JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Truffle Aroma Analysis by Headspace Solid Phase Microextraction

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An experimental design has been used to optimize the extraction of volatile compounds from summer truffle aroma (*Tuber aestivum*) by using headspace solid phase microextraction. The extracted compounds have been analyzed by gas chromatography with a flame ionization detector and by gas chromatography−mass spectrometry (GC-MS). In an attempt to develop an objective method to fully characterize truffle aroma, a fiber of medium polarity (for flavors) was used to avoid discrimination toward very nonpolar and polar volatile compounds. To optimize the extraction conditions, a response surface experimental design was applied considering three factors such as extraction temperature, equilibrium time, and extraction time. From the statistical analysis of the experimental design, it was possible to determine that the most important factor influencing the abundance of aroma compounds was the extraction temperature. Optimal extraction temperature was established at ∼50 °C. By using GC-MS, it was possible to identify 37 compounds, most of them previously described as responsible for truffle aroma.

KEYWORDS: Aroma; experimental design; HS-SPME; optimization; response surface; solid phase microextraction; truffle; *Tuber aestivum*

INTRODUCTION

Tuber aestivum (summer truffle), *Tuber melanosporum* (black Perigord), *Tuber magnatum* Pico (white truffle), and other truffles belonging to the genus *Tuber* F.H. Wigg are subterranean fungi highly appreciated for their unique and characteristic aroma. Their culinary and commercial value is mainly due to their organoleptic properties, such as their aroma, the quality of which clearly provides the economic value of such edible fungi. In general, the demand for truffles greatly exceeds their availability because only ∼20 tons is produced worldwide. Their value can reach >U.S.$3000/kg (considering that white truffles are the most expensive species of truffles). At present there are under development large plantations in countries of southern Europe and in others such as New Zealand, Australia, and the United States (*1*).

Tuber aestivum, also called summer truffle, has a moderately intense aroma and is appreciated because of its moderate cost as compared to other *Tuber* species and its aroma quality. *T. aestivum* is widely distributed in numerous European countries but mainly in Spain, France, and Italy. It is of considerable commercial interest because *T. aestivum* has a lower cost than other truffles of the same genus, such as *Tuber melanosporum* and *Tuber magnatum*. Therefore, an objective evaluation of its aroma is desired in order to identify the above different truffle species. This includes, for example, the ability to detect *T. aestivum* in products involving a mixture of different truffles (to guarantee the authenticity of such products) or even to determine the influence of different growing parameters on the aroma fraction of such valuable fungi.

Some research has been devoted to the study of the aroma quality of different truffle species and their changes as a function of both species and preservation methods (*2*−*4*). The study of truffle aroma has also been suggested as a way of authenticating different truffle species, for example, to detect the presence of *Tuber borchii* used as adulterant for the more highly prized truffle species, *T. magnatum* (*5*).

The most used analytical techniques to extract and concentrate the volatile components of food aroma have been those based on headspace analysis (*6*). For truffle aroma, several papers have been published dealing with the identification of volatile compounds and the study of the effect of processing on the original aroma of different *Tuber* species (*2*, *7*−*10*). For example, black Perigord truffle aroma has been studied using dynamic headspace followed by cryogenic adsorption on Tenax gas−liquid chromatography (GLC) (*11*), followed by analysis

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10.1021/jf025609t CCC: $22.00
Published on Web 09/21/2002

by gas chromatography−mass spectrometry (GC-MS). Volatile compounds in Italian white truffles (*T. magnatum* Pico) have also been determined by employing purge and trap GC-MS (*12*). Headspace solid phase microextraction (HS-SPME) combined with GC-MS has been used to detect the volatile organic sulfur compounds in the aroma of white and black truffles (*T. magnatum* Pico and *T. melanosporum*, respectively) (*13*), but no references have been found on the use of HS-SPME to objectively describe the aroma of truffles.

The objective of the present research has been the optimization of the extraction of volatile compounds from summer truffle aroma (*T. aestivum*) by using HS-SPME. A fiber of medium polarity has been used as a way of reducing discrimination toward very nonpolar and polar volatile compounds, thus allowing an objective method to fully characterize truffle aroma. To optimize the extraction conditions, a response surface experimental design was applied examining three factors: extraction temperature, equilibrium time, and extraction time.

EXPERIMENTAL PROCEDURES

Truffles. Truffles used in this work belong to the species *T. aestivum* and were collected in Navaleno (Soria, Spain). These truffles were previously characterized on the basis of carpophore morphology and spore shape using a Nikkon microscope. On the basis of the classification of Riousset et al. (*14*) the sample was identified as *T. aestivum sensu* Châtin *non* Vittadini. The truffles were deep frozen just after their collection and were kept at freezing temperature until extraction.

Immediately before analysis, ∼1.5 g of truffle was cut from the frozen sample, allowed to thaw at ambient temperature for 15 min, and cut into thin slices of truffle flesh using a sharp knife.

Headspace Solid Phase Microextraction. An SPME holder (Supelco, Bellefonte, PA) was used in performing the experiments. A fused silica fiber coated with a 50/30 μm layer of divinylbenzene/carboxen/polydimethylsiloxane (Supelco) was chosen to extract the volatile components from the truffles.

The fiber was conditioned following the manufacturer's instructions previous to its use.

Approximately 1 g of sample was placed in a 4 mL vial closed with a plastic film. Once the desired temperature had been reached in a water bath, the vial was placed inside the bath and was allowed to condition for the equilibrium time (no fiber exposition). After the equilibrium time, the fiber was introduced into the vial and exposed to the headspace of the sample during the corresponding extraction time (depending on the experimental design).

Experimental Design for Headspace Solid Phase Microextraction. The optimization of the HS-SPME conditions for truffle aroma was performed via the use of a second-order rotatable central composite experimental design that consisted of factorial 2^3 plus 6 star points plus 6 replicates in the center of the design, to estimate the experimental error. The variables selected for the HS-SPME process were extraction temperature (T, in °C), equilibrium time (t_{eq}, in min), and extraction time (t_{ext}, in min). To be able to approach the optimal conditions more accurately, five levels were considered, being −1.68179 for the lowest level, 0 for the medium level, and +1.68179 for the highest level. For each factor, the range of physical values were selected mainly to avoid artifact formation and based on previous studies done in our laboratory with different food materials (*15*); therefore, the temperature range studied was between 30 and 70 °C, extraction time was between 5 and 30 min, and equilibrium time was between 0 and 10 min. Twenty experiments were performed in randomized order. Factor levels were converted to experimental values by using, for each factor, its level-physical value correspondences (level −1.68179 values of the factors, T = 30 °C, t_{eq} = 0 min, and t_{ext} = 5 min; level −1 values, T = 38.1 °C, t_{eq} = 2 min, and t_{ext} = 10.1 min; level 0 values, T = 50 °C, t_{eq} = 5 min, and t_{ext} = 17.5 min; level 1 values, T = 61.9 °C, t_{eq} = 8 min, and t_{ext} = 24.9 min; level 1.68179 values, T = 70 °C, t_{eq} = 10 min, and t_{ext} = 30 min). Resulting values were rounded to the nearest integer.

Aroma Analysis by Gas Chromatography—Mass Spectrometry. A Perkin-Elmer Autosystem XL gas chromatograph (Perkin-Elmer, Norwalk CT) equipped with a programmed split/splitless injector (PSS) and a flame ionization detector (FID) was used to perform all of the GC analyses. The system was coupled to a Perkin-Elmer chromatography software system (Turbochrom). A 30 m × 0.25 mm i.d. fused silica capillary column (Perkin-Elmer) coated with a 0.25 μm layer of Carbowax 20 M (PE-WAX20M) was employed. Thermal desorption of the compounds from the fiber coating took place in the GC injector at 200 °C for 15 min in splitless mode for 10 min. Other operating conditions were as follows: detector temperature, 250 °C; oven temperature program, from 40 to 60 °C at 10 °C min^{-1} and then to 200 °C (15 min at constant temperature) at 3 °C min^{-1}. Helium at 15 psig was used as carrier gas with a flow rate of 1.0 mL/min.

GC-MS analysis was carried out using an Agilent-6890 GC system coupled to an Agilent-5973 mass spectrometer. The capillary column used in the GC-MS was a 50 m × 0.25 mm i.d. fused silica capillary column coated with a 0.2 μm layer of Carbowax 20 M. The chromatographic program was as mentioned previously. Compounds were identified by comparison of the spectra with those in a mass spectrometry library (Wiley) and with data found in the literature.

Statistical Experimental Design and Data Analysis. Statistical calculations and analysis were performed using Statgraphics Plus for Windows v. 5.0 software (Statistical Graphics Corp., Manugistics Inc., Silver Spring, MD, 2000).

RESULTS AND DISCUSSION

One of the major drawbacks associated with the use of SPME is the discrimination against compounds having different polarities as a function of the chemical nature of the fiber used. For example, Pelusio et al. (*13*) found that when using the polydimethylsiloxane coating (nonpolar), a strong discrimination was observed against the very volatile compounds in the black truffle and, therefore, a true picture of the relative quantities of the aroma constituents could not be obtained. In an attempt to develop an objective method to fully characterize summer truffle aroma, a fiber of medium polarity (for flavors) was used to reduce discrimination toward very nonpolar and polar volatile compounds. Even though SPME always shows discrimination (that is, different relative recoveries for each compound in the sample) when applied to multicomponent analysis, the fiber of medium polarity seems to be the best choice for characterizing truffle aroma samples from their volatile composition.

As was mentioned above, the experimental values of the variables (factors) were selected to cover a wide range of conditions and combined by means of experimental design techniques (a rotatable central composite design). To be able to maximize the extraction of volatile components of *T. aestivum*, a response based on the sum of areas of the GC analysis of the HS-SPME of truffle at the conditions of the design (*R1*) was used. This response provides information that can be related to the intensity of the extracted aroma.

The responses obtained after performing the 20 experiments established by the experimental design are shown in **Table 1** along with the physical parameters of the different experiments evaluated.

The design was evaluated by means of an analysis of variance (ANOVA). By using this analysis it is possible to study the statistical significance of each effect and interactions between the different factors. **Figure 1** shows a standardized Pareto chart for the response *R1*. The bar length in the graph provides information about the importance of the contribution to the model of each experimental factor and interaction. As can be seen, only the extraction temperature (T) and the square of the extraction temperature (T^2) have an important effect on the truffle aroma extraction. The vertical line in the chart sets the

Table 1. Experimental Conditions and Response Values (*R1*) of the Rotatable Central Composite Design Used To Study *T. aestivum* Aroma Extraction by HS-SPME

expt	*T* (°C)	t_{ext} (min)	t_{eq} (min)	*R1* (area counts)
13	50.0	17.50	0.00	11635.5
18	50.0	17.50	5.00	11870.1
5	38.1	10.07	7.97	6122.6
11	50.0	5.00	5.00	11395.6
6	61.9	10.07	7.97	10148.9
20	50.0	17.5	5.00	9848.3
1	38.1	10.07	2.03	5799.1
3	38.1	24.93	2.03	8926.9
19	50.0	17.50	5.00	9608.7
17	50.0	17.50	5.00	9209.2
15	50.0	17.50	5.00	11823.7
12	50.0	30.00	5.00	12671.7
14	50.0	17.50	10.00	12356.1
10	70.0	17.50	5.00	6928.8
7	38.1	24.93	7.97	8419.2
9	30.0	17.50	5.00	4766.5
8	61.9	24.93	7.97	10939.2
4	61.9	24.93	2.03	10439.6
2	61.9	10.07	2.03	7814.9
16	50.0	17.50	5.00	9302.6

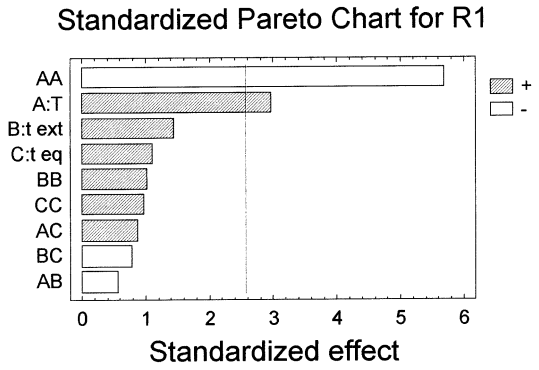

Figure 1. Standarized Pareto chart for the response (*R1*, sum of areas of the GC analysis of the HS-SPME of truffle at the different conditions tested) considered in the study.

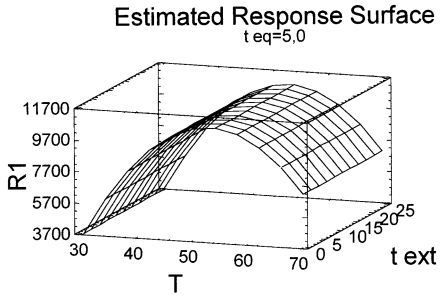

Figure 2. Response surface plot for *R1* (sum of areas of the GC analysis of the HS-SPME of truffle at the different conditions tested) versus *T* (extraction temperature, °C) and t_{ex} (extraction time, min) at constant equilibrium time (t_{eq} = 5 min).

limit for significance at 95% confidence level. The experimental error has been calculated from the central replicates (experiments 15−20) of the experimental design as 12% (coefficient of variation, CV), which provides information about the reproducibility of the whole method (extraction and analysis). Also, the central replicates allow one to ascertain the lack of fit to the experimental model. Because the probability associated with the lack of fit to the model (*p* value) is 0.4263 (that is, >0.05), the model appears to be adequate for the observed data at the 95% confidence level. The *R*-squared statistics indicate that the model as fitted explains 83% of the variability in the response, whereas the standard error of the estimate is found to be equal to 1237 (which represents 13% of the average value of the response *R1*). By retaining only the significant factors (95% confidence level) the following regression equation was obtained:

$$R1 = -27227.4 + 1436.97T - 13.5254T^2$$

In this case, the model adequately describes the observed data (lack-of-fit, $p = 0.510$) at the 95% confidence level. The *R*-squared statistics indicate that the model can explain 68% of the variability in the response. The standard error of the estimate is equal to 1397 (which represents 14.7% of the average value of the response *R1*, that is, 9501.36). Also, the root-mean-square error of prediction by cross-validation (RMSEP) has been calculated as an approximation of the prediction error. RMSEP is defined by the equation

$$\text{RMSEP} = \sqrt{[\sum_{i=1}^{n}(R_i - \hat{R}_{(i)})^2]/n}$$

where *n* is the number of samples in the study ($n = 20$), R_i is the true sum of areas, and $\hat{R}_{(i)}$ is the predicted sum of areas when the regression model is constructed without the sample *i*.

Table 2. Relative Percentages (Percent Normalized Areas) and Retention Times (t_r) of *T. aestivum* Aroma Compounds Extracted by HS-SPME and Tentatively Identified Using MS Databases

n	t_r (min)	compound	% normalized area
1	3.74	acetaldehyde	0.77
2	3.94	dimethyl sulfide	0.48
3	4.32	2-propanone	0.67
4	4.92	ethyl acetate	0.66
5	5.10	2-butanone	2.53
6	5.26	2-methylbutanal	
7	5.31	3-methylbutanal	8.88
8	5.47	ethanol	
9	6.95	2-butanol	0.27
10	7.50	2-butenal + methylbenzene	8.10
11	8.36	hexanal	18.23
12	8.74	3-hydroxybutanal	
13	8.71	2-methyl-1-propanol	0.70
14	8.77	2-methyl-2-butenal	2.12
15	9.38	ethylbenzene	0.32
16	11.09	heptanal	5.53
17	12.51	2-methyl-1-butanol	
18	12.56	3-methyl-1-butanol + 2-methyl-1-butanol	4.50
19	13.55	3-octanone	0.36
20	14.12	unknown (see text)	0.89
21	14.87	octanal	0.70
22	15.50	octa-1,5-dien-3-ol	0.31
23	16.60	2-heptenal	2.11
24	16.91	3-octen-2-one	
25	17.92	1-hexanol	0.48
26	19.16	nonanal	0.95
27	20.94	2-octenal	9.13
28	21.57	1-methoxy-3-methylbenzene	
29	21.82	1-octen-3-ol	1.88
30	22.98	acetic acid	3.21
31	23.49	2-ethyl-1-hexanol	1.38
32	25.23	benzaldehyde	6.51
33	30.01	2(3*H*)-dihydrofuranone	4.62
34	30.24	phenylacetaldehyde	4.27
35	40.29	2,6-bis(1,1-dimethylethyl)-4-methylphenol	3.94
36	40.63	phenylethanol	4.06
37	44.24	phenol	1.40

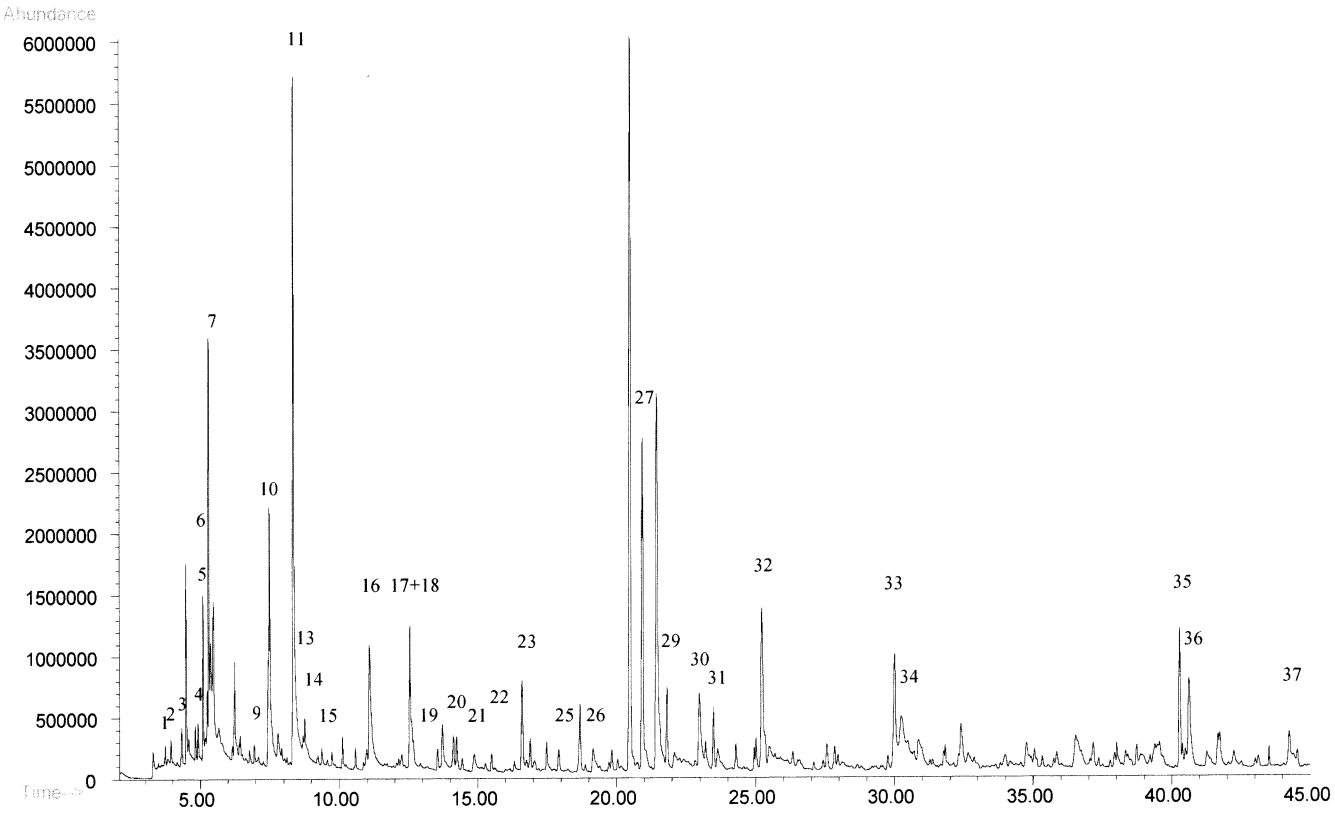

Figure 3. TIC mass chromatogram of an HS-SPME of summer truffle. Chromatographic conditions: injector temperature, 200 °C for 15 min in splitless mode for 5 min; detector temperature, 250 °C; oven temperature program, from 40 to 60 °C at 10 °C min^{-1}, to 200 °C (15 min constant) at 3 °C min^{-1}. Peak assignment is as in **Table 2**.

For the model described above, the RMSEP was 1496.8, which represents a 16% relative error in prediction.

Figure 2 shows the response surface plot for *R1*. By means of this graph it is possible to know the combination of factor levels that maximizes the truffle aroma recovered using the HS-SPME technique over the experimental region, considering an equilibrium time equal to 5 min (average of the equilibrium time tested in the design).

Temperature is the main variable that influences aroma extraction by HS-SPME. Its effect can be associated with a change in the partition coefficients of the compounds both between the sample and the headspace and between the headspace and the fiber, as well as the change in the vapor pressure of the compounds in the sample. As can be seen in **Figure 2**, the maximum extraction of the aroma compounds of the truffle is obtained at intermediate temperatures (∼54 °C), a decreasing response being obtained at lower and higher temperatures. This can be explained by a lower concentration of volatile components of the aroma in the headspace when working at lower temperatures and by a desorption of volatiles from the fiber, which occurs when the temperature is increased. As for the other factors studied, equilibrium time has no influence on the response and, therefore, was not considered in the plot of the surface response. The effect of the extraction time was not significant in the experimental region tested (from 5 to 30 min); therefore, a short extraction time can be used with no detriment on the final response.

By using the model fitted to the present experiments, an optimum extraction of the *T. aestivum* aroma can be obtained. The experimental conditions that lead to this optimum response are the following: extraction temperature of 53 °C, extraction time equal to 13.6 min, and an equilibrium time of 5 min. The fact that the optimum, in terms of extraction temperature, was found to be ∼50 °C has an additional advantage: use of such an extraction temperature avoids production of artifacts caused by overheating of the sample.

To determine the identity of the compounds in the summer truffle aroma, an analysis by GC-MS was performed. **Figure 3** shows the total ion current (TIC) mass chromatogram of an HS-SPME of summer truffle. The first consideration that has to be made is that no peaks appeared in the blank runs, thus indicating that no compounds due to the fiber or contamination can be expected. The 37 identified compounds are listed in **Table 2** along with their relative percentages (as normalized areas). Among the compounds detected and tentatively identified in the present work, acetaldehyde, dimethyl sulfide, 2-butanone, 2-methyl-1-propanol, 2-methylbutanal, 3-methylbutanal, 2-methyl-1-butanol, and 3-methyl-1-butanol have been previously found by other investigators (*8*) in the aroma of *T. aestivum*. Of the above-mentioned compounds, the most characteristic compound of *Tuber* spp. is dimethyl sulfide, which has been described as responsible for the detection of such fungi by animals (*8*−*10*); it has also been detected in different species of truffles, such as *T. melanosporum*, in which it is also the only quantitatively important sulfur volatile organic compound (VOC).

In the present work, other compounds such as ethyl acetate, 2-propanone, 2-butanol, hexanal, heptanal, 3-octanone, 1-hexanol, 1-octen-3-ol, phenylethanol, and phenylacetaldehyde have also been detected. Such compounds have been previously described in *T. melanosporum*, but this is the first time that they have been conclusively identified in *T. aestivum* (*2*, *9*, *10*, *16*). Two of them, 1-octen-3-ol and 3-octanone, have been

described as responsible of the characteristic mushroom odor of such fungi.

Other compounds were also found in the summer truffle aroma such as benzaldehyde, 1-methoxy-3-methylbenzene, 2(3*H*)-dihydrofuranone, and phenol. These compounds have been detected in stored truffle samples; thus, their presence in the samples analyzed can be due to the temperature used during the extraction process (50 °C). The compound 2,6-bis(1,1-dimethylethyl)-4-methylphenol has been cited as a volatile component of the mycelium of *T. borchii* Vitt. (*17*), but this is the first time that it has been detected in a different *Tuber* species. The compound labeled as unknown in **Table 2** has a molecular weight of 112 and the structural formula $C_7H_{12}O$, but its identification could not be confirmed on the basis of its mass spectrum.

As a final conclusion, the present study has demonstrated the usefulness of HS-SPME combined with GC-MS to extract and objectively describe the aroma of summer truffle. This technique seems to be appropriate to effectively study the evolution of the aroma in such fungi and the influence of different growing conditions on their aromatic fraction.

ACKNOWLEDGMENT

We thank Dr. Martin-Alvarez for helpful discussion of the statistical part of this study.

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Received for review April 22, 2002. Revised manuscript received July 12, 2002. Accepted July 12, 2002. This work was supported by Project FP01-41 of the Agricultural and Food Research Institute of Madrid (IMIA, Spain).

JF025609T