JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLES

Characterization and Quantification of Grape Variety by Means of Shikimic Acid Concentration and Protein Fingerprint in Still White Wines

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Protein profiles, obtained by high-performance capillary electrophoresis (HPCE) on white wines previously dialyzed, combined with shikimic acid concentration and multivariate analysis, were used for the determination of grape variety composition of a still white wine. Six varieties were studied through monovarietal wines elaborated in the laboratory: Chardonnay (24 samples), Chenin (24), Petit Manseng (7), Sauvignon (37), Semillon (24), and Ugni Blanc (9). Homemade mixtures were elaborated from authentic monovarietal wines according to a Plackett−Burman sampling plan. After protein peak area normalization, a matrix was elaborated containing protein results of wines (mixtures and monovarietal). Partial least-squares processing was applied to this matrix allowing the elaboration of a model that provided a varietal quantification precision of around 20% for most of the grape varieties studied. The model was applied to commercial samples from various geographical origins, providing encouraging results for control purposes.

KEYWORDS: Proteins; dialyze; shikimic acid; high-performance capillary electrophoresis; grape varietal recognition; still white wine; chemometric; partial least-squares (PLS)

INTRODUCTION

Proteins found in wine have mainly a vegetal origin. Other protein sources have been identified: yeast origin (*1–3*), fungus origin, and possibly bacterial origin, depending on the grape sanitary conditions (*4*). Moreover, at a very low concentration, some proteins can come from enological treatments (*4*). For 50 years, scientists have looked at protein behavior through a "magnifying glass" because protein aggregation and flocculation are responsible for white wine turbidity (*5*). To understand the protein precipitation phenomenon, many works were dedicated to protein fraction characterization (*6–10*), most of the studies being performed by electrophoretic techniques and recently reviewed (*11*).

Many authors have reported that the protein profile is related to the grape variety, important information in terms of white wine grape variety identification that is the focus of this paper. This research objective is reinforced by European régulation EU 753-2002 that allows grape variety specification on wine bottle labels even if another grape variety, up to 15%, is incorporated into the wine (*12*). Thus, it is important to have a reliable method able to quantify a relative part of each variety involved in a grape mixture wine. Different methods have been explored such as shikimic acid concentration (*13*), volatile compounds (*14*), amino acid analysis (*15*), wine near-infrared measurements (*16*), and DNA/PCR analysis (*17, 18*). Other works were dedicated to variety recognition through protein characteristics by electrospray mass spectrometry technique (*19, 20*) and by electrophoretic techniques (*21–26*). Most of these research papers limited their studies to varietal classification on grape must, which is a protein-rich medium, to only two different grape varieties wines, or even between red and white wine; until now, no study has considered a quantification approach of the grape variety present in a wine. Thus, it was important to realize a study bringing together most of the grape varieties used for commercial monovarietal wines in a unique model and to check the model's ability to quantify the percentage of each grape variety involved in a wine.

Despite its wide application for food authenticity (*27–30*) and

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10.1021/jf800117k CCC: $40.75
Published on Web 07/15/2008

its ability to provide quantitative results (*31*), high-performance capillary electrophoresis (HPCE) has been very seldom used for varietal identification in wine (*32, 33*). The aim of this work was to investigate the potential of HPCE technique to discriminate between six grape varieties (Chardonnay, Chenin, Petit Manseng, Sauvignon, Semillon, and Ugni Blanc) using wine protein profile as well as shikimic acid (SHA) concentration determined by HPLC. Multivariate data treatment was applied to protein profile and SHA concentration results to elaborate a quantification model that was applied to commercial still white wines.

MATERIALS AND METHODS

Reagents. Shikimic acid (98%), KOH, phosphoric acid, hydroxymethylcellulose, and arginine were provided by Sigma-Aldrich. Sulfuric acid (0.05 M) was purchased from VWR. Dry fermentation yeasts (*Saccharomyces cerevisiae*), potassium bisulfite (18 g/L), and filters (no. 5) were provided by Laffort Oenologie (Bordeaux, France), and the Clinistix (Bayer) was provided by a local drugstore. HPCE capillaries (μSil-FS, 50 μm i.d. $\times$ 1 m) were purchased from Interchim, and dialysis membrane (Cellu-sep T2, 6000−8000 Da) was purchased from Bioblock Scientist.

Samples. Each year, microvinifications are realized in the laboratory for different grape varieties from northwestern to southwestern French wine areas. This is our contribution to the annual databank performed by all wine producer EU member states for sugar enrichment control using wine isotopic data (*34*). Grapes were pressed using a hydropneumatic press with an approximate capacity of 25 kg. Around 9 L of must was extracted in a plastic tank, and 0.2 g/L of yeasts was added for sugar fermentation. Fermentation was followed by density measurements to a volumetric mass of around 993 g/L; negative Clinistix ensured a residual sugar content lower than 4 g/L. Wines were then filtered, sulfited (free SO_2 final concentration = 100 mg/L for dry wine and 300 mg/L for wines with residual sugar), left during 6 days at −4 °C for tartaric acid precipitation, and then bottled (*35*). The studied varieties are Chardonnay (24 samples), Chenin (24), Petit Manseng (7), Sauvignon (37), Semillon (24), and Ugni Blanc (9) from different vintages: 1992−2003. Some mixtures were prepared, according to the Plackett−Burman sampling plan (*36*), from monovarietal wines in various proportions listed in Table S1 (Supporting Information). Finally, commercial wines from various origins were studied to test the model; an electrophoretic response was detectable for 10 of 16 samples: 6 from France, 1 from Slovenia, and 2 from Argentina.

HPLC Measurements. Shikimic acid (SHA) concentration was determined according to the OIV official method (*13, 37*) on a Varian ProStar (model 230) apparatus equipped with a UV−visible diode array detector (ProStar, model 330). Data treatment was achieved with StarVarian software. Compound separation was obtained by two columns in series, an Alltech C18 type (15 cm $\times$ 4.6 mm) with a 5 μm particle size followed by a Supelcogel H S-DVB cation exchange column (30 cm $\times$ 7.8 mm), 9 μm particle size, warmed at 65 °C. Wine was previously filtered on a 0.45 μm filter before injection (20 μL). Elution was performed by an aqueous solution of sulfuric acid (0.02 N), and the elution rate was 0.6 mL/min. SHA detection was realized at λ = 220 nm. Determination of SHA concentration was achieved through an external standard method and, every eight samples, a standard solution was analyzed to detect any apparatus drift.

Capillary Electrophoresis Determinations. Protein profiles were obtained by HPCE on a Beckman apparatus (P/ace MDQ) controlled by 32 Karat software and equipped with a diode array detector. Before injection, wines were first dialyzed by placing 20 mL of wine in a cellulose membrane with a cutting size of 6000−8000 Da. This membrane was then poured into a 10 L flask filled with 18 MΩ water (Elga water) and gently agitated for 24 h (one renewal of water after the first 8 h). Then, to 5 mL of the 20 mL dialyzed wine (no concentration step), 1 mL of arginine (500 mg/L) was added as an internal standard. Arginine migration time was 12.2 min. For each electropherogram, protein areas were expressed in terms of arginine mass and then normalized according to $\sum_1^n \text{peak}_n = 100$. Injection was carried out at the anode using N_2 pressure (0.5 psi) during 30 s. Separation was performed at V = 15 kV and $A \sim 60$ μA at 25 °C using a fused silica capillary column of 75 cm effective length and 50 μm internal diameter. Protein detection was achieved at 200 nm. Buffer solution was an aqueous solution (Elga water) of phosphoric acid (80 mM) and hydroxypropylmethylcellulose (0.1%) at pH 2 (adjusted with KOH). One separation took around 45 min, and after each separation, the capillary was rinsed by the buffer solution during 2 min at 40 psi.

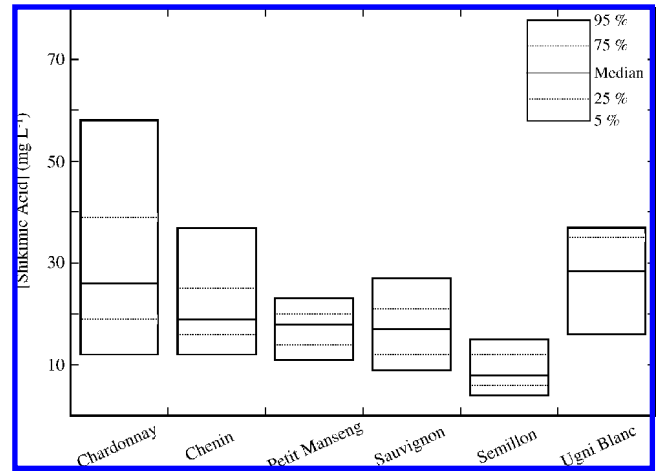

Figure 1. Percentile plot representation of shikimic acid concentration for each studied variety. One box includes 90% of the samples as bottom and top box lines correspond to sample presence probabilities of 5 and 95%, respectively. Inside the box: black line corresponds to median value, amd dashed lines correspond to 25 and 75% of the data.

Data Processing. Analysis of variance were performed to assess variations of shikimic acid concentration for the Sauvignon variety. Values of $P < 0.05$ were considered to be statistically significant. The statistical analysis was carried out using the Microsoft Excel software package. Computation for wine variety composition has been performed with a partial least-squares processing (PLS). The matrix is composed of rows and columns: each row corresponds to a single sample, and the columns are filled with the normalized protein data of the sample and SHA concentration; the last six columns correspond to the effective percentage of each variety involved in the sample. The data matrix was first transformed using a "log 10" function, then PLS computation was performed with a "mean center" preprocessing, a maximum factor of 3, a cross-validation, and a probability threshold of 0.95. Mathematical data processing was performed with the software Pirouette (Infometrix, version 3.11).

RESULTS AND DISCUSSION

Determination of Shikimic Acid Concentration. SHA concentration was determined by HPLC directly on filtered wines using an external calibration curve. Percentile plots for each variety are presented in **Figure 1**; each box delimits a 90% presence as the bottom and top of each box represent, respectively, 5 and 95% of the data. SHA concentration varies from 3 mg/L for Semillon to 73 mg/L (outside the box limits) for Chardonnay; these results are in accordance with previous study on some of these varieties (*13*). The results scattering is quite important in most of the varieties, but because this study is based on authentic samples, treated under the same experimental conditions, this scattering can be attributed to a natural phenomenon. In **Figure 2** is plotted SHA concentrations of Sauvignon variety versus four vintages (from 2000 to 2003) in relation to geographical origin (Bordeaux and Val de Loire, two French wine areas). Variance analysis was performed on the data of these two wine areas for the various vintages: differences are not significant ($P < 0.05$) for the Val de Loire area, whereas vintage had an influence for Bordeaux wine SHA concentration.

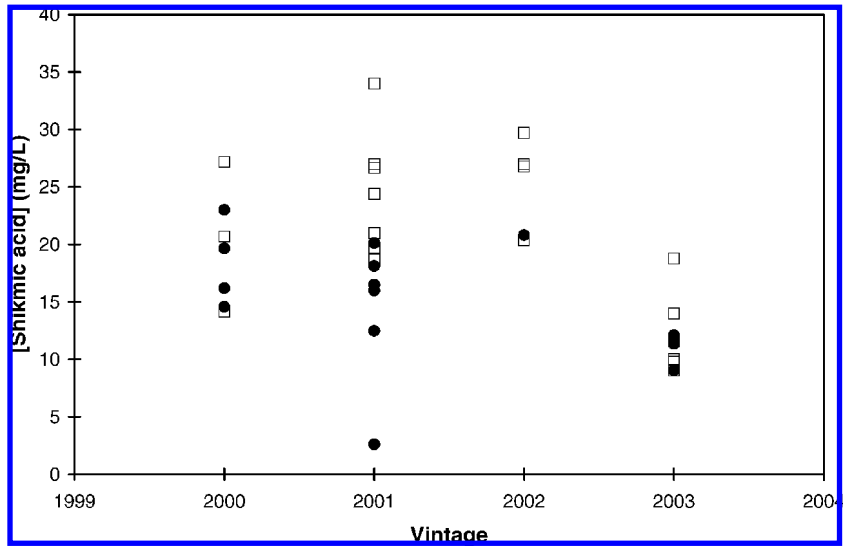

Figure 2. Variations of shikimic acid in Sauvignon samples according to the vintage and the wine-growing areas: Bordeaux (●) and Val de Loire (□).

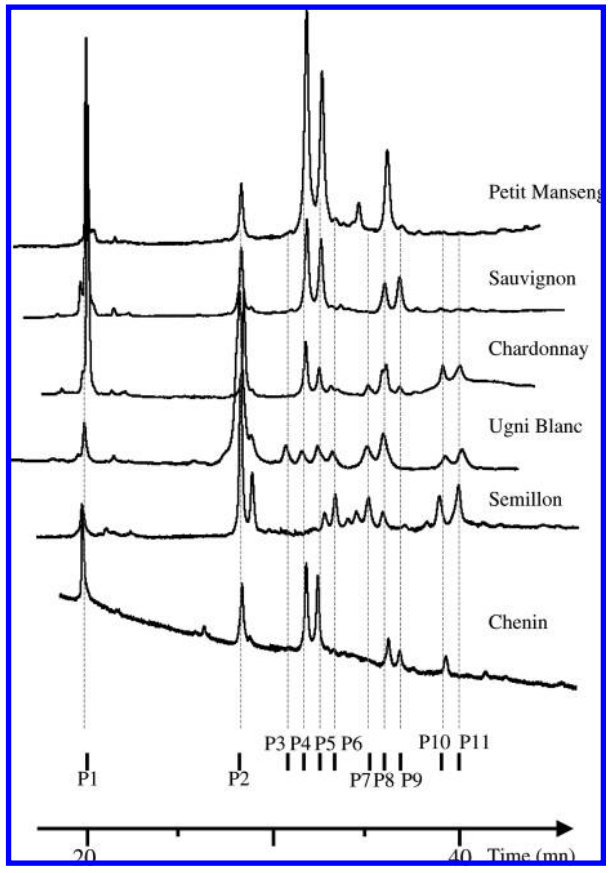

Figure 3. Reconstructed electropherograms obtained for each grape variety. Protein peak correspondence has been achieved by analysis of Sauvignon/varietal mixtures according to experimental conditions detailed under Materials and Methods.

Geographical origin impact was then assessed for three vintages: no significant difference was observed for vintage 2001, whereas for vintages 2000 and 2003, vine growth geographical localization had a significant impact on Sauvignon SHA concentration. From these results, it appears that vintage and vine growth geographical localization have an impact on SHA concentration for the Sauvignon grape variety. Due to the lack of data, this variance analysis could not be realized with other varieties. This trend needs to be confirmed by a specific study considering a consequent number of analysis. From these observations, it appears that the discriminating power of SHA concentration is low as it cannot fully separate white wine varieties, with the one exception for the Semillon variety. For this reason, SHA concentration has been, nonetheless, considered in the model elaboration.

Protein Fingerprint by HPCE. Wine protein profiles were determined by HPCE according to the protocol defined under Materials and Methods. The first step of the process consisted of sample dialysis as it allows the extraction of salts, free amino acids, and low molecular weight peptides that are not grape variety characteristic. After this dialysis step, relatively long (24 h) but necessary, the samples were analyzed by capillary electrophoresis without any concentration step. Most of the samples of each variety provide reproducible peak profiles (**Figure 3**), given in **Table 1**. As an example, on the Semillon electropherogram (**Figure 3**) the peak, close to peak P2, has not been selected as it is not always observable. In **Table 1** are listed the percentage values and the number of samples providing the selected electropherogram. Nonobservation of the protein profile for some samples is quite surprising as it is observed for wines of the same vintage, geographical area, and elaborated in similar conditions in the laboratory (fermentation and stabilization conditions, i.e., potassium bisulfite stabilization). Nevertheless, and the Chenin variety excepted, the selected profiles are observable in >70% of the studied samples (**Table 1**), which is a satisfying result. To correct the model, it was necessary to incorporate in the matrix some data of various varietal known mixtures. As a limited combination of mixtures can be elaborated, a Plackett−Burman sampling plan was applied as it allows the number of possible mixtures to be minimized with an optimal efficiency (*36*). This experiment design indicates that, with six variables (i.e., grape varietals), the study of eight mixtures provides a good insight of all the possible combinations. Concrete mixtures investigated are listed in Table S1 provided as Supporting Information.

The capillary electrophoresis technique is quite sensitive to experimental conditions (column aging, buffer, temperature variations...); thus, peaks retention times can vary drastically. To make links between the protein profile of the grape varieties and to visually identify common proteins, the Sauvignon variety has been used as a reference as its protein peak areas are much higher than those of the other varieties. Thus, mixtures of

Table 1. Protein Peak Selection for Grape Variety Study: Mean Values of Normalized Peaks Area and Standard Deviation in Parentheses

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	%[a]
Chardonnay	36 (18)	23 (12)		10 (2)	7 (5)		4 (3)	10 (8)	2 (2)	3 (3)	4 (3)	75
Chenin	26 (13)	17 (6)		25 (1)	15 (6)			8 (3)	6 (3)	3 (5)		40
Petit Manseng	43 (13)	5 (2)		27 (6)	14 (7)			11 (3)				100
Sauvignon	38 (18)	17 (8)		20 (6)	11 (5)			8 (4)	6 (3)			80
Semillon	23 (18)	43 (23)			7 (6)	10 (6)	3 (2)	2 (2)		3 (3)	10 (11)	70
Ugni Blanc	7 (4)	50 (10)	4 (1)	3 (2)	3 (1)	2 (1)		9 (2)	14 (5)	3 (1)	5 (1)	90

[a] Percentage of the studied samples with the selected peak profile.

Table 2. Prediction of Variety Percentages Computed for Certified and Commercial Wines with the PLS Model

		Chardonnay	Chenin	Petit Manseng	Sauvignon	Semillon	Ugni Blanc
	RMSEP[a]	22	24	18	32	11	18
	test 1[b]	7 (0)	18 (0)	9 (0)	66 (100)	0 (0)	4 (0)
	test 2	5 (0)	18 (0)	13 (0)	65 (100)	1 (0)	3 (0)
	test 3	81 (100)	6 (0)	0 (0)	19 (0)	2 (0)	0 (0)
	test 4	84 (100)	6 (0)	0 (0)	19 (0)	1 (0)	0 (0)
	test 5	43 (40)	3 (0)	0 (0)	11 (0)	51 (60)	6 (0)
(1)	Chardonnay	88	<[c]	nd[d]	18	nd	nd
(2)	Chardonnay	85	<	nd	18	nd	nd
(3)	Chardonnay	70	<	nd	<	40	nd
(4)	Chardonnay	69	<	nd	19	nd	nd
(5)	Sauvignon	<	18	<	66	nd	<
(6)	Sauvignon	<	18	<	66	nd	<
(7)	Sauvignon	<	18	<	65	<	<
(8)	Sauvignon	<	18	<	65	<	<
(9)	Sauvignon	<	18	<	66	nd	<
(10)	Sauvignon	<	18	<	65	nd	<

[a] RMSEP values determined with sets of certified samples. [b] Test number, certified samples run with intermediate models; in parentheses is the effective variety percentage in the sample. [c] <, computed value under 10%. [d] nd, not detected by the model.

Sauvignon and other grape varieties (1:1 v/v) were prepared and analyzed by HPCE. From these electropherogram comparisons, common protein peaks were identified and electrophoretic profiles of each variety were reconstructed (**Figure 3**). The experimental protocol, that is, filtration and sulfite addition right after the end of alcoholic fermentation, did not allow yeast protein liberation; any perturbation of the electropherogram can result from this protein source. Thus, for all samples, visual observation of the electrophoretic profile allows, by comparison with **Figure 3**, an easy variety fingerprint recognition without any protein identification. Each peak area is expressed in terms of milligrams of arginine, and differences are observable from one sample to another even within a varietal group. However, after normalization, protein peaks were nearly constant within a variety (**Table 1**). Thus, normalized peak values are used to elaborate the matrix for mathematical data treatments. As a result, added arginine solution could be avoided; nevertheless, in all of the samples 1 mL of arginine was added before CE experiments as an electrophoretic separation condition witness. The selected protein peaks are expressed in terms of arginine equivalent and then normalized for data computation.

Data Treatment. *Partial Least-Squares (PLS).* The matrix was constructed with analytical data of monovariety, 1:1 (v/v) variety mixtures and the eight mixtures containing all of the studied varieties at various percentages. PLS treatment was applied to this matrix, providing a quantification equation for each variety (coefficients attributed to the variables, SHA concentration and protein ratio, are provided as Table S2 of the Supporting Information). To determine the accuracy of the PLS model, intermediate models were elaborated after the exclusion of five samples, a step repeated for all of the samples. The intermediate models were run on the corresponding set of omitted samples providing a predicted value of the variety composition. Some examples are listed in **Table 1**. As an example, test 1 is a pure Sauvignon; the intermediate model predicted that test 1 was a mixture of Chardonnay (7%) Chenin (18%), Petit Manseng (9%), Sauvignon (66%), and Ugni Blanc (4%). These predicted values and the real percentages have been used to compute a prediction uncertainty related to each variety using the root-mean-square error prediction (RMSEP) formulas (*38–40*). RMSEP values, listed in **Table 2**, give insight into the model ability to quantify the variety amount involved in a wine elaboration; RMSEP varies from 11 to 32%, values higher than the initial objective of quantification, that is, 15% addition of a different grape variety. Nevertheless, in this first approach, the predicted values can still be very useful to encourage the antifraud inspector to more deeply investigate or not the wineries.

This model was applied to commercial monovarietal wines elaborated with the most frequently used grape variety: Chardonnay and Sauvignon. Sixteen commercial wines from different geographical origins were analyzed in terms of shikimic acid concentration, but only 10 samples had an exploitable protein profile: 4 Chardonnay and 6 Sauvignon wines. Predictions computed by the PLS model are listed in **Table 2**. In this table, computed values negative or equal to zero are replaced by "not detected" as it is an indication of the nondetection of the gape variety. Moreover, computed values under 10% are not mentioned and replaced by the sign "<" as these values are in the range of the directive. The model does not detect the presence of Petit Manseng, Ugni Blanc, and Semillon (with one exception for Chardonnay wine 3) in the commercial samples. Predictions for Chardonnay (69−88%) and Sauvignon (65−66%) seem to indicate the predominance of the bottle-specified variety. Considering the uncertainty values provided by the RMSEP,

22% for Chardonnay and 32% for Sauvignon, the analysis confirms the grape monovariety specified on the bottle. Some interferences perturb the model as in Chardonnay samples around 18% of Sauvignon is quantified. Moreover, Chardonnay 3 (**Table 2**) appears to be different from the three others as the presence of 40% of Semillon seems to be detected by the model. Thus, for this last sample, field investigations should be performed to understand the origin of this predicted value. Predictions for Sauvignon wine always provide an 18% value of Chenin. This result is not surprising as these two varieties differ by only one protein. For the six samples of Sauvignon wines, a similar value of Chenin is predicted; thus, this 18% value can be considered as a background model in Sauvignon variety quantification.

Finally, it seems that discrimination of the grape variety involved in wine elaboration can be envisaged through shikimic acid concentration and protein profiles, together with chemometric techniques. The PLS model seems to provide coherent results for grape variety evaluation. To our knowledge, this is the first study attempting a real quantification of a wine variety, but if the results provide satisfactory results, some problems should not be concealed. (1) Except for Semillon, the uncertainty related to the results given by the model is relatively high regarding the initial objective that was 15%. As a result, under these conditions, it would seem to be difficult to provide enough accurate grape variety quantification for the control of the so-called 85/15 regulation. These results can still provide some precious information for fraud suspicion. (2) Some interferences between the various grape varieties are observed. Thus, the matrix needs to be corrected by the addition of new certified samples from various origins and/or by inserting new discriminating parameters; wine near-IR data could be a possible way (*16*). (3) To prevent any protein haze formation, commercial white wines are submitted to various enological treatments, such as bentonite. If these treatments are used, protein concentrations can be under the detection limit of the HPCE method. Thus, the compatibility of concentration processes (*41, 42*) with this application needs to be tested to lower protein detection limits.

ACKNOWLEDGMENT

We thank Dr. Benedicte Laborde for comments and suggestions on the manuscript.

Supporting Information Available: Table S1 provides the concrete mixture ratios elaborated for Plackett−Burman sampling plan, and Table S2 lists the coefficient attributed by the PLS model to the variables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review January 14, 2008. Revised manuscript received June 2, 2008. Accepted June 10, 2008.

JF800117K