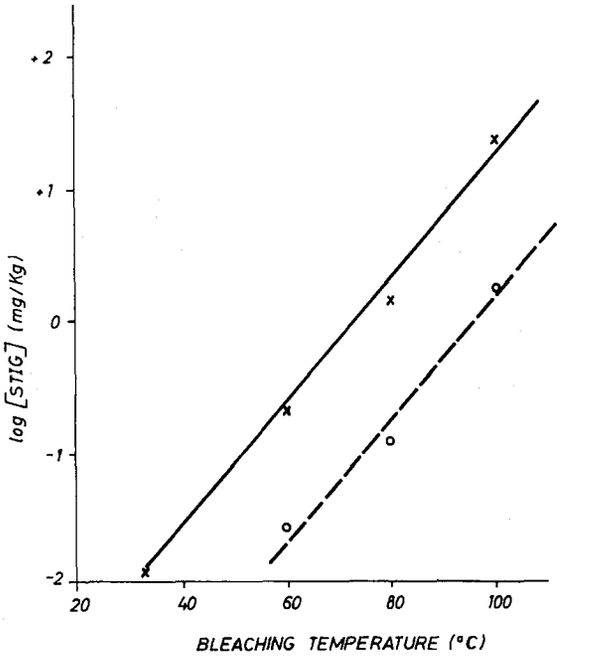

Fig. 5. Variation of the stigmasta-3,5-diene concentration according to the bleaching temperature in an olive oil. Decoloration with 1% of earth during 15 min, (o, Gador-C; x, Tonsyl).

with low STIG level (3 mg/kg) and usual content of C18 : 1, C18 : 2 and C18 : 3 trans-isomers of fatty acids (0·04%, 0·02% and 0·00% respectively) was deodorized under the most extreme conditions applied in the refineries (with steam at 260°C for 6 h under 1·33 kPa pressure). The refined oil showed an increase of STIG content (4·1 mg/kg), and trans-isomer percentages (0·12%, 0·42% and 0·22%) exceeding the maximum official limits (EEC Commission Regulation 1429/92, 1992). These results suggest that the STIG eliminated by distillation is compensated in excess by that generated by heating, and that the attempt to remove STIG during the deodorizing process leads to very high trans-isomer values due to the application of high temperatures for long times (Mariani *et al.*, 1991).

Effect of some treatments on refined olive oils

The addition of maleic anhydride to olive oils is a manipulation for reducing the K270 absorption, based on the Diels–Alder cyclo-addition to conjugated double bonds. STIG contains a *cis–trans* conjugated double bond that cannot react with the anhydride because the reaction is *cis–cis* stereo specific. In spite of this, an experiment using this substance was carried out. The STIG concentration (7·7 mg/kg) of a refined oil remained unchanged by treatment with 0·2% of maleic anhydride at 60°C for 15 min.

To test the possible sequestrant effect of activated charcoal on STIG, 0·2% of this agent was added to Tonsyl earth in a bleaching experiment done with 1% of the bleaching mixture at 80°C for 15 min. The oil obtained showed a STIG concentration (1·6 mg/kg) similar to that (1·5 mg/kg) of the oil refined with only the earth. In the treatment of the refined oil with 0·1% of activated charcoal, no STIG decrease was observed.

Commercial refined olive oils

The above results indicate that decoloration is the step yielding highest amounts of STIG, and explain the considerable differences in STIG content found in the commercial oils. In chemical refining, a partial decoloration without STIG production, achieved by neutralization with sodium hydroxide, is followed by a mild bleaching earth treatment which produces a low STIG concentration. On the other hand, in physical refining, a single decoloration is performed by means of a strong bleaching earth process yielding considerable amounts of STIG. This is consistent with STIG data of oils obtained in various Spanish refineries, working under usual operating conditions, which range between 2 and 10 mg/kg for chemically refined oils and between 15 and 45 mg/kg for physically refined ones.

Refined olive pomace oils

Crude olive pomace oils usually have an undesirable intense colour and consequently their refining requires

a strong decoloration process which yields oils with very high STIG content. Examination of 20 samples from five Spanish refineries showed a very wide STIG concentration range (7–200 mg/kg), even in the same factory, although most values (14 samples) were under 120 mg/kg.

Refined seed oils

Most common vegetable oils, except cocoa, palm, palmseed and teaseed oils, show sterol contents (1400-20000 mg/kg) similar to or greater than olive oil (about 1400 mg/kg), beta-sitosterol being the main component (40–89%) (Homberg and Bielefeld, 1989). Therefore, STIG will be produced in the refining process if bleaching treatments or high temperatures are applied. As expected, laboratory bleaching of normal and high oleic sunflower oils with 1% of Tonsyl earth at 80°C for 15 min yielded oils with abundant STIG (6·0 and 5·6 mg/kg, respectively). Analysis of sunflower, rapeseed and soya oils obtained in refining plants showed the presence of STIG concentrations between 2 and 85 mg/kg.

CONCLUSIONS

Stigmasta-3,5-diene, a hydrocarbon derived from beta-sitosterol by dehydration, is found in refined vegetable oils as a consequence of the refining process. Low concentrations (0·01–2·0 mg/kg) of this compound can be determined by a method involving saponification, fractionation on silica gel column and capillary GC analysis. For concentrations greater than 2 mg/kg a simple method omitting the saponification step is presented.

In virgin olive oil and crude seed oils, STIG is absent (less than 0·01 mg/kg) irrespective of the olive's origin and the production processes, provided that the usual treatments and temperatures below 100°C are applied. In the case of crude olive pomace oils, low STIG concentrations (0·3–0·5 mg/kg) arise from heating the solid residue during the drying step. Long term storage, even under extreme illumination, oxidation and temperature conditions, does not produce STIG. Consequently, the STIG found in some commercial oils labelled 'virgin' is due to manipulation or accidental contamination with refined oils.

STIG appears mainly from the action of bleaching earth, with earth activity and decoloration temperature being the most influential variables. In the deodorizing step, small amounts are produced by heating alone at temperatures over 150°C, although losses by distillation are also observed. However, noticeable amounts of STIG remain under the usual deodorizing conditions. If drastic deodorizing is applied in an attempt towards greater STIG removal, an increase in the trans-fatty acid isomers is observed. Under the usual refining conditions, STIG concentrations in refined olive oils lie in the range 2–45 mg/kg.

The results confirm that STIG detection is a usual procedure for establishing the presence of small amounts of refined vegetable oils (lower than 1% in the worst case) in virgin olive and crude seed oils, whereas trans-fatty acid determination is a complementary method for detecting strongly deodorized oils.

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