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Triacylglycerol Profile as a Chemical Fingerprint of Mushroom Species: Evaluation by Principal Component and Linear Discriminant Analyses

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ABSTRACT: Mushrooms are becoming relevant foods due to their nutritional, gastronomic, and pharmacological properties, namely, antioxidant, antitumor, and antimicrobial properties. However, although several mushroom species have been chemically characterized, the evaluation of the triacylglycerol (TAG) profile remains nearly unknown. Because TAG was formerly used to assess the authentication of highly valued commercial oils, and the distribution of fatty acids on the glycerol molecule is genetically controlled, the potential of the TAG profile to act as a taxonomical marker was evaluated in 30 wild mushroom species. Principal component analysis and linear discriminant analysis were used to verify the taxonomical rank (order, family, genus, or species) more related with the detected TAG profile. The results pointed out that the ability of the TAG profile to discriminate mushroom samples increased for the lower taxonomical ranks, reaching a maximal performance for species discrimination. Because there is a high resemblance among mushroom species belonging to the same genus and considering that conservation techniques applied to mushrooms often change their physical properties, this might be considered as a valuable outcome with important practical applications.

KEYWORDS: *wild mushrooms, triacylglycerols, PCA, LDA*

INTRODUCTION

Northeastern Portugal, with its climatic conditions and flora diversity, is one of the European regions with higher wild edible mushrooms diversity, some of them with great gastronomic relevance. Studies conducted on mushrooms proved their antioxidant,[1] antitumor,[2] and antimicrobial properties, as well as their interesting contents in nutraceuticals.[3] Furthermore, mushrooms are becoming important in our diet for their nutritional and organoleptic characteristics.[4] Our research group has been interested in the bioactive properties and chemical profile of wild and commercial mushrooms; with regard to chemical characterization, special attention has been dedicated to the determination of proteins, fat, ash, carbohydrates, individual sugars, fatty acids, phenolic compounds, carotenoids, ascorbic acid, and tocopherols.[5−11] Other authors also analyzed ergosterol, vitamin D2, nucleosides, and nucleobases in mushrooms.[12−15]

The determination of selected lipid species is of considerable interest because it allows conclusions on metabolic processes.[16] Furthermore, the lipidic fraction of a natural product has a characteristic pattern of triacylglycerols (TAGs), comprising highly specific information due to the genetic control of the stereospecific distribution of fatty acids (FAs) on the glycerol molecule, which is typical for each species.[17] Analysis of TAGs in oils and fats has gained increasing attention in the past decades. In food research, it is used to study crystallization phenomena, to detect adulteration of specialty fats and oils, and for recognition of oils' origin.[18] Nevertheless, studies dealing with TAGs in mushrooms are rather scarce and based on highly specific features, for instance, the neurolysin inhibitory ability of agaricoglycerides (a class of aromatic triacylglycerols) produced by some Basidiomycetes.[19] The evaporative light-scattering detector (ELSD) is a mass-sensitive detector that responds to any analyte less volatile than the mobile phase, which is a suitable solution for TAG analysis. It has a low background signal and a nonspecific response (unlike a flame ionization detector), is compatible with gradient elution (unlike a refraction index (RI) detector) and with a broad range of solvents, and has a signal independent of the degree of saturation and chain length (unlike an ultraviolet detector). From a theoretical point of view, the response of the ELSD is sigmoidal upon increasing analyte concentrations.[18] Partition number, equivalent carbon number, theoretical carbon number, and matrix models are proposed methods to identify TAG peaks from HPLC-ELSD analysis. These methods are relatively well fit when reversed-phase (RP)-HPLC is used as an analytical tool.[20]

Due to the high commercial value of mushrooms, finding an analytical parameter that might act as a chemical fingerprint is a mandatory subject. Herein, 30 different species of mushrooms are characterized with regard to their TAG profile to define this parameter as a taxonomical marker. The results were scrutinized through an analysis of variance, a principal component analysis as pattern recognition unsupervised classification method, and a stepwise-based linear discrimination analysis as a supervised classification technique.

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Table 1. Fatty Acid Composition (Percent) in Some Selected Studies Using the Species Herein Studied[a]

order	family	species	palmitic acid	oleic acid	linoleic acid	ref
Agaricales	Agaricaceae	*Agaricus silvaticus*	11.7 ± 0.1	6.67 ± 0.01	74.78 ± 0.01	3
Agaricales	Agaricaceae	*Agaricus silvicola*	10.0 ± 0.2	3.5 ± 0.2	76.5 ± 0.2	3
Agaricales	Amanitaceae	*Amanita caesarea*	12.4 ± 0.4	54 ± 1	26 ± 2	10
Tricholomatales	Tricholomataceae	*Armillaria mellea*	11.0 ± 0.1	47.7 ± 0.4	27.7 ± 0.3	11
Boletales	Boletaceae	*Boletus edulis*	10.0 ± 0.3	40 ± 2	44 ± 2	3
			9.6 ± 0.2	42.1 ± 0.2	41.3 ± 0.1	8
			9.8	36.1	42.2	27
			21.6	31.1	33.8	28
Boletales	Boletaceae	*Boletus erythropus*	21 ± 1	15 ± 1	49 ± 1	6
			11.20	18.00	63.00	27
Boletales	Boletaceae	*Boletus fragrans*	14.9 ± 0.1	20 ± 1	57 ± 1	6
Boletales	Boletaceae	*Boletus impolitus*	16.8 ± 0.4	14 ± 1	61 ± 1	9
Boletales	Boletaceae	*Boletus reticulatus*	11.0 ± 0.1	47.2 ± 0.1	32.83 ± 0.01	8
Lycoperdales	Lycoperdaceae	*Bovista aestivalis*	21 ± 2	12.6 ± 0.1	42 ± 4	9
Lycoperdales	Lycoperdaceae	*Bovista nigrescens*	17.4 ± 0.1	21.0 ± 0.2	38.3 ± 0.2	9
Tricholomatales	Tricholomataceae	*Calocybe gambosa*	15 ± 1	18 ± 1	58 ± 1	3
			13.6 ± 0.5	33 ± 1	43.9 ± 0.3	11
Cantharellales	Cantharellaceae	*Cantharellus cibarius*	7.2 ± 0.1	8.13 ± 0.01	50.0 ± 0.1	5
			13.1 ± 0.1	10.8 ± 0.3	53.6 ± 0.1	3
			18.30	35.40	17.30	28
Agaricales	Agaricaceae	*Chlorophyllum rhacodes*	16.4 ± 0.3	5.7 ± 0.1	72.6 ± 0.5	9
Clavariales	Clavariadelphaceae	*Clavariadelphus pistillaris*	17 ± 1	49.1 ± 0.2	25 ± 1	9
Cortinariales	Cortinareaceae	*Cortinarius violaceus*	14.02 ± 0.04	15 ± 1	66 ± 1	10
Polyporales	Fistulinaceae	*Fistulina hepatica*	10 ± 1	31.5 ± 0.1	52 ± 1	7
Tricholomatales	Hydnangeaceae	*Laccaria amethystina*	6.9 ± 0.4	14 ± 1	74.4 ± 0.2	29
Tricholomatales	Tricholomataceae	*Lepista nuda*	11.8 ± 0.1	29.53 ± 0.04	51.5 ± 0.1	5
Agaricales	Agaricaceae	*Leucoagaricus leucothites*	12.2 ± 0.2	6.3 ± 0.4	75 ± 1	9
Agaricales	Tricholomataceae	*Leucopaxillus giganteus*	13.5 ± 0.1	21.1 ± 0.5	46.2 ± 0.5	4
Lycoperdales	Lycoperdaceae	*Lycoperdon molle*	13.7 ± 0.2	8.6 ± 0.1	64.2 ± 0.4	5
Lycoperdales	Lycoperdaceae	*Lycoperdon umbrinum*	19.9 ± 0.1	22.8 ± 0.3	29.4 ± 0.1	9
Agaricales	Lepiotaceae	*Macrolepiota procera*	4.6	17.2	47.0	28
Clavariales	Ramariaceae	*Ramaria aurea*	7.32 ± 0.04	56.9 ± 0.5	25.6 ± 0.2	9
Clavariales	Ramariaceae	*Ramaria botrytis*	9.91 ± 0.03	43.9 ± 0.1	38.3 ± 0.1	5
Russulales	Russulaceae	*Russula cyanoxantha*	13.0 ± 0.2	28 ± 1	44 ± 1	6
			17.20	26.00	47.40	30
Telephorales	Bankeraceae	*Sarcodon imbricatus*	11.14 ± 0.05	45.1 ± 0.2	35.4 ± 0.4	4
Tricholomatales	Tricholomataceae	*Tricholoma imbricatum*	7.4 ± 0.2	51.5 ± 0.4	33.0 ± 0.1	7
Tricholomatales	Tricholomataceae	*Tricholoma portentosum*	5.60 ± 0.01	58.4 ± 0.1	30.9 ± 0.1	4
			7.6	58.0	27.9	31

[a]The results are presented, except when not avaliable, as the mean ± SD.

■ MATERIALS AND METHODS

Standards and Reagents. Triacylglycerols 1,2,3-tripalmitoylglycerol (PPP), 1,2,3-tristearoylglycerol (SSS), 1,2,3-trilinolenoylglycerol (LnLnLn), and 1,2,3-tripalmitoleoylglycerol (PoPoPo), of >98% purity, and 1,2,3-trioleoyglycerol (OOO), 1,2,3-trilinoleoyglycerol (LLL), 1,2-dilinoleoyl-3-palmitoyl-*rac*-glycerol (PLL), 1,2-dilinoleoyl-3-oleoyl-*rac*-glycerol (OLL), 1,2 -dipalmitoyl-3-oleoyl-*rac*-glycerol (PPO), 1,2-dioleoyl-3-stearoyl-*rac*-glycerol (OOS), 1-palmitoyl-2-oleoyl-3-linoleoylglycerol (POL), and 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (POO), of ≈99% purity, were purchased from Sigma (St. Louis, MO, USA). Petroleum ether was of analytical grade and obtained from Fisher Scientific (Leicestershire, UK). Acetonitrile and acetone were of HPLC grade and obtained from Merck (Darmstadt, Germany). The code letters used for the fatty acids are Po, palmitoleic; L, linoleic; Ln, linolenic; M, myristic; O, oleic; P, palmitic; and S, stearic.

Samples. Samples of 30 different wild edible mushrooms (Table 1; their composition in fatty acids was previously reported in the cited references) were collected in Bragança (northeastern Portugal) between 2005 and 2010. Taxonomical identification of sporocarps was made, and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. All of the samples were lyophilized (Ly-8-FM-ULE, Snijders, The Netherlands), reduced to a fine dried powder (20 mesh), and mixed to obtain a homogenate sample.

Triacylglycerol Analysis. The sample (~3 g) was submitted to an extraction with petroleum ether (40−60 °C) performed in a Soxhlet apparatus for 1.5 h. The chromatographic analyses were carried out according to the procedure previously described,[21] with a Jasco (Tokyo, Japan) HPLC system, equipped with a PU-1580 quaternary pump and a Jasco AS-950 automatic sampler with a 10 μL loop. The chromatographic separation of the compounds was achieved with a Kromasil 100 C_{18} (5 μm; 250 × 4.6 mm) column (Teknokroma, Barcelona, Spain) operating at room temperature (≈20 °C). The mobile phase was a mixture of acetone and acetonitrile (70:30), in an isocratic mode, at an elution rate of 1 mL/min. Detection was performed with an evaporative light-scattering detector (ELSD) (model 75-Sedere, Alfortville, France) with the following settings: evaporator temperature, 40 °C; air pressure, 3.5 bar; and photomultiplier sensitivity, 6. With the selectivities (R, relative retention times to LLL) taken into account, peaks were identified according to the logarithms of R in relation to homogeneous TAG standards. Quantification of the

peaks was made by internal normalization of chromatographic peak area, and the results were expressed in relative percentage, assuming that the detector response was the same for all of the compounds. Data were analyzed using Borwin-PDA Controller software (JMBS, France).

Statistical Analysis. Two samples of each mushroom species were used. For each mushroom sample, two extractions were performed, and each extract was injected twice in the HPLC system. Data were expressed as the mean ± standard deviation. All of the statistical tests were performed at a 5% significance level using the SPSS software, version 18.0 (SPSS Inc.).

Analysis of Variance. The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Kolmogorov−Smirnov with Lilliefors correction and Levene tests, respectively. In the cases when statistical significance differences were identified, the dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple-comparison tests, when homoscedasticity was verified or not, respectively.

Principal Component Analysis (PCA). PCA was applied as a pattern recognition unsupervised classification method. PCA transforms the original, measured variables into new uncorrelated variables called principal components. The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on.[18] The number of dimensions to keep for data analysis was evaluated by the respective eigenvalues (which should be >1), by Cronbach's α parameter (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the number of components selected.[22]

Stepwise Linear Discriminant Analysis (LDA). LDA was used to classify the mushroom species according to their TAG profiles. A stepwise technique, using the Wilks' λ method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination procedures; before a new variable is selected to be included, it is verified whether all variables previously selected remain significant.[19,20,23] Discriminant analysis defines a combination of varieties in a way that the first function furnishes the most general discrimination between groups, the second provides the second most, and so on.[24] To verify which canonical discriminant functions were significant, the Wilks' λ test was applied. To avoid overly optimistic data modulation, a leave-one-out cross-validation procedure was carried out to assess the model performance. Moreover, the sensibility and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group.[25] Sensibility was calculated by dividing the number of samples of a specific group correctly classified by the total number of samples belonging to that specific group. Specificity was calculated by dividing the number of samples of a specific group classified as belonging to that group by the total number of samples of any group classified as belonging to that specific group.

RESULTS AND DISCUSSION

In the absence of similar publications reporting TAG profiles of wild edible mushrooms, Table 1 presents the FAs with higher representativeness among the TAGs of the studied species. Table 2 shows the mean values obtained for TAG profiles of each mushroom species. Besides the evaluated compounds, OLLn was also found in *Laccaria amethystina*. The values are presented in relative percentage, because in the particular case of TAG, the existence of high-purity standards with a mixed FA composition is limited. However, even if reference material was commercially available, the diversity of TAG molecules in each oil would make virtually impossible the construction of a calibration curve for each TAG. Accordingly, the relative peak areas might be readily converted into relative TAG concentration, assuming linearity and uniformity of the detector signal, regardless of the TAG molecules and absolute concentration.[18] Using S = saturated, M = monoenoic, D = dienoic, and T = trienoic acids, the following order of chromatographic separation is generally obtained: SSS > SSM > SMM> SSD > MMM > SMD > MMD > SDD > SST > MDD > SMT > MMT > DDD > SDT > MDT > DDT > STT > MTT > DTT > TTT.[26] TAGs found in this work (presented in Table 2 according to their elution time) followed the expected order: PPO (SSM) > POO (SMM) > OOO (MMM) > POL (SMD) > OOL (MMD) > PLL (SDD) > OLL (MDD) > LLL (DDD) > LLLn (DDT) > LLnLn (DTT). Furthermore, and even though this conclusion cannot be drawn so directly, the obtained profiles are generally in agreement with the FA percentages (Table 1) quantified by several researchers in these mushroom species.

Analysis of Variance (ANOVA). With regard to the main purpose of this work, that is, assessing the TAG profile as a mushroom taxonomical marker, the Levene test showed that the assumption of equality among variances could be made only for OLL and OOL. Even so, to facilitate the analysis, and because the statistical differences ($p < 0.05$) were always significant (as detected by the one-way ANOVA test), the differences among mushroom species were classificated by means of the Tamhanes T2 test. The multiple comparisons allowed the conclusion that TAG profiles of the evaluated species were quite dissimilar. For instance, the maximal values for each TAG were exclusive for a single mushroom, except in the case of PPO (LLnLn, *Fistulina hepatica*, 22 ± 1; LLLn, *Lycoperdon umbrinum*, 27 ± 1; LLL, *Leucoagaricus leucothites*, 57 ± 1; OLL, *Leucopaxillus giganteus*, 36 ± 1; PLL, *Chlorophyllum rhacodes*, 35 ± 1; OOL, *Sarcodon imbricatus*, 42 ± 1; POL, *Clavariadelphus pistillaris*, 28.9 ± 0.4; OOO, *Lycoperdon molle*, 60 ± 1; POO, *Amanita caesarea*, 33 ± 1; PPO, *Macrolepiota procera*, 19 ± 1; and *Boletus edulis*, 18 ± 1). As an example, the HPLC-ELSD TAG profiles of *Lycoperdon molle* (A) and *Ramaria aurea* (B) can be observed in Figure 1. The significant differences found among the mean values for each TAG are designated by different letters in each column, and as can be seen, most of the values could be differentiated from each other. These differences were a good preliminary indicator of the ability of TAG profile to act as a taxonomical marker. This assumption was checked through a PCA, as an unsupervised classification technique, and LDA, as a supervised classification technique.

Principal Component Analysis. PCA was applied using different labeling variables: order, family, or genus. In each case, the first two dimensions were considered. The reliability of these dimensions was assured by the value of Cronbach's α parameter (first dimension, 0.769; second dimension, 0.558) and the related eigenvalue (first dimension, 3.251; second dimension, 2.008). The selected dimensions account for most of the variance of all quantified variables (32.5 and 20.1%, respectively). Third and fourth dimensions were also reliable (Cronbach's α third dimension, 0.286, and fourth dimension, 0.090; eigenvalue third dimension, 1.347, and fourth dimension, 1.088) and would include 77% of the variance instead of 53%, but the correspondent output would not allow a meaningful interpretation. The effects of the variables more correlated with each considered dimension (LLL, PLL, OOO, OOL, and POO for the first; OLL, POL, LLLn, and OOO for the second) allowed higher separation when genus was used as a labeling variable. With regard to the relationship between the objects and variables (Figure 2), it is clear that *Lycoperdon*, *Clavariadelphus*, and *Chlorophylum* are characterized for having high LLLn, POL, and PLL percentages, respectively (dashed ellipses),

Table 2. Triacylglycerol Composition (Percent)[a]

		LLnLn	LLLn	LLL	OLL	PLL	OOL	POL	OOO	POO	PPO
species	*Agaricus silvaticus*	9.4 ± 0.3 e	nd	29 ± 1 f	12 ± 1 ij	17 ± 1 d	12 ± 1 no	4.3 ± 0.2 lmn	11.7 ± 0.4 k	nd	4.2 ± 0.2 de
	Agaricus silvicola	1.2 ± 0.1 jkl	0.4 ± 0.1 gh	47 ± 1 b	8 ± 1 no	26 ± 1 b	9 ± 1 p	0.5 ± 0.1 qr	2.8 ± 0.3 no	1.7 ± 0.3 i	3.2 ± 0.3 efg
	Amanita caesarea	nd	nd	3.2 ± 0.1 qr	5.0 ± 0.2 q	1.6 ± 0.1 mno	23 ± 1 gh	5.5 ± 0.5 kl	24 ± 1 f	33 ± 1 a	4.3 ± 0.2 de
	Armillaria mellea	0.18 ± 0.02 no	0.19 ± 0.02 ghi	3.5 ± 0.2 q	12 ± 1 hi	2.4 ± 0.3 jklmn	25 ± 1 g	9 ± 1 ef	30 ± 1 e	16 ± 1 d	1.3 ± 0.1 ijkl
	Boletus edulis	nd	nd	9.2 ± 0.3 lm	11.0 ± 0.3 ijk	3.1 ± 0.1 jkl	21 ± 1 ijk	22 ± 1 b	15.2 ± 0.5 ij	nd	18 ± 1 a
	Boletus erythropus	nd	nd	19.2 ± 0.2 j	9.4 ± 0.3 lm	7.3 ± 0.3 h	32 ± 2 d	9.5 ± 0.2 ef	5.8 ± 0.3 m	9 ± 1 f	7 ± 1 c
	Boletus fragrans	1.2 ± 0.3 jkl	2.0 ± 0.2 e	34 ± 1 d	11 ± 1 jkl	8.8 ± 0.4 g	16 ± 1 m	8 ± 1 fgh	4.9 ± 0.3 m	6.8 ± 0.5 g	8 ± 1 c
	Boletus impolitus	1.7 ± 0.2 ij	0.41 ± 0.04 gh	24 ± 1 h	5.5 ± 0.4 pq	5.9 ± 0.5 i	15.8 ± 0.5 m	7.0 ± 0.3 ij	24.8 ± 0.4 f	13.6 ± 0.3 e	1.2 ± 0.1 jkl
	Boletus reticulatus	1.3 ± 0.2 jkl	1.6 ± 0.1 e	6.9 ± 0.5 no	8.8 ± 0.4 mn	0.13 ± 0.01 p	39.4 ± 0.5 b	2.5 ± 0.3 op	36 ± 1 c	nd	4.0 ± 0.3 de
	Bovista aestivalis	10.8 ± 0.4 d	4.4 ± 0.2 b	13.6 ± 0.3 k	18 ± 1 def	2.3 ± 0.2 jklmn	19.3 ± 0.5 kl	4.2 ± 0.3 mn	20 ± 1 h	nd	8 ± 1 c
	Bovista nigrescens	12.0 ± 0.4 c	3.6 ± 0.3 c	19 ± 1 j	16.6 ± 0.4 fg	2.0 ± 0.3 klmn	18.2 ± 0.5 l	5.2 ± 0.2 kl	15.9 ± 0.5 i	nd	7.7 ± 0.3 c
	Calocybe gambosa	nd	nd	32 ± 1 e	17 ± 1 efg	6.0 ± 0.4 i	19 ± 1 l	4.8 ± 0.3 klm	13.7 ± 0.4 j	6.2 ± 0.4 g	2.2 ± 0.3 ghij
	Cantharellus cibarius	0.8 ± 0.1 klmn	0.28 ± 0.05 ghi	8 ± 1 mno	6.8 ± 0.5 op	3.4 ± 0.3 j	20 ± 1 jkl	5.1 ± 0.4 klm	40 ± 1 b	13.4 ± 0.5 e	2.6 ± 0.4 fgh
	Chlorophyllum rhacodes	1.39 ± 0.02 jk	0.06 ± 0.01 hi	31 ± 1 ef	19 ± 1 d	35 ± 1 a	11 ± 1 op	1.4 ± 0.1 pq	0.25 ± 0.02 q	0.23 ± 0.03 jk	0.36 ± 0.02 l
	Clavariadelphus pistillaris	1.6 ± 0.2 j	nd	5.0 ± 0.2 pq	18.3 ± 0.4 de	7.8 ± 0.3 gh	34 ± 1 cd	28.9 ± 0.4 a	2.0 ± 0.2 op	1.6 ± 0.3 ij	1.1 ± 0.2 jkl
	Cortinarius violaceus	0.6 ± 0.2 mn	1.1 ± 0.2 f	25.0 ± 0.4 gh	22 ± 1 c	21 ± 1 c	10 ± 1 op	11 ± 1 d	5.1 ± 0.4 m	nd	4.7 ± 0.2 d
	Fistulina hepatica	22 ± 1 a	nd	43 ± 1 c	13 ± 1 hi	2.2 ± 0.2 klmn	6.7 ± 0.3 q	6.0 ± 0.4 jk	4.2 ± 0.2 mn	nd	3.5 ± 0.2 def
	Laccaria amethystina	7.2 ± 0.2 f	nd	26 ± 1 g	10.3 ± 0.3 jklm	3.1 ± 0.2 jk	15.8 ± 0.4 m	8.4 ± 0.5 fg	14.2 ± 0.5 j	9.9 ± 0.4 f	0.5 ± 0.1 l
	Lepista nuda	4.7 ± 0.2 g	1.0 ± 0.1 f	22.2 ± 0.5 i	27 ± 1 b	5.0 ± 0.3 i	22 ± 1 hi	10 ± 1 de	5.1 ± 0.2 m	nd	2.5 ± 0.2 fghi
	Leucoagaricus leucothites	nd	nd	57 ± 1 a	10 ± 1 klm	14.6 ± 0.5 e	11 ± 1 op	3.5 ± 0.3 no	1.7 ± 0.2 opq	nd	2.2 ± 0.1 ghij
	Leucopaxillus giganteus	0.4 ± 0.1 no	nd	30 ± 1 ef	36 ± 1 a	11 ± 1 f	14 ± 1 mn	7.1 ± 0.1 hij	0.42 ± 0.04 pq	0.21 ± 0.03 jk	nd
	Lycoperdon molle	2.3 ± 0.2 hi	0.39 ± 0.02 ghi	30 ± 1 f	3.1 ± 0.3 r	1.6 ± 0.2 mno	1.2 ± 0.1 r	nd	60 ± 1 a	1.1 ± 0.2 ijk	0.8 ± 0.2 kl
	Lycoperdon umbrinum	13.8 ± 0.5 b	27 ± 1 a	34 ± 1 d	4.9 ± 0.5 q	5.1 ± 0.4 i	3.2 ± 0.2 r	1.2 ± 0.2 qr	9.3 ± 0.3 l	nd	1.9 ± 0.3 hijk
	Macrolepiota procera	0.9 ± 0.1 klm	nd	1.6 ± 0.3 r	8.7 ± 0.4 mn	2.6 ± 0.2 jklm	22 ± 1 hi	8 ± 1 ghi	9 ± 1 l	28 ± 1 b	19 ± 1 a
	Ramaria aurea	1.7 ± 0.1 j	nd	6.0 ± 0.2 op	11.1 ± 0.4 ijkl	1.3 ± 0.1 nop	29 ± 1 e	3.9 ± 0.1 mn	39.7 ± 0.3 b	6.9 ± 0.5 g	0.4 ± 0.1 l
	Ramaria botrytis	1.2 ± 0.2 jkl	0.5 ± 0.1 g	7.9 ± 0.1 mn	13 ± 1 h	1.8 ± 0.2 lmn	25 ± 1 fg	4.6 ± 0.2 lmn	33 ± 1 d	10 ± 1 f	3.0 ± 0.4 efgh
	Russula cyanoxantha	4.3 ± 0.3 g	1.6 ± 0.1 e	13 ± 1 k	10.1 ± 0.4 jklm	5.0 ± 0.5 i	22 ± 1 hij	8.9 ± 0.5 fg	22 ± 1 g	nd	14 ± 1 b
	Sarcodon imbricatus	2.4 ± 0.2 h	2.7 ± 0.2 d	1.5 ± 0.2 r	7.8 ± 0.4 no	1.8 ± 0.3 lmn	42 ± 1 a	13 ± 1 c	4.2 ± 0.4 mn	24.5 ± 0.5 c	nd
	Tricholoma imbricatum	0.4 ± 0.1 mno	nd	9.8 ± 0.2 l	15.8 ± 0.5 g	1.4 ± 0.1 mno	27 ± 1 f	7.8 ± 0.4 ghi	29 ± 1 e	9 ± 1 f	0.4 ± 0.1 l
	Tricholoma portentosum	0.19 ± 0.05 no	1.7 ± 0.2 e	4.0 ± 0.4 q	11 ± 1 ij	0.4 ± 0.1 op	36 ± 1 c	3.6 ± 0.5 no	37 ± 1 c	4.3 ± 0.2 h	2.3 ± 0.4 ghij
homocedasticity[c]	*P* value	0.004	<0.001	0.004	0.428	0.001	0.176	0.048	0.022	<0.001	<0.001
one-way ANOVA[d]	*P* value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a]The results are presented as the mean ± SD. Means within a column with different letters differ significantly ($p < 0.05$). The results were evaluated using either the multiple-comparison Tukey's HSD or Tamhane's T2 test, depending on the fulfillment or not of the homoscedasticity requirement. [c]Homoscedasticity among cultivars was tested by means of the Levene test. [d]$P < 0.05$ meaning that the mean value of the evaluated parameter of at least one cultivar differs from the others (in this case multiple comparison tests were performed).

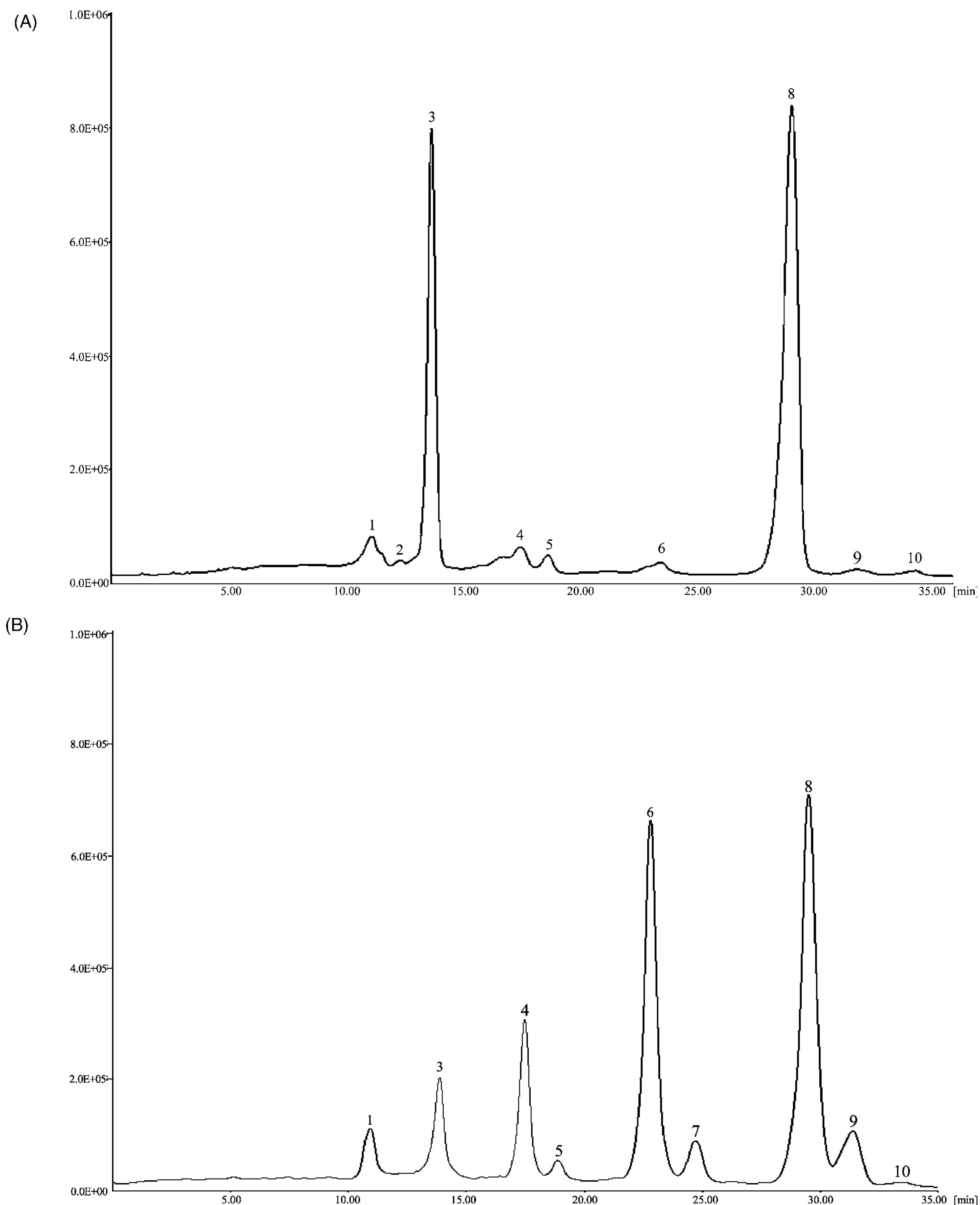

Figure 1. Individual chromatogram of TAG profile in (A) *Lycoperdon molle* and (B) *Ramaria aurea*. Peaks: 1, LLnLn; 2, LLLn; 3, LLL; 4, OLL; 5, PLL; 6, OOL; 7, POL; 8, OOO; 9, POO; 10, PPO.

but the remaining genera are somehow difficult to characterize. Although the lower dimensional solutions often conceal differences among variables, PCA results were satisfactory, and there was no need to increase the number of dimensions. In fact, the results plotted in Figure 2 show that, in general, the TAG profiles recorded for different mushroom genera

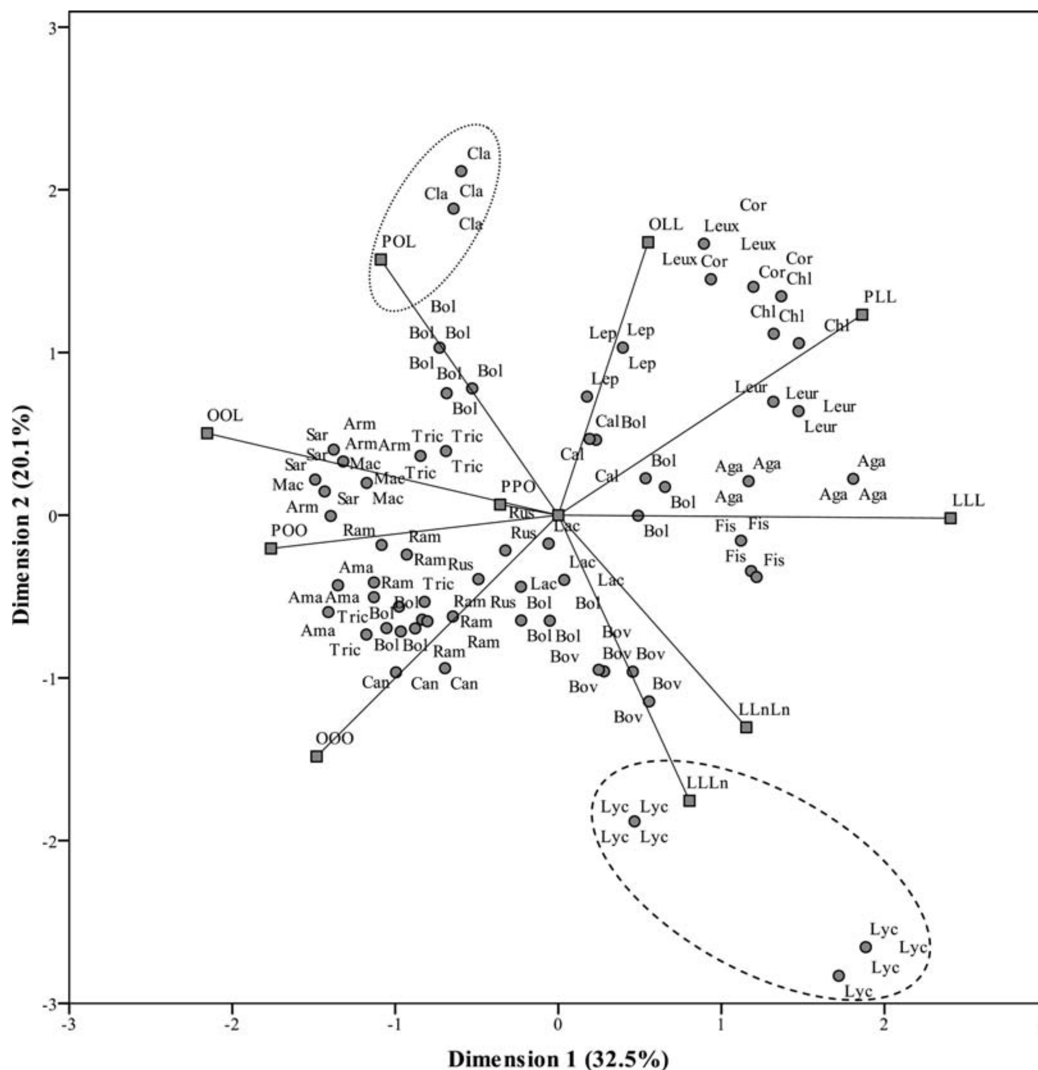

Figure 2. Biplot of objects and component loadings using genus as labeling variable. Aga, *Agaricus*; Ama, *Amanita*; Arm, *Armillaria*; Bol, *Boletus*; Bov, *Bovista*; Cal, *Calocybe*; Can, *Camtharellus*; Chl, *Chlorophyllum*; Cla, *Clavariadelphus*; Cor, *Cortinarius*; Fis, *Fistulina*; Lac, *Laccaria*; Lep, *Lepista*; Leur, *Leucoagaricus*; Leux, *Leucopaxillus*; Lyc, *Lycoperdon*; Mac, *Macrolepiota*; Ram, *Ramaria*; Rus, *Russula*; Sar, *Sarcodon*; Tric, *Tricholoma*.

evaluated in this study contain valuable information that may be used as an effective tool for their differentiation. Actually, the spatial distribution of the object points was improved with the lowering of taxonomical rank, indicating that TAG profile is most related with the lowest ranks. This is in accordance with the genetic control of the stereospecific distribution of fatty acids (FAs) on the glycerol molecule, which is typical for each species.[17]

Linear Discriminant Analysis. To confirm this hypothesis a LDA was also performed, attempting to separate the assayed mushroom species on the basis of their taxonomical ranks. The significant independent variables (TAG) were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only those that showed a statistically significant classification performance ($p < 0.05$) were kept for analysis. The analysis was applied considering order, family, genus, or species as grouping variables. As it would be expected after the performed PCA, the classification performance decreased from lower to higher taxonomical ranks (Table 3). In fact, when mushrooms were grouped by species, 100.0% of the samples were correctly classified for the originally grouped cases, as well as for the cross-validated grouped cases, but due to practical reasons, the presented output (Figure 3) is the one obtained using genus as grouping variable. The three plotted functions integrated 89.2% of the observed variance (first, 59.1%; second, 15.8%; third, 14.3%). As can be observed, although the clusters are well individualized, the model joined (dot and dashed ellipses) genera belonging to the same family (*Armillaria*, *Calocybe*, *Lepista*, *Leucopaxillus*, and *Tricholoma* belonging to Tricholomataceae; *Agaricus*, *Chlorophylum*, and *Leucoagaricus* belonging to Agaricaceae; *Bovista* and *Lycoperdon* belonging to Lycoperdaceae).

Table 3. LDA Parameters Considering Different Grouping Variables

		correctly classified groups		
grouping variable	no. of functions (Wilks' λ test)	original grouped cases	cross-validated grouped cases	variables not in the analysis
order	$p < 0.001$	75.8	64.2	OLL
family	$p < 0.001$	95.8	93.3	LLL
genus	$p < 0.001$	99.2	99.2	OOO
species	$p < 0.001$	100.0	100.0	POL

In summary, the set of analyzed mushrooms presented very particular intrinsic differences in their TAG profiles. Hence,

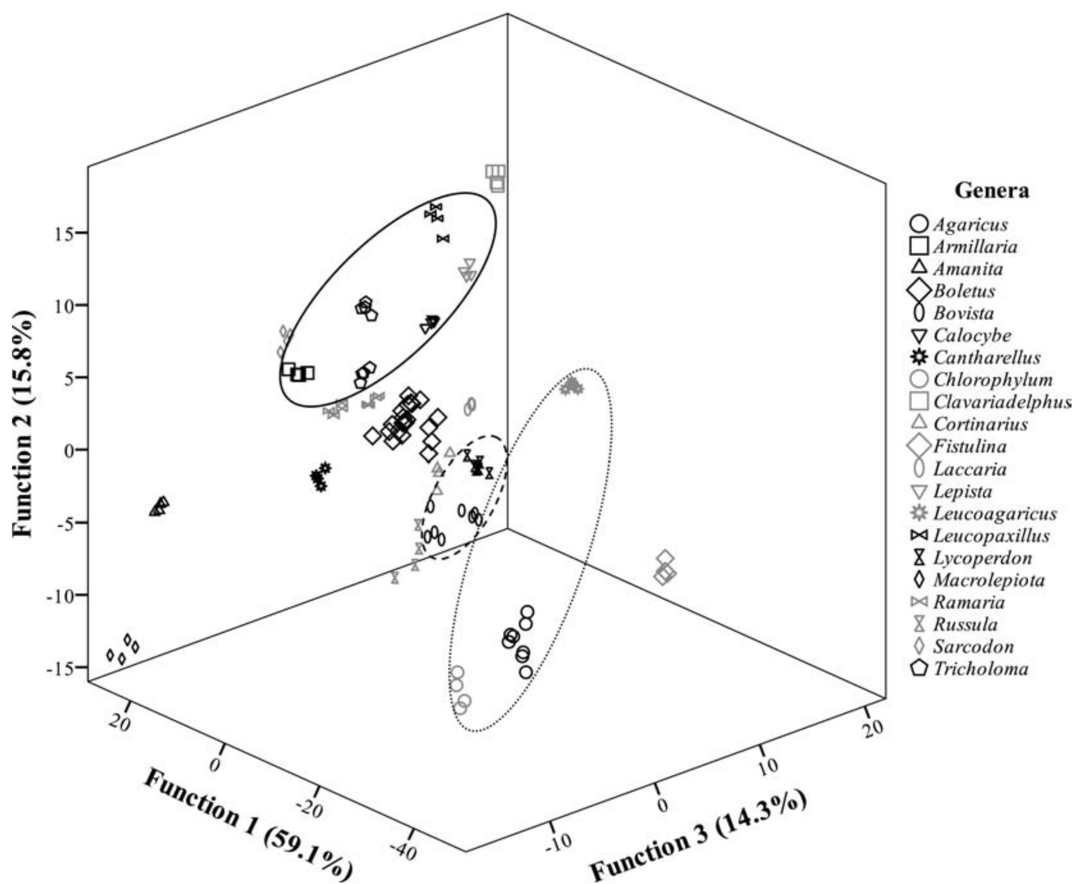

Figure 3. Canonical analysis of mushroom genera based on triacylglycerol profiles.

chemical assessment linked to stereospecific analysis of TAG can be very useful in checking mushroom species. In fact, the usefulness of stereospecific analysis of TAG as a potential species discriminator was already indicated in vegetable oils.[32] Herein, the results obtained for TAG analysis showed the ability to assemble the tested mushroom species within single groups, indicating a high degree of specificity possibly derived from the genetic control of the stereospecific distribution of FA on the glycerol molecule.[17] Therefore, the TAG profile seems to be related with the most specific taxonomical rank, proving that it might be used as a practical tool to identify a particular mushroom species. Because the conservation techniques applied to mushrooms often change their physical properties, the TAG profile might be a feature for species identification.

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Notes

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