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Variety differentiation of virgin olive oil based on *n*-alkane profile

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Abstract

An on-line LC–GC method was used to assess the *n*-alkane composition of 40 olive oil samples obtained from three different cultivars from a restricted grove zone in Croatia. Olive samples were handpicked at three different ripening stages during four consecutive years. No significant differences were found in relation to the variable ''period of harvesting'', while the effects ''variety'' and ''year'' as well as their interaction caused significant differences for most of the *n*-alkane components ($p \leqslant 0.05$). Despite the influence of the production year, linear discriminant analysis based on the *n*-alkane components was able to correctly identify the variety of 97.5% of the samples.

Keywords: Virgin olive oil; Variety differentiation; *n*-Alkanes; Discriminant analysis

1. Introduction

The determination of the paraffinic fraction of vegetable oils is normally used as a marker to reveal contamination with mineral oil residues widely diffused in most of the seed oils present in the market (Grob & Bronz, 1995; Moret, Populin, Conte, Grob, & Neukom, 2003; Tan & Kunton, 1993; Wagner et al., 2001). Mineral oil contamination can be easily recognized by the presence of a complex mixture of isoalkanes, forming a ''hump'' which cannot be resolved by GC analysis, and/or by the fact that there is no odd carbon number predominance for *n*-alkane (as for naturally occurring hydrocarbons).

Except for heavily contaminated samples, the same analysis can be used to assess natural *n*-alkane composition in order to characterize different vegetable oils (Bastic, Bastic, Jovanovic, & Spiteller, 1978; McGill, Moffat, Mackie, & Cruickshank, 1993; Moret, Populin, Conte, & Koprivnjak, 2002). Webster, Simpson, Shanks, and Moffat (1999) demonstrated that olive oil can be differentiated from other vegetable oils using principal component analysis applied to the *n*-alkane profiles, and that it is also possible to identify adulteration with rapeseed or sunflower oil at levels of only 0.5% w/w.

Differences in total *n*-alkane content and composition were also found among virgin olive oils of different cultivars from a restricted grove zone (Koprivnjak & Conte, 1996; Koprivnjak, Procida, & Favretto, 1997), as well as of several representative cultivars from different Spanish grove zones (Guinda, Lanzon, & Albi, 1996). Nevertheless, the composition of vegetable products depends not only on genetic factors, but can also be influenced by ripening grade of fruits or specific climatic conditions of the year of production. As no information dealing with this topic was found in literature, the aim of our work was to verify the influence of these potential sources of variations, in order to examine the possible application of the *n*-alkane fraction in olive oil authentication.

The analysis of oil samples was performed applying an on-line LC–GC method (Moret et al., 2003). With respect to the traditional methods involving saponification and/or tedious passage on packed silica gel column,

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this method allows to perform a rapid automated analysis, with optimal reproducibility characteristics, reducing the time for sample preparation to a few minutes and minimizing the amount of solvent used for analysis.

2. Materials and methods

2.1. Samples

Samples of virgin olive oil were extracted from olive fruits of three cultivars – Leccino, Buza and Bjelica, growing in restricted zone of Istria (Croatia) characterized by homogeneous pedoclimatic conditions. The fruits were handpicked three times during the ripening period, from the middle of October until the end of November with a 3-week interval between each picking, during four consecutive years (1997–2000). Batches of 50 kg of fruits were processed within 24 h of harvesting. Olive paste was malaxed for 30 min at 20 °C and oil was extracted by a two-phase centrifugation system equipment. After filtration, the oils were stored in dark closed bottles at 0 °C until analysis. In total, there were 17 samples of Leccino, 11 samples of Buza (in 1999 there was not sufficient amount of fruits for the third date of harvesting), and 12 samples of Bjelica olive oil.

2.2. On line LC–GC analysis

60 mg of oil sample, diluted in pentane after the addition of an adequate amount of internal standards (aliphatic hydrocarbons C13:0 and C14:1) was injected into an LC–GC–FID apparatus (Dualchrom 3000, Fison/C.E. Instrument, Milan, Italy) equipped with a syringe pump (Phoenix 30) and two switching valve in the ISS 300 unit. A 100×4.6 mm i.d. LC silica column (Sperisorb, Lab Service Analytica, Anzola dell'Emilia, Italy) separated the hydrocarburic fraction from triglycerides and other components. The mobile phase consisted of redistilled pentane at a flow rate of 600 $\mu l\,min^{-1}$. After the elution of the fraction of interest, the column was backflushed with 6 ml of CH_2Cl_2 to remove the residual fat. GC transfer occurred through the wire interface (Grob & Bronz, 1995). The vapour exit was closed 10 s after the end of the transfer that started 1:45 min after injection and lasted for 1 min. During transfer, the LC pump drives the fraction of interest into a vaporizing chamber (consisting of a short fused silica transfer line) thermostated at 350 °C. The carrier gas supply is stopped and vapours are discharged by overflow through a 3 m $\times$ 0.53 retaining pre-column (immobilized PS-255) and a 0.53 mm i.d. vapor exit. Separation was performed on a 12 m $\times$ 0.25 mm i.d. capillary column coated with 0.3 μm of immobilized PS-255. Transfer occurred at 49 °C oven temperature (op-

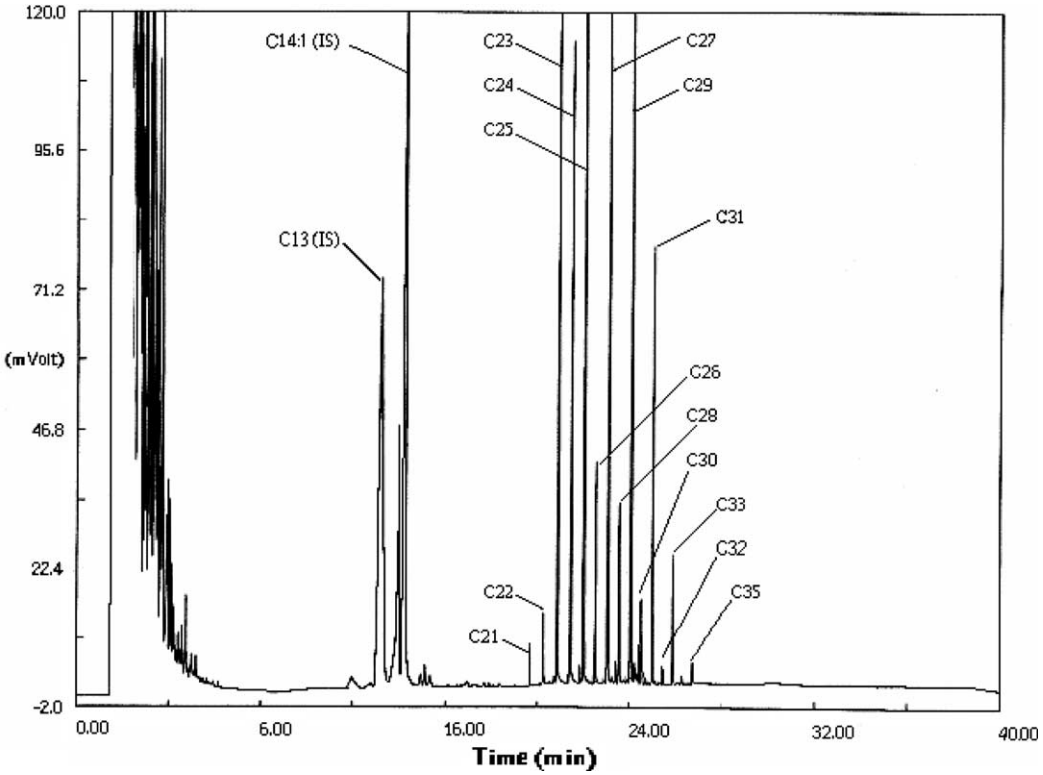

Fig. 1. LC–GC chromatogram obtained from a Bjelica oil sample.

timised for a maximum retention power in the retaining pre-column); 8 min after the transfer the temperature was programmed at 15 °C min^{-1} to 320 °C. The carrier gas (helium) inlet pressure was 70 kPa.

2.3. Statistical analysis

Unsaturated *n*-alkanes were not included in the data submitted to statistical analysis, as they were not found in all samples. The homogeneity of variance was tested by Levene's test. Logarithmic transformation of original data was performed, when needed, in order to increase the homogeneity of variance. The results were submitted to two way and one way analysis of variance at 5% significance level. Statistical differences between mean values were determined by Tukey's honest significant difference test for unequal number of cases. Linear discriminant analysis was used as a tool to identify the olive cultivar on the basis of the *n*-alkane profile.

All statistical analyses were performed using the software package Statistica 6.0 (StatSoft Ltd., Tulsa, USA).

3. Results and discussion

All the olive oil samples submitted to the LC–GC analysis were found to be free from mineral oil contamination. Fig. 1 shows a typical LC–GC chromatogram obtained from a Bjelica sample.

Single *n*-alkane concentrations (mean and standard deviation for the four years) in relation to the period of harvesting are shown in Fig. 2 for each of the three cultivars. The *n*-alkane profile of all samples is characterized by predominance of odd carbon number alkanes, among which the main components are those from C23 to C29, in accordance to previously reported data for olive oils in general (McGill et al., 1993; Moret et al., 2002; Webster et al., 1999). Bjelica samples present a considerably lower content of total hydrocarbons (13.68–26.41 mg kg^{-1}) with respect to Buza (28.00–62.28 mg kg^{-1}) and Leccino samples (27.00–80.39 mg kg^{-1}). The presence of unsaturated components (from C23:1 to C35:1) was observed only in Bjelica samples and in Buza samples produced in 1997.

Bjelica oils also present a peculiar *n*-alkane profile with respect to the other two cultivars, the main components being C29, C25 and C27, while for the other two cultivars the main components are C25, C23 and C24. Leccino samples show a decreasing trend during ripening, especially for components with less than 30 carbon number. Nevertheless, a general decreasing trend for the all three cultivars cannot be shown.

Moreover, the one-way analysis of variance applied separately to each component gave no significant differences among the means of the three periods of harvesting, which were then considered as replicates in the subsequent analyses.

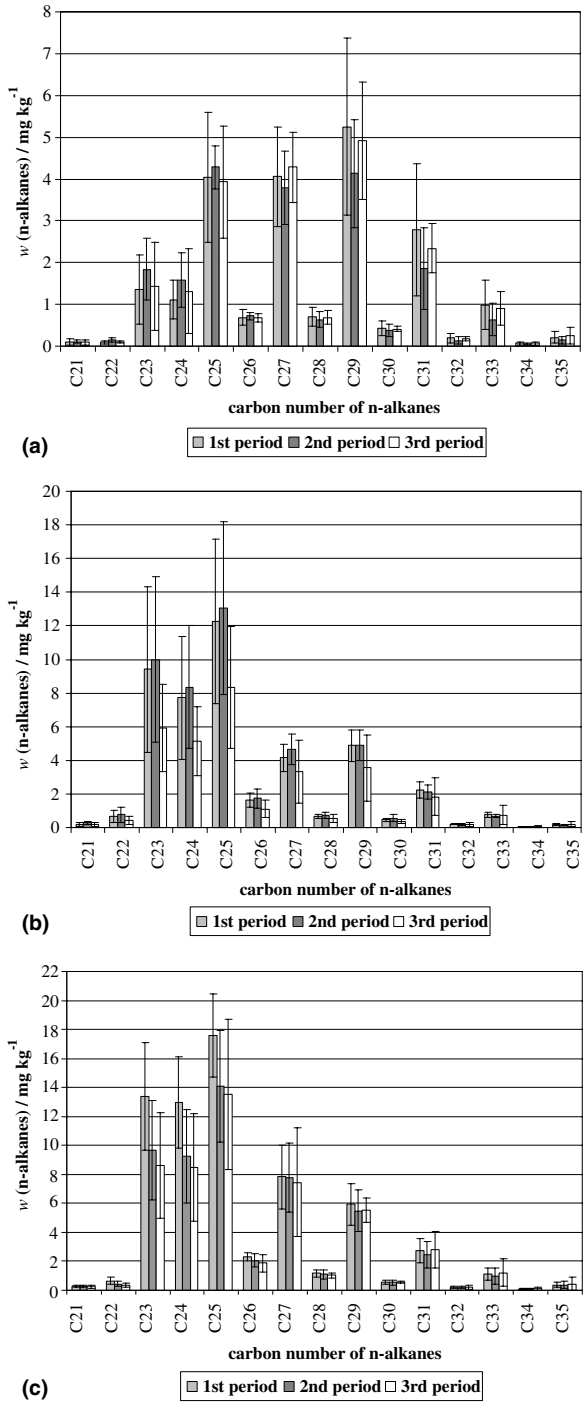

Fig. 2. (a) Changes of single *n*-alkanes content in Bjelica oil samples during harvesting period. Error bars represent standard deviation relative to the four years of production. (b) Changes of single *n*-alkanes content in Buza oil samples during harvesting period. Error bars represent standard deviation relative to the four years of production. (c) Changes of single *n*-alkanes content in Leccino oil samples during harvesting period. Error bars represent standard deviation relative to the four years of production.

In order to evaluate eventual differences among cultivars and years, the next step in data examination was a two-way analysis of variance. The two variables had a significant effect on most of the components, and their interaction was also significant for *n*-alkane from C21 to C27 (Table 1). This means that each cultivar had a specific response to the climatic conditions of different years. The production years were then compared separately for each cultivar with the Tukey honest significant difference test (Table 2). Six of 15 *n*-alkanes were significantly influenced by production year in the case of Bjelica samples, five in Buza samples, while the variety with the least number of influenced components was Leccino with four. It is important to underline that no significant differences were found between the first two years of production, for the entire *n*-alkane profile in the three cultivars.

The whole set of samples was submitted to linear discriminant analysis in order to verify the efficiency of *n*-alkane composition as a means of variety differentiation, despite the above discussed influence of the production year and year-variety interaction. As the number of groups (varieties) considered was three, two discriminant functions were calculated by the program as linear combinations of the chemical descriptors. All variables were included in the model, but the variables that gave the major contribution to the discrimination power were C28, C22 and C25, since these are the only alkanes with p values of <0.05.

In Fig. 3 the distribution of data, whose coordinates represent the discriminant scores derived from the discriminant functions, show that the three groups (varieties) are well separated. As it can be observed from the classification matrix reported in Table 3, all olive oil samples except one Leccino sample received the correct assignment. Therefore the percentage of total correct predictions was 97.5%. In order to better evaluate the classification ability of the two discriminant functions, the leave-one-out method was used as a validation procedure. The proportion of correctly predicted cases was equal to 87.5%, showing a good performance of the method.

Table 1
Two-way analysis of variance – F values and significance level for the effect of year and variety on *n*-alkane composition

Carbon number	Factors		
	Year	Variety	Year × variety
C21	10.0***	28.1***	6.38***
C22	10.3***	37.5***	4.96**
C23	7.90***	44.3***	3.17*
C24	5.35**	47.6***	2.46*
C25	14.8***	76.0***	4.86**
C26	16.8***	94.7***	3.48**
C27	10.4***	28.2***	5.67***
C28	5.95**	21.3***	0.908 ns
C29	8.26***	2.02 ns	1.72 ns
C30	9.73***	6.44**	1.32 ns
C31	3.56*	0.842 ns	1.50 ns
C32	3.21*	1.88 ns	1.03 ns
C33	1.85 ns	1.57 ns	0.897 ns
C34	2.93 ns	5.58**	1.12 ns
C35	1.85 ns	4.90*	0.594 ns
Total	14.5***	66.7***	2.48*

ns, not significant.
* $p \leqslant 0.05$.
** $p \leqslant 0.01$.
*** $p \leqslant 0.001$.

Table 2
Analysis of variance – significance of differences in *n*-alkane composition of olive oils related to the production year within each single variety

Carbon number	Bjelica				Buza				Leccino			
	1997	1998	1999	2000	1997	1998	1999	2000	1997	1998	1999	2000
C21	a[A]	a	a	b	×[B]	×	×	×	f	f	f	g
C22	××[C]	××	××	××	c	c	d	e	×	×	×	×
C23	×	×	×	×	c	c	d	d	×	×	×	×
C24	×	×	×	×	c	c	d	d	×	×	×	×
C25	ab	ab	b	a	c	c	d	d	f	f	g	fg
C26	×	×	×	×	c	c	d	d	f	f	g	fg
C27	a	a	b	ab	××	××	××	××	fg	f	g	h
C28	a	a	b	a	×	×	×	×	×	×	×	×
C29	a	a	b	ab	××	××	××	××	×	×	×	×
C30	a	a	b	a	××	××	××	××	×	×	×	×
C31	×	×	×	×	×	×	×	×	×	×	×	×
C32	×	×	×	×	×	×	×	×	×	×	×	×
C33	×	×	×	×	×	×	×	×	×	×	×	×
C34	×	×	×	×	×	×	×	×	×	×	×	×
C35	×	×	×	×	×	×	×	×	×	×	×	×
Total	×	×	×	×	c	c	d	d	fg	f	g	fg

[A] Different letters denote a significant difference among years (a,b for Bjelica samples; c–e for Buza samples; f–h for Leccino samples) at $p \leqslant 0.05$ according to Tukey test.
[B] ×Difference is not significant.
[C] ××Significance of differences was not tested because of poor homogeneity of variance according to Levene test.

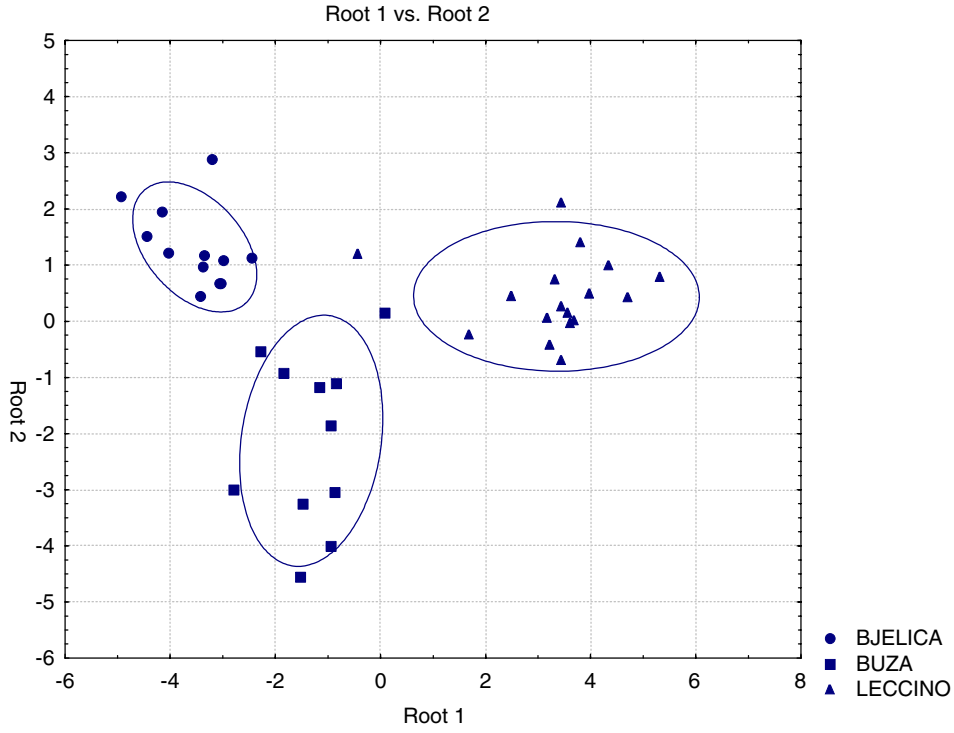

Fig. 3. Discriminant analysis – scatterplot of canonical scores (range, coefficient 0.95).

Table 3
Discriminant analysis – classification matrix

Observed classification	Predicted classification			Correctly classified cases (%)
	Bjelica	Buza	Leccino	
Bjelica	12	0	0	100
Buza	0	11	0	100
Leccino	1	0	16	94.1
Total	13	11	16	97.5

To take into account variations due to the year of production and to the year-variety interaction, we repeated the linear discriminant analysis four times, excluding each year in turn from the estimation of the discriminant functions and evaluating the prediction ability on the excluded cases (this validation method is known as the leave-*k*-out procedure). A very good performance was obtained for 1998 and 1999 (perfect or nearly perfect classification), a good one for 1997 (66.7% correctly classified), and only cases from 2000 were poorly classified (36.4%). This means that the ability to identify the cultivar from the *n*-alkane profile depends, at least partially, on the year of production, even if in most cases the results are very good. Furthermore, when interpreting these results, we must take into account that the elimination of all the cases of one year (≈25% of the complete data set) corresponds to an important reduction in the information available, which in turn results in a lower discriminant power.

Considering the above discussed results it can be concluded that the period of fruit harvesting and consequently the ripening grade of fruits have minor influence on *n*-alkane composition of olive oils. Different production years significantly influence this composition, but different olive cultivars have specific response to this source of variation. Despite this, the *n*-alkane composition of olive oils is useful to distinguish the variety regardless the production year, at least in most cases, if discriminant analysis is applied.

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