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USE OF INNOVATIVE ANALYTICAL METHODOLOGIES TO BETTER ASSESS THE QUALITY OF EDIBLE VEGETABLE FATS AND OILS

Francesco Caponio*, Tommaso Gomes, Antonella Pasqualone

Dipartimento di Progettazione e Gestione dei Sistemi Agro-Zootecnici e Forestali - Sezione di Industrie Agro-Alimentari, Università di Bari, Bari, Italy

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An experimental investigation was conducted to test the use of non-conventional analyses for determining the actual level of oxidative and hydrolytic degradation of edible oils and fats. For this purpose three extra-virgin olive oils, three refined oils recovered from in-oil preserves, and three refined fats extracted from margarines were considered. Besides the determination of the free fatty acids, the peroxide value, the spectrophotometric constants, the fatty acid composition, and the *trans* isomers content – prescribed by the EC Regulations – the high performance size-exclusion chromatography of polar compounds – separated from oils and fats by silica gel column chromatography following an IUPAC method – was carried out to separate and quantify the main classes of oxidation and hydrolysis substances.

The results obtained show that the routine analyses can enable an effective measurement of quality only for extra-virgin olive oils. On the other hand, for determining the actual degree of the oxidative and hydrolytic degradation of oils and fats subjected to treatments requiring high temperatures (refining, hydrogenation, *etc*.) the HPSEC (High Performance Size Exclusion Chromatography) analysis of polar compounds can be successfully used. The determination of *trans* isomers contributes to the assessment of the quality of different oils.

INTRODUCTION

The use of edible fats has experienced, over the last decades, deep changes subsequent to nutritional, commercial, economic and technological factors. The main issue of these changes has been the gradual replacement of animal lipids by vegetable lipids in the diet as a mean to suppress cholesterol, harmful for consumers' arteries, and to increase the level of unsaturated fatty acids to the detriment of saturated ones. Vegetable oils have the following main applications: 1) as a seasoning; 2) as an ingredient in the preparation of many first and main courses; 3) as a liquid medium for in-oil preserved food; and 4) as a major constituent in margarine preparation. In the Mediterranean countries, extra-virgin olive oils (*i.e.* oils obtained from olives by only mechanical extraction) are used for the first two purposes, while mostly refined oils are employed for the two last applications. The analytical methodologies used to assess oil quality are those prescribed by EC Regulation N° 2568/91 and its subsequent modifications and integrations. Nevertheless, while these determinations can enable an effective measurement of hydrolytic and oxidative degradation of virgin oils, they cannot apply the same for refined oils. During oil refining, the fatty acids are removed and hydroperoxides are degraded and/or transformed; therefore, their determination can only offer indications about the neutralisation or the resumption of the oxidation process. Moreover, it is well-known that the values of spectrophotometric constants (K_{232} and K_{270}) are a function of the applied refining conditions [Amati *et al.*, 1978; Tiscornia *et al.*, 1982].

Much more difficult is the assessment of the genuineness and quality of the same oils when they are used both as liquid media for preserving food and as a major constituent in the production cycle of food preparations (*e.g.* margarine, bouillon and flavouring cubes). Actually, it is well-known that in these cases the traditional analytical parameters employed to assess the quality of lipids lose much of their significance at the high temperatures required in some phases of the product processing or during the preservation of food preparations.

As to preserved fish and vegetables, the literature reports that during preservation one can observe both a reduction in the number of peroxides and an increase in both the level of free fatty acids and spectrophotometric indices; these values were in some cases higher than those prescribed by law for the marketing of olive oils [Bocca *et al.*, 1990; Paganuzzi *et al.*, 1995]. Moreover, it is also known that the contact between oil and food causes a transfer of the main constitutents from the food to the oil used as medium; this is especially noteworthy to preserved fish. Different authors have concluded that the *trans* isomers analysis is the sole technique that one can use to assess the genuineness of oils employed as liquid media for preserving foods [Bizzozero & Carnelli, 1996; Mucciarella & Marsilio, 1997]. It is equally difficult to assess the quality of margarines' lipid fraction, as it is represented by refined fats, which are often hardened by hydrogenation.

Hence, the purpose of this work was to investigate the possibility of employing non-conventional assays to provide a more reliable and realistic assessment of the hydrolytic and oxidative status of edible fats and oils.

*Author's address for correspondnece: Francesco Caponio, University of Bari, Dept. PRO.GE.S.A., Sezione di Industrie Agro-Alimentari,Via Amendola, 165/A, I – 70126, Bari, Italy; tel.: (39 080) 544 22 35; fax: (39 080) 544 29 39; e-mail: francesco.caponio@agr.uniba.it

MATERIALS AND METHODS

For this study several samples were purchased from different supermarkets, including three extra-virgin olive oils; three refined oils recovered from in-oil preserves; and three samples of margarine. Upon arrival in the laboratory, the oil used as a liquid medium to preservation was separated from foods by filtration on anhydrous sodium sulphate, whereas the samples of margarines were warmed up to 60°C in a vacuum separatory funnel and the lipid fraction was filtered over anhydrous sodium sulphate. All samples for investigation were stored in a freezer at -18°C until analysed. Besides the determination of the free fatty acids (FFAs) content, the peroxide value (PV), the spectrophotometric constants, the fatty acid composition, and the *trans* isomers content – as provided by EC Regulations – the polar compounds (PCs) were separated from oils and fats by silica gel column chromatography following an IUPAC method [IUPAC, 1987]. The PCs were subsequently analysed by a high performance size-exclusion chromatography (HPSEC) technique: the main classes of substances of oxidation and hydrolysis of the constituent triacylglycerols, such as triacylglycerol oligopolymers (TGPs), oxidised triacylglycerols (ox-TGs), diacylglycerols (DGs) were separated and quantified.

Trans isomers were determined after methylation of the fatty acids, as described in EC Regulation N° 2568/91 [Official Journal European Communities, 1991], following the indications of EC Regulation N° 1429/92 [Official Journal European Communities, 1992] and using a chromatographic system composed of a Fisons high retention gas chromatograph (HRGC) (Milan, Italy) with a SP™ 2340 fused-silica capillary column, film thickness 0.20 µm, 60 m length × 0.25 mm i.d., from Supelco (Bellefonte, PA, USA). The carrier gas was hydrogen; the temperature of the split injector was 210°C with a split ratio of 1:100; the temperature of the flame ionisation detector was 220°C. The oven temperature was programmed from 160 to 200°C with increments of 1.3°C/min and a final isothermal of 15 min.

The analysis of fatty acid methyl esters was performed using a Fisons HRGC (Mega 2 series) with a flame-ionisation detector and equipped with a WCOT fused-silica capillary column, FFAP-CB coating, film thickness 0.30 µm, 25 m in length × 0.32 mm i.d. from Chrompack (Middleburg, The Netherlands). Oven temperature was isothermal at 180°C, while the temperature of the split-splitless injector was 270°C with a splitting ratio of 1:17 and detector temperature of 300°C. The carrier gas was hydrogen at a flow rate of 2 mL/min. The accuracy of the method, expressed as CV%, was about 5% for each gas-chromatographic determination.

The PCs, separated by silica gel column chromatography following the IUPAC method, were recovered in CH_2Cl_2 and analysed by means of an HPSEC system, which consisted of a series 10 pump (Perkin-Elmer, Norwalk, CT, USA) with a Rheodyne injector, a 50-µL loop, a PL-gel guard column (Perkin-Elmer, Beaconsfield, UK) of 5 cm length × 7.5 mm i.d., and a series of three PL-gel columns (Perkin-Elmer, Beaconsfield, UK) of 30 cm length × 7.5 mm i.d. each. The columns were packed with highly cross-linked styrene-divinylbenzene copolymer with particles of 5 µm and a pore diameter of 500, 500 and 100 Å, respectively. The detector was a deflection-type differential refractometer (RID 6A, Shimadzu, Osaka, Japan) connected to an integrator. Dichloromethane was utilised as eluant at a flow rate of 1 mL/min. Peaks on the chromatograms were identified by polystyrene standards (Supelco, Milan, Italy) of known molecular weight (MW = 4000 and 2000 g/mol) as well as tristeartin, distearin, and monostearin standards (Sigma-Aldrich, St. Louis, MO, USA). For each standard, the elution volume was measured under the same conditions as used in our analysis. The log of MW, as a function of elution volume, was plotted and the line of best fit was plotted by the least square method. From the elution volume of each separated peak in a chromatogram, the corresponding MW could then be obtained [Gomes, 1992]. Known amounts of TGPs, ox-TGs, and DGs were obtained by preparative gel permeation chromatography of PCs derived from a refined peanut oil and then used as standards in the HPSEC method. The amount collected for each standard, corresponding to a given class of compounds, was used to prepare a stock solution in CH_2Cl_2 and a solution containing different concentrations after successive dilutions. These solutions were analyzed by HPSEC following the analytical method developed. The calibration curves were obtained by plotting the amounts of standards (µg) that had been injected into the HPSEC system loop against the areas of the corresponding chromatogram peaks [Gomes & Caponio, 1999]. In order to identify the free sterols and triterpene diols, the unsaponifiable matter of an olive oil was fractionated by TLC. The bands corresponding to sterols and triterpene diols were recovered in CH_2Cl_2 and analysed by HPSEC analysis: these substances were eluted together as one peak before the FFAs peak. Afterwards, the HRGC analysis of these bands showed a typical gas chromatogram of sterols and triterpene diols of the olive oil [Gomes & Caponio, 1996]. The FFAs were identified by a standard of oleic acid but the quantitative determination was carried out by acid-base titration [Official Journal European Communities, 1991]. The accuracy of the method, expressed as CV%, was 2.1% for PCs, 1.6% for TGPs; 1.8% for ox-TGs, and 1.7% for DGs.

RESULTS AND DISCUSSION

Table 1 shows the values of the fatty acid composition of tested samples. For extra-virgin olive oil samples all values determined comply with those prescribed by law for the marketing of these oils [Official Journal European Communities, 1991]; oleic acid is contained in the highest amount (71.84-79.58% range). As to the oils used as liquid media for preserving food, instead, the results obtained reveal in some cases the presence of highly unsaturated fatty acids ($C_{20:4}$, $C_{20:5}$, $C_{22:5}$, $C_{22:6}$) that are typical of fish lipids. This highlights the lipid supply contributed by foods to the liquid medium. Lastly, for margarines, the composition of fatty acids is quite diversified due to both the use of vegetable oils of different botanical origin and of the different hardening processes of fats. The overall results show that the determination of the acid composition is not sufficient to assess the genuineness of edible oils and fats when used as liquid media for preserving food or when subjected to a hardening treatment for producing, for instance, margarines.

TABLE 1. Fatty acid composition of the examined samples.

Fatty acids (%)	Margarines' lipid fraction			Oil used in preserved food			Extra-virgin olive oil		
	Sample n°			Sample n°			Sample n°		
	1	2	3	1	2	3	1	2	3
$C_{8:0}$	0.75	0.02	0.10	-	-	-	-	-	-
$C_{10:0}$	0.42	0.00	0.02	-	-	-	-	-	-
$C_{12:0}$	5.10	0.30	0.40	-	-	-	-	-	-
$C_{14:0}$	2.45	1.06	0.20	0.08	tr	0.09	tr	tr	0.01
$C_{16:0}$	34.05	42.78	31.13	7.83	12.77	10.95	9.36	11.08	14.34
$C_{16:1}$	0.04	0.13	0.10	0.34	0.93	1.07	0.33	0.39	1.72
$C_{17:0}$	0.10	0.10	0.04	0.06	0.05	0.06	0.05	0.04	0.05
$C_{17:1}$	0.00	0.03	0.03	0.07	0.05	0.10	0.09	0.08	0.03
$C_{18:0}$	3.79	6.00	7.10	3.72	2.60	3.17	2.48	1.90	2.04
$C_{18:1}$	31.03	47.04	42.40	35.22	68.55	73.26	79.58	77.29	71.84
$C_{18:2}$	18.68	0.99	12.70	50.89	12.23	6.04	6.49	7.67	8.26
$C_{18:3}$	2.49	0.13	4.50	0.10	0.56	0.58	0.74	0.71	0.61
$C_{20:0}$	0.25	0.41	0.40	0.24	0.39	0.38	0.21	0.17	0.28
$C_{20:1}$	0.12	0.35	0.10	0.18	0.32	0.32	0.19	0.22	0.32
$C_{20:4}$	-	-	-	0.05	0.04	0.02	-	-	-
$C_{20:5}$	-	-	-	0.17	0.05	0.16	-	-	-
$C_{22:0}$	0.25	0.17	0.40	tr	0.01	0.03	0.03	0.03	0.01
$C_{22:5}$	-	-	-	0.02	0.03	0.02	-	-	-
$C_{24:0}$	0.22	0.18	0.10	0.16	0.05	tr	0.05	0.03	0.02
$C_{22:6}$	-	-	-	0.27	0.69	3.11	-	-	-
Others	0.27	0.31	0.28	0.60	0.68	0.64	0.40	0.39	0.48

tr- traces

Table 2 shows the values of the routine analytical determinations obtained for the samples being tested. The determination of the FFAs revealed values always below 10 g/kg, which is the maximum allowable value for the marketing of extra-virgin olive oils [Official Journal European Communities, 1991]. The mean contents are higher in the lipid fraction of margarines (5.1 g/kg) than in extra-virgin olive oils (2.8 g/kg) and in refined oils used as liquid media for preserving food (2.3 g/kg). Nevertheless, this does not mean that extra-virgin olive oils are of the same quality as other oils, since for the latter the observed values are a consequence of the refining process to which they have been subjected. Actually, the FFAs are removed during the neutralisation of crude oil. The determination of the PV showed higher values in extra-virgin olive oils (mean value of 9.0 meq O_2/kg) than in the other oils tested (*i.e.* mean values of 3.0 and 3.5 meq O_2/kg for refined oils used as liquid media for preserving food and for the margarine lipid fraction, respectively). This pattern was largely expected because during refining hydroperoxides are degraded and/or transformed. On the other hand, the spectrophotometric constants at the typical oil wavelengths showed higher values in refined oils than in extra-virgin olive oils. However, for the determination of the level of oxidative degradation of refined fats and oils this determination could be unable to give reliable results.

The determination of the PCs, including all the substances of oxidative and hydrolytic degradation of the fats with a higher polarity than unaltered triacylglycerols, and their subsequent HPSEC analysis may be an effective tool to assess the quality of edible oils and fats. This is because through this technique one can separate and quantify the products of triacylglycerol oxidation and hydrolysis which are stable and cannot be removed by subsequent treatments. Figure 1 depicts the HPSEC chromatograms of the PCs relating to an extra-virgin olive oil (A), a refined vegetable oil used as liquid media for preserving food (B), and the lipid fraction of one of the analysed margarines (C). All chromatograms were obtained by recovering the PCs, separated by silica gel chromatography from 1 g of oil with 25 mL of CH_2Cl_2, and by injecting 50 µL of the obtained solution onto the column. The figure clearly shows that the PCs are markedly reduced in extra-virgin olive oils than in the other tested samples. Moreover, the TGPs were absent in the tested extra-virgin olive oils, but were always present in the lipid fraction of the two other samples being investigated. Through this analysis it is also possible to estimate the triacylglycerol trimers that are classes of substances able to indicate a strong oxidative degradation of oils and fats since they are formed during heat treatments such as frying [Cuesta *et al.*, 1993].

Table 3 shows the values of PCs and the main classes of constituent substances obtained for the tested samples. The PCs were found in higher amounts in margarines' lipid fraction and in refined oils used as liquid media for preserving food, as compared to extra-virgin olive oils, whose contents were even about 65% lower as compared to refined oils. This

TABLE 2. Results of routine analyses of the examined samples.

Samples	FFAs	PV	K_{232}	K_{270}	ΔK
Margarines' lipid fraction					
1	2.8	4.2	4.090	1.579	0.045
2	2.9	3.2	2.889	0.967	0.021
3	1.1	3.0	3.968	1.383	0.057
Mean	2.3	3.5	3.649	1.310	0.041
Oil used in preserved food					
1	2.8	3.1	2.872	0.826	0.053
2	7.1	3.1	2.787	0.967	0.052
3	5.5	2.7	1.996	0.517	0.012
Mean	5.1	3.0	2.552	0.770	0.039
Extra-virgin olive oil					
1	2.1	7.7	1.768	0.180	0.000
2	2.7	9.2	1.769	0.223	0.000
3	3.6	10.1	1.859	0.260	0.000
Mean	2.8	9.0	1.799	0.221	0.000

FFAs - free fatty acids (g/kg oil); PV - peroxide value (meq O_2/ /kg oil); K_{232} - specific absorption at 232 nm; K_{270} - specific absorption at 270 nm; ΔK - measure of the peak height at maximum absorption around 270 nm.

TABLE 3. Polar compounds and classes of the main constituent substances.

Samples	PCs	TGPs	ox-TGs	DGs
Margarines' lipid fraction				
1	63.1	7.6	15.7	35.4
2	69.4	10.5	7.2	46.7
3	55.0	9.1	13.3	28.0
Mean	62.5	9.0	12.1	36.7
Oil used in preserved food				
1	39.2	6.4	7.3	19.7
2	55.0	7.3	8.5	29.9
3	54.3	4.6	79	33.7
Mean	49.5	6.1	7.9	27.8
Extra-virgin olive oil				
1	23.2	nd	3.1	15.6
2	27.1	nd	3.9	18.2
3	30.7	tr	4.9	20.3
Mean	27.0	-	4.0	18.0

PCs - polar compounds (g/kg); TGPs - triacylglycerol oligopolymers (g/kg); ox-TGs - oxidized triacylglcyerol (g/kg); DGs - diacylglycerol (g/kg); nd - not detected; tr - traces.

indicates a more evident overall degradation in the former as compared to the latter. The TGPs – *i.e.* all triacylglycerol polymerisation substances that are formed as a result of the high process temperatures (refining, hydrogenation) – were absent in virgin oils and constantly present in refined oils showing values till 10 g/kg. Even the ox-TGs were found to be higher in margarines' lipid fraction (mean value 12.1 g/kg) and in refined oils used as liquid media for preserving food (mean value 4.0 g/kg). The values determined for TGPs and ox-TGs point out a higher oxidative degradation of refined oils, as compared to virgin ones; their simultaneous determination can thus provide more reliable indications than routine analyses on the actual oxidative degradation of edible oils and fats subjected to refining. The products of triacylglycerol oxidation and polymerisation in high doses have potential harmful effects on human health [Alexander, 1978; Chang *et al.*, 1978]. Lastly, the DGs were determined at levels ranging from 15.6 g/kg to 46.7 g/kg, with values definitely higher in refined than in virgin oils, thereby providing an indication of the actual hydrolytic degradation of oils.

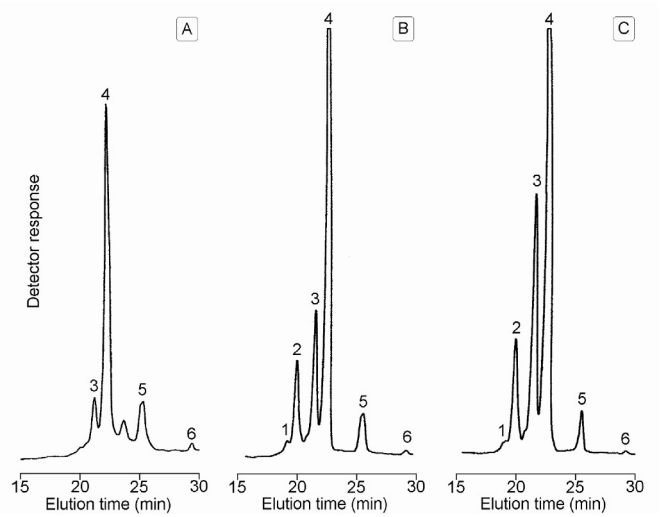

FIGURE 1. HPSEC analysis of polar compounds from extra-virgin olive oil (A); oil used for preserving food (B); and margarines' lipid fraction (C). (1) Triacylglycerol trimers, (2) triacylglycerol dimers, (3) oxidized triacylglycerols, (4) diglycerols, (5) free sterols and triterpene diols, (6) free fatty acids.

Figure 2 shows the mean values of *trans* oleic and *trans* linoleic + *trans* linolenic isomers obtained for the samples of different oils under analysis. They are indeed absent in virgin oils whereas they are always present in the oils submitted to refining and hydrogenation processes. The sharply higher values of *trans* isomers observed in margarines' lipid fraction as compared to the contents determined in refined oils used as liquid media are attributable to the fat hardening process, which is often performed through hydrogenation, thus causing a conspicuous isomerisation of unsaturated fatty acids. Hence, the determination of *trans* isomers contributes to assess the oil quality, given they are supposed to have detrimental effects on consumers' health, as they are responsible for the increase in the LDL/HDL ratio [Valenzuela *et al.*, 1995; Combe *et al.*, 2000].

CONCLUSIONS

Based on the overall results the following conclusions may be drawn:

1. the traditional routine analyses used to assess oils' quality provide reliable results only in virgin olive oils;

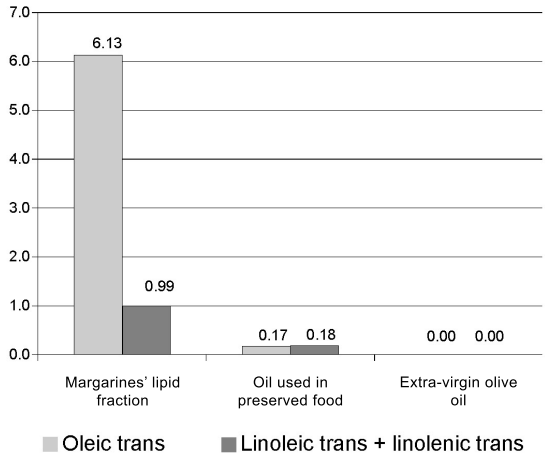

FIGURE 2. Mean values of *trans* isomers determined in different categories of oils tested.

2. the determination of the PCs showed sharply diversified contents in tested samples, with values that were 65% lower in virgin than in refined oils;
3. the TGPs, which are substances formed due to the high process temperatures, were absent in virgin oils and constantly present in refined oils showing values up to 10 g/kg;
4. the ox-TGs and the DGs resulted to be higher in refined oils as compared to the virgin ones;
5. the determination of *trans* isomers aids in assessing oil quality.

In order to determine the actual degree of the oxidative and hydrolytic degradation of oils and fats subjected to treatments necessitating high temperatures (refining, hydrogenation, *etc*.) the HPSEC analysis of PCs can be successfully employed.

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