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Stereospecific Analysis of Triacylglycerols as a Useful Means To Evaluate Genuineness of Pumpkin Seed Oils: Lesson from Virgin Olive Oil Analyses

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In Slovenia two superb vegetable oils with high added nutritional value are produced: "Ekstra deviško oljčno olje Slovenske Istre (extra virgin olive oil from Slovene Istra)" and "Štajersko prekmursko bučno olje (pumpkin seed oil from Slovenia)". Their quality and genuineness must be monitored as adulteration can easily be undertaken. Olive oil genuineness determination experiences can show how analyses following an experience data-driven decision tree gathering several chemical determinations (fatty acids, *(E)*-isomers of fatty acids, sterol and tocopherol determinations) may be helpful in assessing the pumpkin seed oil from Slovenia genuineness. In the present work a set of HPLC triacylglycerol determinations was performed, based on the nine main triacylglycerols (LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL, and SLS) on a limited number of different pumpkin seed oils from northeastern Slovenia. The performed determinations showed that stereospecific analyses of triacylglycerols together with other chemical determinations can be useful in building a protocol for the evaluation of the genuineness of pumpkin seed oil from Slovenia.

KEYWORDS: Chemical assessment; *Cucurbita moschata* D.; *Cucurbita pepo* L.; genuineness; HPLC; principal component analysis; pumpkin seed oil; stereospecific triacylglycerol determination; virgin olive oil

INTRODUCTION

Pumpkin, the New World *Cucurbita* spp., has been known in Europe since the early 16th century (*1*) and soon became appreciated in the Štajerska and Prekmurje regions in northeastern Slovenia (mostly *Cucurbita pepo* L. and to a lesser extent *Cucurbita moschata* D.) as well as in southern Austria as a vegetable and as a source for excellent seed oil pressed from roasted seeds (*2*, *3*). On the other hand, olive has been known in Europe and in Slovenia or better in the part of Slovenia geographically known as the peninsula of Istra since ancient times.

In Slovenia two superb edible vegetable oils with high added nutritional and hedonistic value are produced, extra virgin olive oil from Slovene Istra (*4*) and pumpkin seed oil from the Štajerska and Prekmurje regions (*3*, *5*, *6*). Both oils contain certain pharmacodynamically active substances of non-triacylglycerol origin, mainly phytosterols (*3*, *7*). It was shown that unroasted pumpkin seeds contain dietary lignans, namely, secoisolariciresinol, isolariciresinol, and lariciresinol; however, secoisolariciresinol is destroyed after 20 min of roasting (*8*). Maybe with the pumpkin oils of a new era, the ones "cold" pressed from dry seeds without roasting or chemical treatments (*9*), lignan content may change. In the case of olive drupes dietary lignans are 1-acetoxypinoresinol, pinoresinol, and 1-hydroxypinoresinol (*10*), and some are retained in extra virgin olive oils (*11*). Both oils are known for their hedonistic, cultural, nutritional, and health-promoting aspects (*12*, *13*), which result in a relatively high price if compared to other seed oils. In 2005 the plant oil market in Slovenia was worth >26 million €, with olive oil taking third place with 4 million € and pumpkin seed oil with a 43% price increase for the period 2004−2005 (*14*). With respect to this it is logical to expect adulteration with cheaper seed oils. In the field of olive oil, adulteration can be revealed following the excellent and unique European Commission regulations (*15*, *16*). They give inter alia the methods of analysis, the decision tree for establishing the conformity of the declared type of olive oil with the analyzed parameters, and the limit values needed to assess the characteristics of olive and olive pomace oil.

Seven different and rather tedious analytical procedures from an instrumental point of view must be performed before the sample can be classified as a sample of extra virgin olive oil. All of these procedures are the result of long and cooperative work performed over many years by experts from the olive field and chemical analysts who gathered their knowledge and experiences (*14*).

The present situation in Slovene pumpkin seed oil production is not as strict, and to our best knowledge we are not aware of such

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regulations and procedures one can follow to establish/assess an oil's quality or purity parameters with an exemption of sensorial analyses and some quality determinations. Only genuine pumpkin seed oils can fully preserve the nutritive value the consumer has paid for, and the stereospecific analysis of triacylglycerols can add a value in assessing them. Other not so well-defined parameters are the roasting temperature and roasting time, which greatly influence the pressed roasted pumpkin seed oil aroma and therefore the quality (*17*).

Butinar and co-workers (*14*) tried to establish a procedure to assess a pumpkin seed oil's genuineness, performing a set of analyses described in the olive oil purity decision tree procedure (*15*). These were (*E*)-isomers of fatty acid content, fatty acid content, and composition and quantity of sterols. The tocopherols by normal phase HPLC were determined as well. It was shown that all determinations can lead to a very clear picture of the pumpkin seed's oil genuineness, especially if the final sterol composition is challenged (Δ-5 to Δ-7 sterol ratio). However, sterol composition is the major drawback in genuineness estimation due to complexity of the determination and because of the rather demanding procedure, which can cause problems even for trained sterol analysis personnel.

The efforts of the present work were aimed at another genuineness determination technique widely used in the virgin olive oil purity determination, which is basically a stereospecific analysis of triacylglycerols and is known as a ΔECN 42. It was implemented in 2006 and 2008 (*18*, *19*). The history of the ΔECN 42 determination in olive oils lies in the problem of the olive oils, which were in the past extensively adulterated with admixtures of other extraneous and cheaper seed oils. The method is based on the HPLC triacylglycerol determination. The chromatographic separation follows the equivalent carbon numbers (ECN) rule, where ECN is the number of carbon atoms in a triacylglycerol (not counting the glycerol carbons) minus twice the number of double bonds in a triacylglycerol. The "problematic" triacylglycerols are those with the ECN 42, especially LLL (trilinolein). Thus, the percentage of LLL in the examined oils was adopted as a criterion to detect seed oils in olive oils. Lately, LLL content was substituted by a new parameter, the difference between experimental and theoretical values of triacylglycerols with ECN equal to 42, thus giving more space to the genuine olive oils with higher (but still acceptable) content of linoleic acid (*20*). The theoretical value of ECN 42 is calculated from the fatty acid composition of the purified sample (purification removes possible oxidized products that could interfere with the lower ECN peaks) using a calculation procedure or a programmed macro (*19*). It was developed on the basis of fatty acid and triacylglycerol composition of pure olive oils with proven genuineness assuming a 1,3-random, 2-random distribution of fatty acids in the triacylglycerol, with restrictions for saturated fatty acids in the 2-position (*18*). The method uses a silica column to purify the sample, which is divided into two parts. One part is used to determine fatty acid composition and the other one to determine the practical triacylglycerol composition (HPLC). The liquid chromatographic separation is performed on an octadecylsilyl reverse phase column using almost equivoluminal mixtures of acetone and acetonitrile, thus allowing the optimal separation of all peaks of interest. The mobile phase composition is crucial. The detection is based on the refractive index change and monitored.

To follow the newer adulteration approaches using hazelnut oil, the official method was subjected to changes, introducing certain steps that should give more space to possible discovery of olive oils adulterated with oils with similar fatty acid composition. The improvements were the utilization of a silica SPE cartridge, substitution of the acetone/acetonitrile mobile phase with 100% propionitrile, and utilization of the column compartment heater/cooler, thus allowing the analysis to be performed in the stable temperature environment of 20 °C or less. Such a modification produces a much more stable baseline and allows more peaks to be separated and better peak resolution. The propionitrile mobile phase approach with a flow gradient was successful in chemometric characterization and differentiation of French virgin olive oils (*21*, *22*).

To our best knowledge there are not many such works performed; they were mainly focused on triacylglycerol determination in various seed oils with accent on particular chromatographic technique evaluation (*23*, *24*). With respect to this knowledge, the focus of the work was on triacylglycerol determination in the chosen pumpkin seed oil samples with known origin and previously determined status of adulteration/genuineness (fatty acids determination, (*E*)-isomers of fatty acids, Δ-5 to Δ-7 sterol ratio, tocopherols). Finally, their PCA elaborated stereospecific triacylglycerol composition data were questioned, in view of a possible complementary analytical tool to assess pumpkin seed oil genuineness. Consequently, we see the purpose of this research oriented to the development of a tool that will have discriminating capacity for closely related/adulterated pumpkin seed oils.

MATERIALS AND METHODS

Materials. *Pumpkin Seed Oil Samples.* Seven different pumpkin seed oil samples were examined. They were bought in local stores in the Prekmurje region or purchased locally at the pumpkin seed oil roasting/pressing facilities. All samples were gathered in June and July 2006 and analyzed between June and October 2006. After arrival at the laboratory, the samples were immediately divided into 50 mL portions and deep frozen at −30 °C until analysis. Just prior to analysis, they were simply room thawed and used according to the chosen procedure. **Table 1** gives detailed information about the samples. It should be emphasized that definition of the terms cold-pressed/warm-pressed oils is still very ambiguous.

Chemicals and Reference Material. All chemicals and materials needed to perform sample analyses were chosen and used in accordance with the laboratory's quality system accredited in accordance with ISO 17025.

TAG standards (LLL, LnLnLn, LLO, LLP, LOO, POL, OOO, POO) were purchased from Sigma-Aldrich Chemie Gmbh (Munich, Germany).

TAG CRM IRMM-801 standard (PPS, POP, PLP, POS, POO, PLS, PLO, SOO, SLS, SLO) was purchased from EC-JRC-IRMM (Geel, Belgium).

Olive oil reference samples with certified TAG composition were from International Olive Council (IOC, Madrid, Spain).

Various fat reference samples with certified TAG composition were from BIPEA (Gennevilliers, France).

Analytical Methods. The official method for the ΔECN 42 determination in olive oils (*19*) is COI/T.20/Doc. 20/Rev. 2 - 2008, Determination of the difference between actual and theoretical content of triacylglycerols with ECN 42.

The global method for the detection of extraneous oils in olive oils (*18*) is COI/T.20/Doc. No. 25 - 2006.

Analytical Method and HPLC Equipment Used. An SPE silica gel cartridge (1 g/6 mL) was washed with 6 mL of hexane, not allowing the cartridge to dry. Approximately 0.12 g of the oil dissolved in 0.5 mL of hexane was loaded into the cartridge; the solution was pulled through and then eluted with 10 mL of hexane/diethyl ether (87:13 v/v) under vacuum. The eluted solution was homogenized and divided into two equal parts. Both solutions were separately evaporated under reduced pressure, and the part for HPLC analysis was dissolved in 1 mL of acetone of such a purity that no extra spikes or interferences were seen when it was injected into the HPLC system. The other part was needed for GC fatty acid analysis after it had been dissolved in *n*-heptane, but this step was not performed because of the pumpkin seed oil specificity and because the fatty acid composition was established previously (*14*).

Ten microliters of acetone solution was injected into an Agilent HPLC 1100 HPLC apparatus equipped with a BinPump G1312A binary pump used together with a thermostabilized ALS G1329A autosampler, an ALS-Therm G1330B autosampler thermostat, a thermostabilized COLCOM

Table 1. Information about Samples Including the Code, Origin, Declared Type of Pumpkin Seed Oil (from the Label or from Personal Communication Gained When the Sample Was Obtained), and Previously Determined Adulteration Status[a]

sample	purchased in	label, explanation, origin	adulteration status
S1	food store A	pumpkin oil from Prekmurje region	A
S2	PO facility A	oil from dehulled seeds of *Cucurbita moschata* D., second pressing	G
S3	PO facility A	oil from dehulled seeds of *Cucurbita moschata* D., first pressing	G
S4	PO facility A	oil from dehulled seeds of *Cucurbita moschata* D., third pressing	A
S5	food store B	pumpkin seed oil ("Kmečko bučno olje")	A
S6	PO facility B	warm-pressed pumpkin seed oil	A
S7	PO facility B	cold-pressed pumpkin seed oil	G

[a] Food store A(B), two different local food stores; PO facility A(B), two different pumpkin oil roasting/pressing facilities; first pressing, the ground and roasted seeds were pressed in the freshly prepared unheated clean press; second pressing, the ground and roasted seeds were pressed in the heated press immediately following the first pressing; third pressing, the ground and roasted seeds and previously obtained seed cake (pumpkin seeds from first and second pressing) were mixed together in nonrevealed proportion and hot pressed (possibly with the aid of cheap seed oil to maximize the extracted oil quantity); warm-pressed pumpkin seed oil, ground and roasted pumpkin seeds were pressed in a heated medium press; cold-pressed pumpkin seed oil, according to the PO facility B owner ground pumpkin seeds were pressed in an unheated press without prior roasting; adulteration status, A (adulterated)/G (genuine), based on a set of chemical analyses described and performed in ref *14*.

G1316A column compartment, and a refractive index detector operating at 35 °C. A Phenomenex LiChrospher/Superspher RP18 80A (4 μm i.d., 250 × 4.0 mm) column was used and propionitrile as the mobile phase. To achieve better resolution, the column compartment temperature was kept at 15 °C. The chromatographic system was run with Agilent Chemstation v. 10.01 software. The software was used for the integration and elaboration of the chromatographic data as well.

Peak identification was performed on the basis of TAG standards, olive oil reference samples used in our accredited laboratory for performing the "Determination of the difference between actual and theoretical content of triacylglycerols with ECN 42" analysis and which are traceable to the International Olive Council (IOC, Madrid, Spain). IOC is (besides other important activities) an organizer of the Proficiency Test (PT) schemes from the olive oils area. Peak identification was performed as well on the basis of our laboratory's participation in PT schemes organized by BIPEA (Gennevilliers, France) for different fat and oil samples with certified triacylglycerol compositions and on the basis of our observations comparing certain pumpkin seed oil samples with the literature data dealing with triacylglycerol composition of pumpkin seed oils or with oils with similar fatty acid composition (*23*−*25*). The areas of integrated peaks were assigned as triacylglycerols and were evaluated with the assumption with identical reference factors (chromatographic peak vs peak area ratio) (*19*). All triacylglycerol areas were summed, and each triacylglycerol was expressed as a percent ratio versus the sum of all triacylglycerols (normalization method).

Each sample was determined in parallel, the results for each triacylglycerol were averaged, and the standard deviation was calculated.

Statistical Analysis. The analysis of chromatographic data was performed as one-way analysis of variance (ANOVA) for each triacylglycerol species in all seven samples using Statgraphics Plus 4.0 software. The differences for major triacylglycerols among samples (group means) were estimated using the Tukey honestly significant difference (Tukey HSD) test at $p < 0.05$. The principal component analysis (PCA) from the same software package was used as well.

Triacylglycerol Nomenclature. The triacylglycerols (TAG) are designated by letters corresponding to abbreviated names of fatty acid carbon chains that are linked to the glycerol. The fatty acids abbreviations together forming the TAG are as follows: P, palmitoyl; S, stearoyl; O, oleoyl; L, linoleoyl; and Ln, linoleolenyl (*21*).

RESULTS AND DISCUSSION

HPLC Approach. Using the official method (*19*) with propionitrile as mobile phase and column temperature control, we were able to separate 29 different triacylglycerols within 70 min as shown in **Figure 1**. Twenty-five of them were assigned, and the four unidentified triacylglycerols (marked NAS1, NAS2, NAS3, and NAS4) were almost evenly grouped across triacylglycerols with ECNs of 42 (NAS1), 48 (NAS2), and 50 (NAS3 and NAS4), and their sum ranged from 0.85 wt % (S2) to 1.10 wt % (S7). From these data it can be concluded their contribution to the overall triacylglycerol amount for the analyzed samples was not

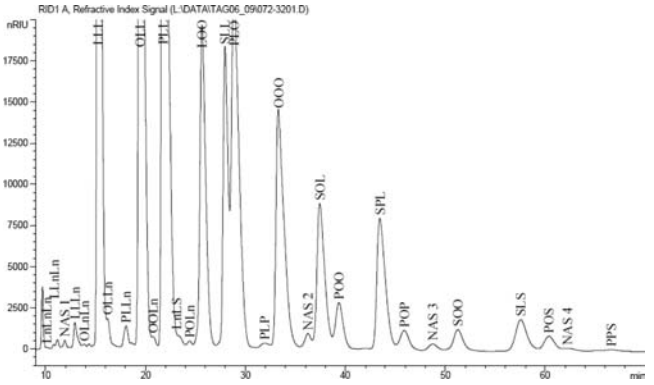

Figure 1. HPLC chromatogram of sample S2 using propionitrile as mobile phase. Chromatographic conditions: detection, RID (35 °C); flow, 0.638 mL/min; chromatographic column, Phenomenex LiChrospher/Superspher RP18 80A (4 μm i.d., 250 × 4.0 mm); column temperature, 15 °C; signal in nRIU, time in minutes. The assigned peaks are respective triacylglycerols.

relevant, because of their low amount and because of the rather small variations across the samples.

The triacylglycerol elution order was as follows: LnLnLn, LLnLn, NAS1, LLLn, OLnLn, LLL, OLLn, PLLn, OLL, OOLn, PLL, LnLS, POLn, LOO, SLL, PLO, PLP, OOO, NAS2, SOL, POO, SPL, POP, NAS3, SOO, SLS, POS, NAS4, PPS.

Elaboration of HPLC Data. From an examination of **Table 2** it can be concluded the number of 29 separated triacylglycerols is greater than previously reported (*23*, *24*). References *23* and *24* both report 12 triacylglycerols each. From the number of separated triacylglycerols it is obvious the separation using the thermostabilized column compartment and propionitrile can give much better results. A work dealing with the antioxidant compounds in commercial oilseeds for food use found 20 different triacylglycerol species in pumpkin seed oil (*26*). A comparable HPLC determination was used (without sample precleaning); however, the mobile phase used was acetone/acetonitrile in a volume ratio of 65:35. Unfortunately, the paper shows no chromatograms, so further comments would be purely speculation, although experience from our laboratory demonstrates the approach using propionitrile and temperature control gives more accurate and more repeatable data with greater baseline stability. They give data for a single pumpkin seed oil bought in a Polish market in the year 2004 or 2005 and analyzed in triplicate. Comparison of the six main triacylglycerols in their pumpkin seed oils with the S2, which our previous work showed to be genuine (four criteria of four) (*14*), thus being comparable, shows there exist similarities between our and their work as follows: PLL, 26.1 versus 16.95 wt %; LLL,

Table 2. Differences in Triacylglycerol Composition for Seven Pumpkin Seed Oils (S1−S7) in Weight Percent from Northeastern Slovenia Determined by HPLC Using Propionitrile as Mobile Phase[a]

TAG	S1	SD	S2	SD	S3	SD	S4	SD	S5	SD	S6	SD	S7	SD
LnLnLn	0.30	0.01	0.03	0.00	0.03	0.00	0.01	0.00	0.02	0.00	0.02	0.00	0.02	0.00
LLnLn	0.10	0.02	0.09	0.01	0.22	0.00	0.78	0.00	0.10	0.00	0.09	0.00	0.17	0.01
NAS1	0.05	0.00	0.09	0.02	0.18	0.00	0.06	0.00	0.11	0.01	0.13	0.00	0.22	0.01
LLLn	2.34	0.00	0.36	0.04	0.43	0.02	0.18	0.00	0.20	0.02	0.25	0.01	0.24	0.01
OLnLn	0.08	0.00	0.05	0.02	0.15	0.01	0.50	0.01	0.13	0.02	0.16	0.01	0.23	0.01
LLL	**16.09**	0.09	**16.95**	0.08	**17.36**	0.00	**26.17**	0.11	**14.51**	0.05	**16.47**	0.04	**11.11**	0.02
OLLn	2.41	0.01	0.36	0.07	0.38	0.01	0.17	0.02	0.35	0.02	0.39	0.01	0.31	0.01
PLLn	1.19	0.00	0.42	0.07	0.58	0.02	0.39	0.02	0.38	0.01	0.34	0.03	0.33	0.01
OLL	**19.97**	0.11	**16.26**	0.08	**16.64**	0.02	**23.31**	0.15	**20.17**	0.09	**21.69**	0.04	**18.26**	0.04
OOLn	1.83	0.01	0.15	0.02	0.23	0.00	0.43	0.04	0.23	0.02	0.26	0.02	0.26	0.01
PLL	**9.88**	0.04	**19.42**	0.07	**20.09**	0.01	**13.36**	0.08	**12.46**	0.07	**11.54**	0.02	**10.13**	0.01
LnLS	0.76	0.01	0.21	0.03	0.27	0.01	0.29	0.03	0.22	0.01	0.23	0.03	0.25	0.01
POLn	0.24	0.01	0.15	0.03	0.19	0.00	0.28	0.01	0.16	0.00	0.22	0.03	0.09	0.01
LOO	**13.27**	0.02	**7.17**	0.02	**6.41**	0.01	**7.36**	0.01	**14.40**	0.08	**13.83**	0.02	**15.97**	0.01
SLL	3.74	0.01	5.54	0.01	5.57	0.00	5.97	0.02	4.25	0.01	4.19	0.02	3.59	0.00
PLO	**7.58**	0.02	**10.53**	0.00	**10.70**	0.00	**6.81**	0.02	**9.75**	0.05	**9.00**	0.01	**10.64**	0.01
PLP	0.35	0.02	0.20	0.04	0.29	0.01	0.24	0.03	0.22	0.03	0.23	0.01	0.28	0.00
OOO	**8.37**	0.01	**7.37**	0.02	**6.78**	0.01	**3.90**	0.08	**8.23**	0.00	**7.68**	0.03	**10.59**	0.01
NAS2	0.36	0.02	0.40	0.02	0.44	0.01	0.47	0.03	0.35	0.01	0.34	0.00	0.30	0.01
SOL	3.26	0.00	4.37	0.01	4.09	0.02	3.06	0.02	4.55	0.00	3.98	0.01	4.70	0.00
POO	2.56	0.02	1.45	0.04	1.19	0.02	0.83	0.01	2.96	0.05	2.79	0.06	4.41	0.02
SPL	1.51	0.05	4.38	0.04	4.04	0.00	2.23	0.05	1.94	0.01	1.80	0.05	1.95	0.02
POP	0.83	0.01	0.83	0.13	0.89	0.02	0.94	0.04	0.73	0.00	0.88	0.03	1.10	0.04
NAS3	0.30	0.00	0.27	0.04	0.31	0.00	0.20	0.03	0.30	0.03	0.31	0.04	0.38	0.00
SOO	1.29	0.02	0.81	0.01	0.67	0.02	0.50	0.01	1.71	0.04	1.58	0.03	2.56	0.04
SLS	0.51	0.02	1.37	0.00	1.21	0.03	0.90	0.02	0.71	0.07	0.70	0.05	0.68	0.05
POS	0.42	0.00	0.63	0.01	0.52	0.01	0.31	0.03	0.57	0.03	0.52	0.04	0.84	0.02
NAS4	0.28	0.01	0.10	0.00	0.12	0.01	0.32	0.01	0.18	0.01	0.28	0.04	0.21	0.03
PPS	0.15	0.06	0.04	0.00	0.02	0.00	0.03	0.00	0.11	0.02	0.10	0.03	0.18	0.03

[a] SD columns represent the standard deviation in wt % from duplicate determinations. The rows in bold represent the first six triacylglycerols (PLL, LLL, OLL, PLO, OOO, and LOO), which account for >77% of the overall triacylglycerol amount in S2, which was previously found to be genuine (*14*).

9.2 versus 16.95 wt %; OLL, 17.2 versus 16.26 wt %; PLO, 5.1 versus 10.53 wt %; OOO, 5.4 versus 7.37 wt %; and LOO, 11.5 versus 7.17 wt% for their work and our results, respectively. Considering the well-known differences in fatty acid composition among different pumpkin varieties (*3*, *27*−*30*) and the not specified pumpkin variety in the oil analyzed by Tuberoso et al. (*26*), we can assume comparable agreement.

Our goal was to discriminate the analyzed pumpkin seed oils on the basis of their (assumed) adulteration or (assumed) genuineness.

At first glance, the results from **Table 2** show some similarities, and two somehow distinct profiles in the composition pattern for samples S2 and S3 and for samples S5 and S6 can be spotted; however, this is not obvious for the rest of the samples, although PLL seems to dominate the discrimination among profiles. For that reason it is obvious that one should approach from a more focused triacylglycerol amount ratio perspective. Instead of finding clusters of samples with identical triacylglycerol patterns, one should determine which triacylglycerols can act as a discriminating tool for the samples. **Figure 2** thus gives the same data that were shown in **Table 2** but divided among six clusters with the following triacylglycerols in each: cluster A, LnLnLn, LLnLn, NAS1, LLLn, OLnLn; cluster B, LLL, OLLn, PLLn, OLL, OOLn; cluster C, PLL, LnLS, POLn, LOO, SLL; cluster D, PLO, PLP, OOO, NAS2, SOL; cluster E, POO, SPL, POP, NAS3, SOO; and cluster F, SLS, POS, NAS4, PPS. Analyzing **Figure 2** one could see which triacylglycerols might act as a discriminating tool for the samples. Cluster A reveals LLLn as dominating, clearly showing S1's accentuated amount of LLLn with >2 wt %. In cluster B LLL shows discrimination among samples and separates samples S4 and S7 from the rest. Cluster C gives two possibilities: PLL and LOO have samples S2 and S3 in a well-defined group in both cases. PLO and OOO are dominating triacylglycerols in cluster D, whereas cluster E shows discrimination in SPL for samples S2 and S3 again. To a lesser extent this stands for POO as well. Cluster F repeats the grouping mentioned before samples S2 and S3 with SLS.

Inspection of the data from the clusters thus identifies 9 possible triacylglycerols (LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL, and SLS) evenly distributed among all 29 triacylglycerols and covering the amount ratio from 0.18 to 26.17 wt %. These 9 triacylglycerols could act as a useful tool in discriminating the samples according to the triacylglycerol distribution.

A closer inspection of the data plotted in **Figure 3** reveals five different groups with samples S2 and S3 in the first group, samples S5 and S6 in the second one, and samples S1, S4, and S7 each defining its single group. Obviously these data per se cannot be too promising because the existing literature data are true only for defined nonadulterated samples, thus lacking the crucial set of parameters that could help one in assigning a pattern for sample recognition.

It has been shown so far that the different pumpkin seed oils can be discriminated according to some definite triacylglycerol species. However, this procedure could be satisfying only if a well-defined adulterated or genuine sample existed in the series. The analyzed triacylglycerols could then serve as a relative aid if compared to sets of known origin.

Principal Component Analysis (PCA). To find another and more revealing way of discriminating adulterated samples from genuine ones and vice versa, the PCA approach was challenged. PCA is a linear transformation of a set of original data to a set of uncorrelated components in such a way that only a few of the

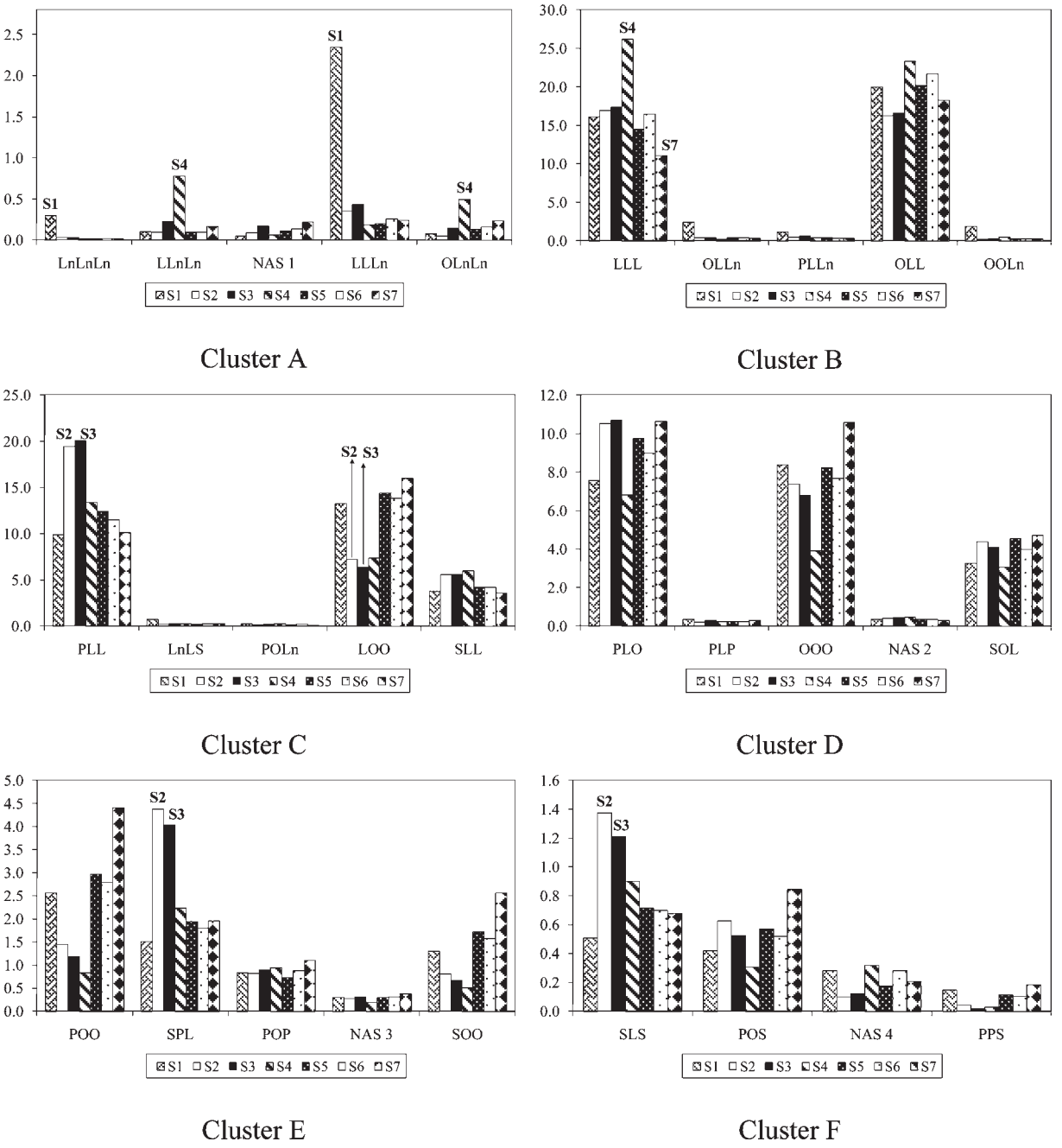

Figure 2. Triacyglycerol composition pattern distribution among seven samples of pumpkin seed oils (S1−S7) from northeastern Slovenia plotted in clusters A−F representing 29 determined triacylglycerols. The *x*-axis denotes triacylglycerols from each cluster and the *y*-axis triacylglycerol amount in wt %. Different letters under the graphs represent different triacylglycerol clusters from the text.

resulting variables account for the majority of the variability observed in the original data. Tsimidou et al. (*31*, *32*) tried to classify and authenticate large sets of virgin olive oils using PCA of triacylglycerols and fatty acid profiles and visualizing the results of PCA by plotting pairs of the first few PCs.

The starting approach was to classify and discriminate the samples according to their triacylglycerol composition with the aid of PCA, deliberately focusing the process solely on triacylglycerol composition and not including other variables (*33*). Understanding the concept of PCA, which is based on a few selected and transformed components, one can face serious doubt that some minor components which are still important for the case are excluded from this very focused approach. It it strongly believed this possible drawback was compensated by including the nine beforementioned triacylglycerols chosen from the whole triacylglycerol set. The PCA approach and stepwise analysis of various triacylglycerols finally elucidated seven variables: LLLn, LLL, sum of PLL + LOO, PLO, OOO, sum of POO + SPL, and SLS. The first two principal components explained 88.8% of the variance (60.3 and 28.5%, respectively), and their comparison to the number of samples judges them as reasonable classifications. With respect to all data harvested in this study, the plot of component weights in the top of **Figure 4** shows good distribution among all selected triacylglycerols. The two chosen variables with the sums PLL + LOO and SPL + POO cover the possible future triacylglycerol combinations in pumpkin seed oil samples as shown in clusters C and E of **Figure 2**. Visual inspection of the scatter plot in the bottom of **Figure 4** analytically confirms the previous supposition of five different groups resulting from the inspection of the data plotted in **Figure 3**.

The first group shown in the bottom of **Figure 4** is composed of samples S2 and S3, which were in fact pressed out from the same pumpkin seeds in a differently driven press and shown previously to be genuine (*14*). The second group is composed of samples S5 and S6, the first being bought in food store B and the second one warm pressed in PO facility B. For them as well it was shown that they are adulterated: three criteria of four for each of them revealed the admixture of the seed oil. The only criterion that was not positive for the adulteration was fatty acid composition, which reveals that the degree of adulteration was not very elevated and thus could not be proven in the fatty acid composition determination. The sample's vicinity in the scatter plot points to possible admixture of the seed oil of the same type. The remaining three groups are composed of a single sample each (S1, S4, and S7) and have very well-defined positions in the scatter plot.

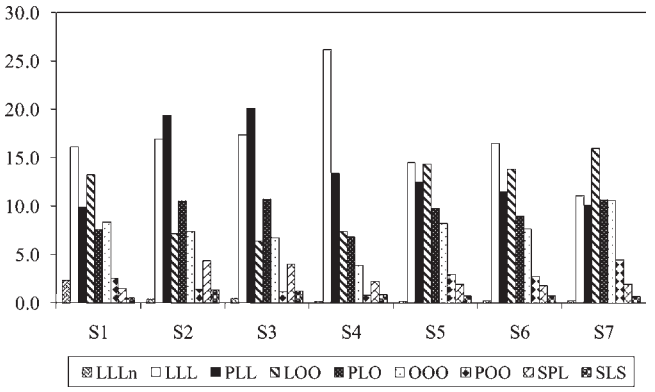

Figure 3. Triacyglycerol composition pattern distribution among seven pumpkin seed oils (S1−S7) from northeastern Slovenia plot based on the selection of nine different triacylglycerols (LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL, and SLS). Legend denotes selected triacylglycerols. The *x*-axis denotes analyzed samples and the *y*-axis triacylglycerol amount in wt %.

This fact could lead us to a conclusion that each sample has a much defined triacylglycerol composition. If we account for the fact that two of them are adulterated (S1 and S4) and for their relative positions (very well apart in the scatter plot), a conclusion can be drawn that either they were pressed from very different pumpkin seeds or they were adulterated with very different seed oils or even both. Indeed, from the tocopherol analysis (S1 has 58 mg/kg of δ-tocopherol and 245 mg/kg of α-tocopherol; on the other hand, S4 has 309 mg/kg of α-tocopherol) it is evident (*14*) that S1 is adulterated with soy oil and S4 with sunflower oil. Usually, in genuine pumpkin seed oils the predominant isomer is γ-tocopherol (>500 mg/kg), followed by α-tocopherol (10−100 mg/kg) and δ-tocopherol (5−15 mg/kg). The Δ-5 to Δ-7 sterol ratio in S1 and S4 is much greater than 0.1, which again is a confirmation of the adulteration with seed oil(s) (*14*). S4 was pressed from the pumpkin seed cake after pressing samples S3 and S2. Evidently, the pumpkin seed cake was abundantly extracted with sunflower oil. This can be concluded after comparing the position of "original" pumpkin seed oil of samples S2 and S3 with the position of S4. The last group goes to S7 with well-defined position in the scatter plot. Its triacylglycerol composition is unique. This is cold-pressed oil and also a genuine one as shown before. Obviously, the pumpkin seeds were of different origin or variety than those for samples S2 and S3. Another fact that should be taken into account is the way pumpkin seeds for S7 were pressed; they were cold pressed. From the data shown one could conclude the roasting process influences the triacylglycerol composition as previously stated (*34*); however, Reference 8 report only very slight differences in linoleic acid composition before and after the roasting process (54.6 vs 54.0%).

These facts could lead us toward reconsideration and even redefinition of the term "cold-pressed pumpkin seed oils". Usually, cold pressing simply denotes the temperature of the press medium, which should not exceed 40 °C but often reaches 60 °C. Recently, this term was enlarged (due to expansion of extra virgin olive oil benefits) to cover seed oils as well (*35*), so the new

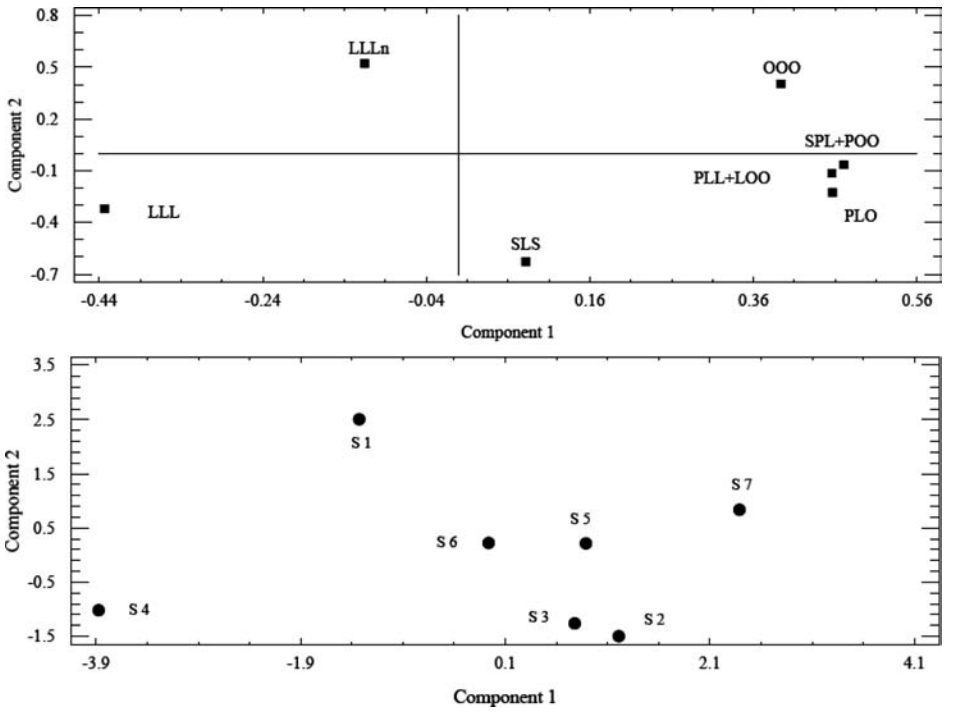

Figure 4. PCA plot of component weights for triacylglycerols LLLn, LLL, PLL + LOO, PLO, OOO, POO + SPL, and SLS for seven pumpkin seed oils (S1−S7) from northeastern Slovenia (top) and PCA scatter plot based on triacylglycerols LLLn, LLL, PLL + LOO, PLO, OOO, SPL + POO, and SLS for seven pumpkin seed oils from northeastern Slovenia (bottom).

term "cold-pressed pumpkin seed oil" AND NOT "cold-pressed roasted pumpkin seed oil" should mean pressed ground seeds were not roasted before pressing (*9*), thus lacking their characteristic flavor.

It was demonstrated that guided chemical assessment can be very useful in checking pumpkin seed oil genuineness, especially if properly linked to stereospecific analysis of triacylglycerols. The set of analyzed pumpkin seed oils (adulterated and genuine) from northeastern Slovenia has well-defined intrinsic differences resulting from their different triacylglycerol compositions. In the future a more profound discrimination between various pumpkin seed oils from the market and/or from the field, based on the nine main triacylglycerols, LLLn, LLL, PLL, LOO, PLO, OOO, SPL, POO, and SLS needed for the PCA, can be undertaken. Stereospecific analysis of triacylglycerols per se gives a profound insight into a sample lipid composition and is less time-consuming if compared to sterol analysis; however, a larger number of samples with stated genuineness and/or adulteration state is needed to establish a comprehensive reference pool.

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LITERATURE CITED

(1) Paris, H. S.; Janick, J. Early evidence for the culinary use of squash flowers in Italy. *Chronica Hortic.* **2005**, *45* (2), 20–21.

(2) Bavec, F.; Gril, L.; Grobelnik-Mlakar, S.; Bavec, M. Production of pumpkin for oil. In *Trends in New Crops and New Uses*; Janick, J., Whipkey, A., Eds.; ASHS Press: Alexandria, VA, 2002; pp 187−190.

(3) Fruhwirth, G. O.; Hermetter, A. Seeds and oil of the Styrian oil pumpkin: components and biological activities. *Eur. J. Lipid Sci. Technol.* **2007**, *109* (11), 1128–1140.

(4) Bandelj, D.; Jakse, J.; Javornik, B. DNA fingerprinting of olive varieties by microsatellite markers. *Food Technol. Biotechnol.* **2002**, *40* (3), 185–190.

(5) Kreft, I.; Stibilj, V.; Trkov, Z. Iodine and selenium contents in pumpkin (*Cucurbita pepo* L.) oil and oil-cake. *Eur. Food Res. Technol.* **2002**, *215* (4), 279–281.

(6) Nakic, S. N.; Rade, D.; Skevin, D.; Strucelj, D.; Mokrovcak, Z.; Bartolic, M. Chemical characteristics of oils from naked and husk seeds of *Cucurbita pepo* L. *Eur. J. Lipid Sci. Technol.* **2006**, *108* (11), 936–943.

(7) Sivakumar, G.; Bati, C. B.; Perri, E.; Uccella, N. Gas chromatography screening of bioactive phytosterols from mono-cultivar olive oils. *Food Chem.* **2006**, *95* (3), 525–528.

(8) Murkovic, M.; Piironen, V.; Lampi, A. M.; Kraushofer, T.; Sontag, G. Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (part 1: nonvolatile compounds). *Food Chem.* **2004**, *84* (3), 359–365.

(9) Bavec, F.; Grobelnik-Mlakar, S.; Rozman, C.; Bavec, M. Oil pumpkins: niche for organic producers. In *Issues in New Crops and New Uses*; Janick, J., Whipkey, A., Eds.; ASHS Press: Alexandria, VA, 2007; pp 185−189.

(10) Oliveras Lopez, M. J.; Innocenti, M.; Ieri, F.; Giaccherini, C.; Romani, A.; Mulinacci, N. HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian *Olea europaea* L. fruits. *J. Food Compos. Anal.* **2008**, *21* (1), 62–70.

(11) Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B.; Bartsch, H. Identification of lignans as major components in the phenolic fraction of olive oil. *Clin. Chem.* **2000**, *46* (7), 976–988.

(12) EC, Publication of an application pursuant to Article 6(2) of Council Regulation (EC) No. 510/2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. *Off. J. Eur. Union* **2009**, *C72* (52), 67−69.

(13) EU, Council regulation (EC) No. 510/2006 − Application for registration according to Article 5 and Article 17(2) − 'Ekstra deviško oljčno olje Slovenske Istre'. *Off. J. Eur. Union* **2006**, *C127*, 16−18.

(14) Butinar, B.; Bucar-Miklavcic, M.; Krumpak, A.; Raspor, P. Experiences in olive oil purity and quality assessment as a tool for pumpkin seed oil evaluation. What can consumers benefit? *Acta Aliment. Hung.* **2009**, *38* (2), 219–227.

(15) EC, Commission Regulation (EC) No. 1989/2003 of 6 November 2003 amending Regulation (EEC) No. 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis. *Off. J. Eur. Union* **2003**, *L295*, 57−77.

(16) EEC, Commission Regulation (EEC) No. 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Off. J. Eur. Union* **1991**, *L248*, 1−83.

(17) Siegmund, B.; Murkovic, M. Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (part 2: volatile compounds). *Food Chem.* **2004**, *84* (3), 367−374.

(18) IOC COI/T.20/Doc. No. 25 − 2006 Global method for the detection of extraneous oils in olive oils; http://www.internationaloliveoil.org/downloads/25.AVELLeng.pdf.

(19) IOC COI/T.20/Doc. 20/Rev. Two − 2008 Determination of the difference between actual and theoretical content of triacylglycerols with ECN 42.; http://www.internationaloliveoil.org/downloads/testing7.pdf.

(20) Moreda, W.; Perez-Camino, M. C.; Cert, A. Improved method for the determination of triacylglycerols in olive oils by high performance liquid chromatography. *Grasas Aceites* (*Sevilla*) **2003**, *54* (2), 175–179.

(21) Ollivier, D.; Artaud, J.; Pinatel, C.; Durbec, J. P.; Guerere, M. Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. *J. Agric. Food Chem.* **2003**, *51* (19), 5723–5731.

(22) Ollivier, D.; Artaud, J.; Pinatel, C.; Durbec, J. P.; Guerere, M. Differentiation of French virgin olive oil RDOs by sensory characteristics, fatty acid and triacylglycerol compositions and chemometrics. *Food Chem.* **2006**, *97* (3), 382–393.

(23) Andrikopoulos, N. K.; Chiou, A.; Mylona, A. Triacylglycerol species of less common edible vegetable oils. *Food Rev. Int.* **2004**, *20* (4), 389–405.

(24) Jakab, A.; Heberger, K.; Forgacs, E. Comparative analysis of different plant oils by high-performance liquid chromatography−atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr., A* **2002**, *976* (1−2), 255–263.

(25) Fiebig, H. J. HPLC separation of triglycerides. *Fett. Wiss. Technol.* **1985**, *87* (2), 53–57.

(26) Tuberoso, C. I. G.; Kowalczyk, A.; Sarritzu, E.; Cabras, P. Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. *Food Chem.* **2007**, *103* (4), 1494–1501.

(27) Applequist, W. L.; Avula, B.; Schaneberg, B. T.; Wang, Y. H.; Khan, I. A. Comparative fatty acid content of seeds of four *Cucurbita* species grown in a common (shared) garden. *J. Food Compos. Anal.* **2006**, *19* (6−7), 606–611.

(28) Murkovic, M.; Hillebrand, A.; Winkler, J.; Leitner, E.; Pfannhauser, W. Variability of fatty acid content in pumpkin seeds (*Cucurbita pepo* L.). *Z. Lebensm. Unters. Forsch.* **1996**, *203* (3), 216–219.

(29) Stevenson, D. G.; Eller, F. J.; Wang, L. P.; Jane, J. L.; Wang, T.; Inglett, G. E. Oil and tocopherol content and composition of pumpkin seed oil in 12 cultivars. *J. Agric. Food Chem.* **2007**, *55* (10), 4005–4013.

(30) Zhong, H. Y.; Bedgood, D. R.; Bishop, A. G.; Prenzler, P. D.; Robards, K. Endogenous biophenol, fatty acid and volatile profiles of selected oils. *Food Chem.* **2007**, *100* (4), 1544–1551.

(31) Tsimidou, M.; Macrae, R.; Wilson, I. Authentication of virgin olive oils using principal component analysis of triglyceride and fatty-acid profiles. 1. Classification of Greek olive oils. *Food Chem.* **1987**, *25* (3), 227–239.

(32) Tsimidou, M.; Macrae, R.; Wilson, I. Authentication of virgin olive oils using principal component analysis of triglyceride and fatty-acid

profiles. 2. Detection of adulteration with other vegetable-oils. *Food Chem*. **1987**, *25* (4), 251–258.
(33) Aranda, F.; Gomez-Alonso, S.; del Alamo, R. M. R.; Salvador, M. D.; Fregapane, G. Triglyceride, total and 2-position fatty acid composition of Cornicabra virgin olive oil: comparison with other Spanish cultivars. *Food Chem*. **2004**, *86* (4), 485–492.
(34) Yoshida, H.; Tomiyama, Y.; Kita, S.; Mizushina, Y. Roasting effects on fatty acid distribution of triacylglycerols and phospholipids in the kernels of pumpkin (*Cucurbita* spp) seeds. *J. Sci. Food Agric*. **2005**, *85* (12), 2061–2066.
(35) Matthaus, B.; Spener, F. What we know and what we should know about virgin oils − general introduction. *Eur. J. Lipid Sci. Technol*. **2008**, *110* (7), 597–601.

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