High-quality Italian rice cultivars: Chemical indices of ageing and aroma quality

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High-quality Italian rice cultivars: chemical indices of ageing and aroma quality

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Abstract

The volatile fractions of six Italian high-quality rice cultivars were investigated by HS-SPME-GC-MS to define fingerprinting and identify chemical markers and/or indices of ageing and aroma quality. In particular, four non-aromatic (Carnaroli, Carnise, Cerere and Antares) and two aromatic (Apollo and Venere) rices, harvested in 2010 and 2011, were monitored over 12 months.

Twenty five aroma components were considered and, despite considerable inter-annual variability, some of them showed similar trends over time, including 2-(*E*)-octenal as a marker of ageing for all cultivars, and heptanal, octanal and 2-ethyl hexanol as cultivar-specific indicators. The area ratios 2-acetyl-1-pyrroline/1-octen-3-ol, for Venere, and 3-methyl-1-butanol/2-methyl-1-butanol, for Apollo, were also found to act as ageing indices.

Additional information on release of key-aroma compounds was also obtained from quantitation and its dependence on grain shape and chemical composition. Heptanal/1-octen-3-ol and heptanal/octanal ratios were also defined as characterizing the aroma quality indices of the six Italian rice cultivars investigated.

KEYWORDS: HS-SPME-GC-MS; rice (*Oryza sativa* L.); cultivars; storage; ageing markers and indices; quantitation; aroma quality; chemometrics

1.INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most widely cultivated cereals in the world. It is a staple food for about half the world's population, in particular for Asian, South-American and African countries (Food and Agriculture Organization of the United Nations (FAOSTAT), United States Department of Agricultural (USDA)). Italy is the largest rice producer in the European Union, producing approximately 50 % of the total EU-27 harvest. Although Italy accounts for less than 1 % of world production, it is currently the fourth-largest rice-exporting country, after Thailand, United States, and India (counting intra-EU trade). Rice cultivation in Italy is mainly located in the northern regions (Piedmont, Lombardy and Veneto).

Rice cultivars can be classified into two major groups: the ecotype "*indica*", which is characterized by long grains, and the ecotype "*japonica*", with short grains. Several cultivars are cultivated in Italy, around 70% of them belonging to the "*indica"* variety (Ariete-Drago, Arborio, Baldo, S.Andrea, Carnaroli) (Istituto di Ricerche Economiche e Sociali per il Piemonte (IRES)). The EU characterizes specific qualities of specific products, through a series of labels (Protected Designation of Origin – PDO, Protected Geographical Indication – PGI, and Traditional Specialty Guaranteed – TSG), that give them a further added value related to their origins, and to the manufacturing and/or processing practices employed (European Commission, Agricultural and Rural Development). The quality of rice grains has great economic interest, characteristics such as yield, shape and defects being important in marketing, while the aroma of the cooked product, in particular when prepared in the Asiatic mode, has a big impact on consumers. The aroma of both aromatic and non-aromatic rice cultivars consists of a complex mixture of odor-active compounds. Several authors have studied the composition of the cooked rice volatile fraction, identifying a large number of components and defining several key-aroma compounds (Champagne, 2008, Jezussek, Juliano, & Schieberle, 2002; Widjaja, Craske, & Wootton, 1996a; Yang, Shewfelt, Lee, & Keys, 2008 a-b; Zeng et al., 2008). These include saturated and unsaturated aldehydes, alcohols, and cyclic compounds; in particular, hexanal, 1-octen-3-ol and 2-pentylfuran are markers of both quality and ageing, while 2-acetyl pyrroline (2-AP) is one of the aroma quality markers for aromatic rice (Buttery, Turnbaugh, Ling 1988; Champagne, 2008; Grimm, Bergman, Delgado, Bryant , 2001; Laguerre, Mestres, Davrieux, Ringuet, & Boulanger, 2007; Mahatheeranont, Keawsa-Ard., Dumri, 2001; Widjaja, Craske, & Wootton, 1996a). It has a characteristic popcorn-like aroma that, together with its low odor threshold, gives aromatic rice a characteristic flavor, whose accumulation is favored by their genetic characteristics (Bradbury et al., 2005; Kovacha, Calingacion, Fitzgerald and McCouch, 2009; Fitzgerald , McCouch and Hall, 2009).

Rice is a seasonal product, harvested during a limited period of a few weeks, but consumed throughout the year. Rice in the field is never uniform, changing at each crop, therefore processing and storage after harvesting have a big impact on yield and quality of the final product (Champagne 2008). During storage, the rice aroma can change, mainly because of oxidation and losses over time.

Headspace solid phase microextraction (HS-SPME) is a well-established and popular technique for headspace sampling, that is used in several fields, (Belliardo et al., 2006), including rice. Because of its flexibility and sensitivity, HS-SPME with a DVB/CAR/PCMS fiber has also been used to monitor the evolution of volatiles directly during storage (Grimm, Bergman, Delgado, & Bryant, 2001; Laguerre et al., 2007; Zeng et al., 2008).

This study aimed to analyze the volatile fractions of six high-quality Italian rice cultivars, by a fully-automated HS-SPME–GC–MS method, so as to define volatiles characterizing fingerprints and to identify reliable chemical markers and indices of ageing and aroma quality. In particular, the study comprised four main parts: i) the first part focused on validating the method of analysis; ii) the second part dealt with the effects of storage and temperature on the composition of the volatile fraction (aroma fingerprinting) of the investigated cultivars, seeking markers or indices correlated with ageing, independently of the inter-annual variability; iii) the third part comprised quantitation of the identified key-aroma compounds and the influence of the physico-chemical characteristics of the grain of the cultivars investigated on the release of the aroma components; iv) the final part concerns the identification of indices to describe the aroma quality of rice.

In particular, four non-aromatic (Carnaroli, Carnise, Cerere and Antares) and two aromatic (Apollo and Venere) rice cultivars, harvested in 2010 and 2011, were investigated over a period of 12 months.

2. MATERIALS AND METHODS

2.1 Reference compounds and solvents:

Pure reference compounds for analyte identity confirmation, and n-alkanes (*n*-C5 to *n*-C25) for linear retention index (I^T) determination, were from Sigma–Aldrich (Milan, Italy), 2-acetyl-2-pyrroline was from BOC Sciences (Shirley, NY, USA). A standard stock solution of *n*-heptadecane (C17) at 63 mg/L was prepared in dibutyl phtalate (Sigma–Aldrich, Milan, Italy) and stored in a sealed vial at – 18°C. C17 was used as Internal Standard for peak response normalization (ISTD). Solvents (cyclohexane) were HPLC-grade from Riedel-de Haen (Seelze, Germany).

2.2 Samples:

Rice (*Oryza sativa* L.) samples, of six high-quality Italian cultivars, were harvested in 2010 and in 2011: four cultivars (Carnaroli, Carnise, Cerere and Antares) were non-aromatic, and two (Apollo and Venere) were aromatic; all were supplied by SA.PI.SE. (Vercelli – Italy). Carnaroli is a high-quality non-aromatic rice that is PDO-labeled as "Riso di Baraggia Biellese e Vercellese" (IT/PDO/0005/0337) and PGI-registered as " Riso del delta del Po" (IT/PGI/0005/0712); Carnise is an agronomically-improved cultivar of Carnaroli, with enhanced ability to absorb condiments, maintaining its texture after cooking. Venere is a patented semi-whole pigmented black aromatic rice produced by SA.PI.SE (EU4481/1999) that has recently been introduced into Italy (2008), after adaptation to the climatic conditions by crossbreeding an Asiatic pigmented rice with a local cultivar, which contains lower relative amounts of key-odor compounds than does Apollo. **Table 1** reports the list of rice cultivars and their characteristics. Rice samples were stored as paddy at different controlled temperatures (i.e. at the conventional temperatures of 25°C and 5°C in air) and times (0-6-12 months), under 13% relative humidity.

A set of commercial Thai rice samples and a standard solution of methyl isobutyl ketone (80 mg/L), carvone (4.75 mg/L), 3-hexanol (40 mg/L), linalool (4.35 mg/L), 1-butanol-3-methyl acetate (17 mg/L), and eucalyptol (3.68 mg/L) in water (EXTD) were stored at -18°C and used as references to standardize the HS-SPME system performance over time (see paragraph 2.5) .

2.3 *Headspace solid phase microextraction (HS-SPME) sampling and optimization*

The SPME device and fibers were from Supelco (Bellefonte, PA, USA). A Divinylbenzene/ Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) d_f 50/30 μm, 2 cm length fiber was chosen, and conditioned before use as recommended by the manufacturer.

Volatiles were sampled by automated headspace Solid Phase Microextraction (auto-HS-SPME) using a Combi-PAL AOC 5000 (Shimadzu, Milan, Italy) on-line integrated with a Shimadzu QP2010 GC–MS system provided with a Shimadzu GC–MS Solution 2.51 software (Shimadzu, Milan, Italy).

The HS-SPME sampling conditions were optimized on a group of characteristic components of the rice volatile fraction of a milled Thai sample, i.e. 3-methyl-butanal, pentanal, hexanal, 3-methyl-1-butanol, 2-pentyl furan, hexanol, and nonanal at three temperatures (40°C, 60°C, 80°C) and times (15, 30, 60 min). ~~(Wongpornchai, Dumri, ongkaewwattana & Siri, 2004; Laguerre et al., 2007; Bryant, McClung, 2011)~~. The adopted HS-SPME fiber (2cm, DVB/CAR/PDMS) and conditions (60°C for 60 min) were a compromise to maximize recovery and repeatability throughout the two years of the project (Figure 1 SD).

Since most studies are based on cooked rice aroma, a group of experiments were carried out preliminarily on rice samples boiled for 20 min. These analyses, run under the conditions reported above, revealed that the volatile fraction was qualitatively identical but quantitatively more abundant than in uncooked rice, indicating that the method adopted can be applied to uncooked or cooked rice alike to study the influence of storage on the volatile aroma profile.

Aliquots of 3.5 g of dry uncooked milled rice of the investigated cultivars, and 5 µL of ISTD (C17), were placed in a 20 mL screw-cap vial, equilibrated for 5 min, and sampled by HS-SPME at 60°C for 60 min. After sampling, the accumulated analytes were recovered by thermal desorption of the fiber for 5 min at 250 °C into the GC injector and then transferred on-line to the chromatographic column. All samples were analyzed in triplicate.

2.4 GC-MS analysis conditions

GC-MS analysis - Chromatographic conditions: injector temperature: 250°C, injection mode: splitless; carrier gas: helium, flow rate: 1 mL/min; fiber desorption time and reconditioning: 5 min; column: 95% polydimethylsiloxane, 5% phenyl SE52 (d_f 0.25 µm, d_c 0.25 mm, length 30 m) (Mega, Legnano (Milan), Italy). Temperature program: from 45°C (5 min) to 170°C at 3°C/min, then to 250 °C (2 min) at 7°C/min.

MSD conditions: ionization mode: EI (70 eV), ion source temperature: 200°C; quadrupole temperature: 150°C; transfer line temperature: 270°C. A Standard Tune was used with a m/z 35-350 scan range and a scanning rate of 1.000 amu/s.

Analytes were identified on the basis of their linear retention indices and EI-MS spectra, compared to those of authentic standards, or were tentatively identified through their EI-MS fragmentation patterns and retention indices.

2.5 HS-SPME-GC-MS Validation

A validation protocol was adopted to assess the following parameters of method performance: specificity, precision (repeatability and intermediate precision), robustness, and Limit of Detection (LOD). A two-week validation scheme was applied for two months; 15 replicate analyses were run during this period. Specificity and precision were assessed on paddy rice and on an external standard mixture. ISTD linearity and matrix response were verified by analyzing ISTD stock solution (63 mg/L in dibutylphthalate) and milled rice and paddy as reported in Table 1 SD - (Supplementary data). Twenty five peaks characteristics of the rice volatile fraction were characterized, identified, and adopted for sample profiling, to evaluate inter- and intra-week variability of retention data (Linear Retention Index I^T_S), Absolute Peak

Areas, Normalized Peak Areas % (calculated vs. C17 as ISTD) and mass spectra match factors. Peak Areas were calculated on the basis of analytes' Target Ion (Ti) response; the ratios Ti/Q1 and Ti/Q2 (Target ion and two qualifiers) were used to confirm analytes' identity and to minimize quantitative differences due to changes in chromatographic performance over time.

2.6 HS-SPME fiber performance evaluation

SPME fiber performance was evaluated in terms of total volatile fingerprint area, to characterize the fiber's sampling capability and selectivity, and to minimize sampling errors/discriminations, in view of the extended duration (24 months) of this study and the large number of samples and replicates (Bicchi et al. 2007). The sampling performance of four DVB/CAR/PDMS fibers from different lots was evaluated on three replicated analyses for each fiber, on Thai rice and paddy samples.

The raw total volatile fingerprint areas from the entire set of analyses were submitted to Analysis of Variance (ANOVA). The One-Way ANOVA analysis on the replicates for each fiber revealed that the null hypothesis (i.e. "*there is no difference among the four fibers in terms of total volatile fingerprint area*") was verified with $p \leq 0.05$. Additional fibers were submitted to the entire testing routine, by analyzing reference samples stored at -18°C.

The fiber's life-span was monitored throughout the study with a set of commercial Thai rice samples and 100 μL of a standard mixture (EXTD), whose composition is reported in paragraph 2.2.

2.7 Data elaboration

ANOVA analysis was run with XLStat® software (Version 7.5.1) copyright 1995–2005 Addinsoft (Paris, France). Principal Component Analysis (PCA), Partial Least Square Discriminant Analysis (PLS-DA) and regression analysis were performed with Pirouette® (Comprehensive Chemometrics Modeling Software version 4.0 - 2009) (Infometrix, Inc. Bothell, WA). The data matrix consisted of as many rows as the number of samples (total objects: 192) and 25 columns (m/z variables). Data for statistical elaboration were pre-treated by baseline correction, through noise subtraction, and by internal normalization of the signal from each sample, and then pre-processed by autoscaling.

3. RESULTS AND DISCUSSION

The goals of this study, which were listed in the introduction, will be discussed in separate sub-sections.

3.1 Method validation

3.1.1 Evaluation of method performance parameters

Specificity was assessed for each target compound, by verifying analytes' identities through the Mass Spectrum Match factor, taking spectra in commercial and in-house databases as reference, together with target ion (Ti), qualifier ratios (Q1 and Q2), and interactive use of analytes' linear retention indices $I^{T}{}_{S}$ (Liberto et al. 2008, Costa, De Fina, Valentino, Dugo & Mondello 2007). Table 2 confirms that the method is specific for matching the target analytes. Retention index allowance for a correct match was set at ±10 units, and in all cases the MS match factors were above the arbitrarily-fixed threshold (75%), with an average value of 90%.

Precision, expressed as repeatability, and intermediate precision of the HS-SPME-GC-MS target profile, were evaluated over the entire validation period. Repeatability was determined on five analyses on rice and standard solution samples (see paragraph 2.5) during one week, while intermediate precision was measured on five repetitions of each samples per day, over three non-consecutive weeks during three months. Repeatability and intermediate precision are here expressed as Relative Standard Deviation % (RSD%) on the absolute areas for each target analyte, for the above validation periods (Table 2). The results show very good average repeatability, which in no case exceeded 11%, and satisfactory average intermediate precision of 12.6%. These values are very satisfactory, in consideration of the low abundance of some components, the large number of experiments, and the long duration of the study.

3.1.2 Limit of Detection and method sensitivity

The limit of detection (LOD) was determined experimentally across the entire set of validation analyses, and was taken as the lowest Absolute Peak area for which analyte identity confirmation was consistent with a fixed acceptable matching factor (75%) established a priori. In particular, minor or trace components were selected, and their identification was checked within the limits adopted (Table 2). The results are expressed as minimum Absolute Peak Area corresponding to 10,000 counts, measured on dec-(2*E*)-enal.

3.2 Volatile fraction fingerprinting

This part concerns the possibility of using volatile fingerprinting as a parameter to study the effect of storage temperatures and times on the volatile fraction composition. In the first year, paddy was also analyzed, and the results were in line with those of processed rice: since it is not the final product destined for the consumer, the study therefore only focused on processed rice. Twenty five volatiles released from processed Italian rice were considered to evaluate variations in the volatile fingerprint (Table 2).

PCA (Principal Component Analysis) carried out on the volatile fraction fingerprints of the investigated samples over the two years clearly shows the influence of storage time on discrimination of the first two principal components (PCs, explained variance 52.3%) (Figure 1a and b). The loadings plot indicated that discrimination at different storage times depended on several components: isopentyl acetate and ethyl hexanoate, which are abundant at T0, 2-butanone, 2 and 3-methyl butanol, pentanal, hexanal 2-pentyl furan, 3-octen-2-one at T6, and heptanal, 2-(*E*)-heptenal, 2-(*E*)-octenal, 1-octen-3-ol, 2-ethyl hexanoate, nonanal, 2-(*E*)-nonenal, 1-octanol, octanal at T12. Some components (heptanal, octanal, 2-ethyl-1-hexanol) increased in both years for all cultivars, although to different extents. Venere was the only cultivar whose composition only changed slightly with storage, probably because it is a semi-whole rice, and because it contains large amounts of phenolic compounds, in particular anthocyanines, which give it great resistance to oxidation over time (Gui-Fang Deng et al. 2013, Frank, Reichardt, Qingyao, Engel, 2012).

Conversely, the effect of storage temperature was slight (Figure 1a). This was also confirmed by PLS-DA (Partial Least Square Discriminant Analysis), which was used to evaluate whether volatile fingerprinting can be correlated to the physical characteristics of the grains of rice in cultivar classification. The results were satisfactory, although Figure 1c shows that some samples did not match any model category (class 0, shown on the x axis) while others actually belonging to class 2 were misclassified as class 1. The ability of the model to correctly classify samples in the proper class was characterized by a sensitivity of 97.8%, and the ability to reject samples that do not belong to a given class had a specificity of 79.2%. Within the defined classes, i.e. the grain shape, the model for samples belonging to long grains type B and round grain had low sensitivity 66.7% but a high specificity (100%), while long grains type A and medium-long grains showed a sensitivity of 91.6% and low specificity (92%).

The year of harvest strongly influences rice fingerprints, although to different extents depending on the cultivar (Figure 2 SD).

On the other hand, storage at different temperatures (25°C and at 5°C) within the same year of harvest, does not significantly affect the total amount of volatiles in each cultivar. In consequence, the following discussion will mainly focus on the usual condition of storage (25°C) unless specified otherwise.

The different climatic conditions occurring during the growing season affected not only the total abundance of the volatile fraction, but also its composition. Between the first and second years' harvest, the volatile fingerprints of the investigated cultivars showed different trends, also depending on the duration of storage.

Antares, Carnise and Venere increased their volatile abundance from 0 to 12 months, although differently. Cerere, Carnaroli and Apollo volatiles tended to increase over time, for rice of the first year's harvest; for that of the second year, they decreased at T6 and increased again at T12; this trend may be due to interaction of some volatiles with the starchy matrix, through the formation of stable inclusion complexes and/or adsorption, mainly for highly polar compounds (Arvisenet, Voilley & Cayot, 2002; Boutboul et al., 2002; Jouquand, Ducruet & Le Bail, 2006). At 25°C ketones were more abundant in the early phases of storage, and decreased or even disappeared after T0 Figure 2. The trend differed depending on the cultivar: in Carnise and Apollo, saturated aldehydes decreased up to T6 and then increased again; in Antares, Cerere and Venere, they increased until T6 and decreased to T12; in Carnaroli, they increased steadily until T12. These trends were similar for all saturated aldehydes, and were closely related to hexanal, which is the most abundant aldehyde. Aromatic and unsaturated aldehydes and alcohols (e.g. benzaldehyde, dec-(2*E*)-enal, 2-ethyl-1-hexanol and 1-octen-3-ol) showed a reduction at T6 and an increase at T12, although to different extents, again depending on the cultivar. This trend may be due to a competitive interaction between the oxidative formation of these volatiles and the starchy matrix.

3.3 Definition of markers of ageing

The change of the volatile fraction over time showed different trends depending on year of harvest and the cultivar; however, it highlighted time-related compounds and enabled markers of ageing to be defined. These can be divided into three groups: i) a universal marker, valid for all cultivars: 2-(*E*)-octenal, ii) markers for all cultivars except Venere: heptanal, octanal, 2-ethyl-1-hexanol, iii) cultivar-specific markers: 1-octen-3-ol (Carnise), 2-pentyl furan (Carnaroli), dec-(2*E*)-enal (Venere).

The Venere aroma fraction was the least susceptible to storage time, despite the inter-annual differences occurring in the two years of harvest.

2-AP has been reported as a possible marker of ageing, because it decreases over time (Wongpornchai, Dumri, Jongkaewwattana & Siri, 2004; Widjaja, Craske, & Wootton, 1996b). The results on Italian rice showed that both the abundance of this compound and its trend over time depend closely on the year of harvest. The 2010 samples showed a cultivar-specific reduction of 2-AP's relative abundance over time, in agreement with the literature, while those of the second year presented an increase during storage, in particular for Apollo aromatic rice. This could be due to a contamination of paddy by microorganisms, in particular *Bacillus cereus,* which is reported to be a possible agent of 2-AP formation (Yoshihashi T., Huong Thi Thu N., Inatomi H., 2002).

A set of ageing indices, expressed as analyte area ratio, were then defined and their trends investigated over time. The aroma compounds most closely correlated with storage, and showing similar trends in all cultivars, were selected by regression analysis. Their mutual area ratios were calculated at each storage time, by means of a routine carried out with a specific visual basic Excel macro. The resulting ratios were multiplied by a factor of 1000 to facilitate data handling, and normalized to those at T0 (i.e. initial storage time). The selected indices were those for which there was both an increase or decrease versus T0, and a correlation coefficient $r^2>0.7$, in both years of harvest. The results showed that some cultivar-specific indices related to ageing can be defined Table 1; indices of ageing cannot be defined for some non-aromatic cultivars (Cerere, Carnaroli, Carnise), although significant variations were detected in function of storage time, because of the great seasonal fluctuation in their absolute values, i.e. the high standard deviation between years. Some ageing indices were found that decrease over time up to 6 months of storage: for Antares (1-hexanol/heptanal) and for Venere (2-acetyl pyrroline/2-(*E*)-nonenal, 2-methyl propanol/pentanal, 2-acetyl pyrroline/2-(*E*)-octenal, 2-acetyl pyrroline/ dec-(2E)-enal) while others, such as pentanal/3-methyl butanol, increased significantly in late storage. Effective chemical indicators of storage for longer than 6 months were 2-acetyl pyrroline/1-octen-3-ol for Venere and 3-methyl butanol /2-methyl butanol for Apollo.

3.4 Aroma quality:

3.4.1 markers quantitation

Analytical procedures suitable to detect, identify, and quantify active odor components, occurring at trace levels and sometimes below ng/kg, are in general rather complex and time consuming. Conventional approaches based on liquid–liquid extraction, or steam distillation–solvent extraction techniques, such as Solvent Assisted Flavor Evaporation

(SAFE), can meet the need for fundamental studies to isolate-identify-quantify key odorants, but their applicability in fully automated systems to fast high-throughput screenings and detailed profiling are often limited.

HS sampling can overcome these limits, in particular for quantitative determination of volatile compounds from solid matrices; however, it is in any case rather complex, because of the strong matrix effect that can affect aroma release. This occurs, for example, for rice whose volatile fraction composition is strongly influenced by the amylose/amylopectin fraction. Multiple Headspace Extraction (MHE) is an approach for the true quantitation of volatile compounds from solid matrices, which overcomes the matrix effect, although this characteristic is not yet generally appreciated (Serrano, Beltrán, Hernández, 2009; Bicchi et al., 2010, Nicolotti et al, 2013). The most widely adopted approach for sample comparison is relative abundance based on peak area % or Internal Standard normalization. Although these approaches are scientifically accepted for several applications, they may be inaccurate (Bicchi et al., 2008) and misleading for profiling, when chemical composition must be correlated to sensory properties. Conversely, MHE is of great interest, not only for quantitation, but also useful to provide information about variations in the release of the same odorant(s) from different matrices into the headspace, and their distribution in the solid matrix (Nicolotti et al., 2013). It may thus be applicable to rice grains with different textures and shapes, or that are characterized by different amylose/amylopectin ratios, or different protein and lipid contents (Champagne E.T., 2008; Guichard E., 2002).

MHE is based on a dynamic gas extraction carried out stepwise; the total peak area, obtained from a series of consecutive extractions, is directly proportional to the total amount of the analytes presents in the sample (Kolbe, Ettre ,1997). The analytes' peak area decreases exponentially with the number of extractions, provided that a suitable amount of matrix is processed. The cumulative instrumental response is obtained from the following equation:

$$AT = \sum_{i=1}^{\infty} Ai = A1\,(\frac{1}{1-e^{-q}}) = \frac{A1}{1-\beta}$$

where *AT* is the total estimated area, *A*1 is the area detected with the first extraction, and q is a constant describing the exponential decay of the area with successive extractions. When a series of relatively homogeneous samples of the same matrix are processed, the β value (i.e. the e-logarithm of - q) is in general constant or, at least, falls within an acceptable range, which can be fixed a priori for a given analyte (Bicchi et al., 2010). True quantitation can be achieved by an external standard calibration with a standard solution of the analyte(s) investigated under the same MHE conditions as for the matrices; in this study, nine key-aroma compounds (table 2) in dibutylphthalate in a 0.5÷100 ppm concentration range for five calibration points were used.

Quantitative analyses were carried out on the four cultivars of most interest for the European and Italian markets, in terms of grain dimension and aroma quality (i.e. Carnaroli, Apollo, Venere and Cerere), at T0 stored at 25°C Table 2. Carnaroli and Apollo are respectively the non-aromatic and the aromatic standard of reference for Italian rice, in terms of organoleptic properties. Carnaroli is the reference variety for the “risotto” (Italian rice dish) because of its behavior when cooked, whereas Apollo may be considered as an “Italian Basmati rice” because of its similar aroma, which persists after cooking, mainly due to its high 2-AP content.

The results showed different matrix effects on specific odor-active compounds, as is clear from their β values Figure 3. Compared to the other cultivars, Venere displayed higher β values for all investigated components, with the sole exception of 2-AP, meaning that the relative release of these markers in the headspace was slowed because of greater retention. This shows that the release of a compound from the matrix is governed by different mechanisms of retention, depending on matrix composition and texture rather than on the volatility of the compound in question. Venere is a semi-whole grain rice, therefore the substantial presence of polysaccharides, proteins, and ash in the caryopsis antagonistically influences the release of the aroma compounds. On the other hand, Apollo contains large amounts of amylose, which may interact with the release of 2-AP in the headspace (Bellato S. et al. 2013).

However, grain dimension does not strongly influence key-aroma release, as is shown by Carnaroli, Apollo, and Cerere; these three cultivars have different grain shape, but their β values are similar. In particular, release of highly volatile compounds (i.e, hexanal, heptanal) is controlled by their volatility, independently of the cultivar, while the distribution of 1-octen-3-ol, nonanal, 2-(*E*)-nonenal, and decanal in the headspace is governed by the partition coefficient between solid matrix and vapor phase, which is cultivar-dependent. Apollo shows a higher β value than Carnaroli or Cerere, probably because of the higher content of amylose fraction (Table 1) which may induce the formation of inclusion complexes with the investigated key-aroma (Arvisenet, Voilley & Cayot , 2002; Boutboul et al., 2002; Jouquand, Ducruet & Le Bail, 2006, Bellato S. et al. 2013).

The true quantitation of rice key-aroma compounds, known as markers of both quality and ageing, (Table 2) provides other additional and diagnostic information: i) Carnaroli is richer than the other non-aromatic cultivars in key-aroma compounds, in particular nonanal and decanal, which mainly originate from oleic acid autoxidation; ii) Apollo contains similar concentrations of hexanal to Venere, but higher concentrations of 2-AP; iii) Venere releases the largest amount of

2-pentyl furan, probably because of enolization and cyclization of 5-ketononanal, i.e. an oxidation product of linoleic acid, as is 1-octen-3-ol (Widjaja, R., Craske, J. D., & Wootton, M. 1996b); a possible explanation is that Venere and Carnaroli cultivars contain the most lipids, in particular Carnaroli has an abundant polyunsatured fraction (Simonelli, Casati, Cormegna, Abbiati, 2013); iv) Cerere contains a lower concentration of hexanal, possibly because of its round-grain conformation, which has a smaller air-exposed surface for the same volume, influencing the oxidation rate.

3.4.2 Tools for aroma quality definition

More than 200 volatile compounds contribute to the aroma and flavor of rice, although few (13 compounds) have been found to characterize the aroma and flavor of cooked rice (Grosch,1993). Some of the key markers were found to be related to odor/off odor depending on storage; most of these are products of lipidic degradation, such as hexanal, octanal, 2-(*E*)-nonenal, dec-(2*E*)-enal, 2-pentylfuran, and (*E*,*E*)-2,4-decadienal (Champagne, 2008). The present results can also be used to define aroma quality indices for Italian rice cultivars; they were determined through systematic study over time and on different years of harvest. These indices can be obtained from the ratio of normalized areas between key aroma components but, if they are to be defined as a characteristic of different Italian rice cultivars, they should remain constant over time and should not vary with the year of harvesting. Again, normalized area ratios were calculated at each storage time, by a routine carried out with a specific visual basic Excel macro. Those indices that did not show significant variations over time, on the basis of an arbitrarily fixed relative standard deviation of ±30% within the two years of harvest, were taken as aroma quality descriptors. The results show that the heptanal/1-octen-3-ol and heptanal/octanal ratios (indices) could be used to characterize rice aroma quality. **Figure** 4 shows the specific index values at 25°C with their standard deviations in all cultivars. *Io toglierei del tutto questa frase oppure metterei: These results will be confirmed on the same rice cultivars from 2013 harvest.* TOGLIEREI

CONCLUSIONS

This study monitors the variations of the volatile profile of six Italian rice cultivars stored under different conditions, through a robust, reliable, and fully-automated analytical method. The study showed that: i) the storage temperature (5°C vs. 25°C) does not significantly influence aroma preservation; ii) fingerprinting depends on the grain shape, and chemical composition and texture of the cultivars investigated; iii) the effects of ageing can be monitored by several components: 2-(*E*)-octenal was identified as an universal marker of ageing for all rice cultivars, while 2-pentyl furan, 1-

octen-3-ol and dec-(2*E*)-enal were cultivar-specific ageing markers. In addition, independently of inter-annual variability, specific indices, such as 2-acetyl pyrroline/1-octen-3-ol for Venere, and 3-methyl butanol/2-methyl butanol for Apollo, were found to be reliable indicators of the different stages of ageing; iv) heptanal/1-octen-3-ol and heptanal/octanal were identified as indices of aroma quality for the Italian cultivars investigated and, more in general, for rice aroma quality.

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Caption to Figures

Figure 1: Scores (a) and loadings plots (b) resulting from the PCA on rice from two different year crops stored during 0 (circle, orange), 6 (square, blue), and 12 months (diamond, fuchsia). (c) PLS-DA class prediction: class 0 no match, class 1 long grain A (brown), class 2 round grain (red), class 3 medium grain (green), class 4 long grain B (fuchsia). Acronym legend: VE5, VE25: samples at T0 of the first year at 5°C and 25°C respectively; 2VE5 and 2VE25: samples at T6 of the first year; 3VE5 and 3VE25: samples at T12 of the first year, 2_2VE25: a Venere sample at T6-25°C of second year crop

Figure 2: Class volatile profiling in the different rice cultivars stored at 25°C, results are expressed as ISTD normalized abundance averaged on the two year of harvest

Figure 3: β values of MHE decaying of some key aroma compounds in four of the investigated Italian rice cultivars.

Figure 4: Average value and standard deviation of the two aroma quality indices

Supplementary data

Figure 1 SD: Comparison of HS-SPME sampling conditions on some selected compounds from rice volatile fraction.

Figure 2 SD: HS-SPME-GC-MS total area volatile profiling normalized to the ISTD (C17) in the two years of harvest for the investigated cultivars.

Caption to Tables

Table 1 List of the rice samples and their commercial and physico-chemical characteristics together with their chemical indices of ageing, expressed as relative target ion area ratio obtained by HS-SPME–GC–MS, reported as average value; in parentheses standard deviation calculated on the two years of harvest at 25°C.

Table 2 List of the 25 target peaks characteristic of the volatile fraction of Italian rice, identified through MS and (linear retention index ITS). Each compound is listed together with its Identity Spectrum Match factor, target ion (Ti) qualifiers (Q1 and Q2) ratios and ITS. Validation data on method precision (repeatability and intermediate precision) expressed as relative standard deviation% calculated over replicates collected over the whole validation period (Week 1–3) or in each validation week (Week 1, Week 2 and Week 3).

Supplementary Table – Table 1 SD: Experimental plan giving the number of replicates per sample and total number of analyses for the validation of the method, aEXTD solution isobutyl ketone (80 mg/L), carvone (4.75 mg/L), 3-hexanol (40 mg/L), linalool (4.35 mg/L), 1-butanol-3-methyl acetate (17 mg/L), eucalyptol (3.68 mg/L) in water ISTD solution C17 63 mg/L in dibutylphthalate

Table 1

Cultivars	Type	Commercial name and Product group*	Characteristics§	Amylose g/100g	Indices	Ti response ratio	T0	T6	T12
ANTARES (AN)	non-aromatic	Ribe Fine	Long grain rice A	n.a.	*1-hexanol/heptanal*	56/70	8074 (±1435)	4588(±449)	4250(±183)
CERERE (CE)	non-aromatic	Original Common	Round grain rice	16.3	-	-	-	-	-
CARNAROLI (CA)	non-aromatic	Carnaroli Superfine	Long grain rice A	22.4	*2-ethyl hexanol/2-(E)-decenal*	57/70	50119(±12334)	71627(±11083)	124732(±64435)
CARNISE (CR)	non-aromatic	Carnaroli Superfine	Long grain rice A	22.9	*1-hexanol /2-(E)-octenal*	56/70	19258(±4920)	12695(±3074)	10432(±3019)
APOLLO (AP)	aromatic	Fragrance Superfine	Long grain rice B	24.2	*pentanal/3-methyl butanol* *3-methyl butanol /2-methyl butanol*	44/70 70/57	3599(±1151) 1938(±461)	8527(±4025) 1197(±192)	19791(±7590) 731(±85)
VENERE (VE)	aromatic	Venere Semifine	Medium grain rice	16.3	*pentanal/2-(E)-heptenal* *2-methyl propanol/pentanal* *2-methyl propanol /1-hexanol* *2-acetyl pyrroline/1-octen-3-ol* *2-acetyl pyrroline /2-(E)-octenal* *2-acetyl pyrroline /2-(E)-nonenal* *2-acetyl pyrroline /2-(E)-decenal*	44/41 43/44 43/56 83/57 83/70 83/70 83/70	3341(±1026) 493(±27) 31(±11) 164(±49) 1521(±1107) 1635(±837) 13125(±8894)	6509(±2729) 218(±66) 18(±5) 73(±11) 264(±31) 413(±288) 2843(±648)	8666(±1747) 177(±57) 14(±3) 43(±5) 224(±84) 296(±9) 2134(±469)

*G.U. n°121, 25.5.2012, § EEC Regulation No 1234/2007, 22.10.2007

Table2

Compounds	MS match factor	I^T_{calc}	I^t_{ref}	Ti	QI_s	average abs area	w1-3	w1	w2	w3	OT in water (ppb)	odor description	concentration (ng/g)			
Ketones													CA	CE	AP	VE
2-Butanone	93	604	609	43	72-57	5064728	6.46	3.40	6.02	2.28	50000	fruity, ethereal [1,2]				
6-Methyl-5-hepten-2-one	87	982	986	43	41-69	31401	13.02	4.53	7.93	19.71	50.0	citrus-like, green[1]				
Oct-(3E)-en-2-one	86	1034	1038	55	43-111	11056	14.08	1.23	19.01	9.59	-	nutty, berry[1]				
Saturated aldehydes																
Pentanal	86	700	697	44	41-58	31219	19.94	1.47	11.70	7.64	12.0	nutty, malty[1,2]				
_Hexanal_*	96	761	770	56	41-44	642612	12.12	0.41	8.55	5.90	4.5	green, fatty[1,2]	68.10	32.90	64.20	53.50
_Heptanal_*	90	897	906	70	41-44	40521	10.63	4.21	7.04	1.14	3.0	fat, rancid[2]	2.20	1.70	1.66	1.70
_Octanal_*	94	998	998	56	57-84	26096	18.00	11.26	20.14	11.89	0.7	fatty, citrus-like[2]	2.80	0.60	0.63	0.30
Nonanal	82	1099	1099	57	41-70	297697	20.80	2.05	7.87	0.76	1.0	fat, citrus, green, waxy[1,2]	40.60	30.00	32.90	13.20
_Decanal_*	94	1200	1208	57	41-70	17031	16.00	2.61	16.47	13.11	0.1	soapy[2]	52.20	37.30	37.70	13.90
Unsaturated aldehydes																
2(E)-Heptenal	86	950	956	41	55-83	16861	17.04	5.48	6.77	2.27	13.0	green,soapy[1,2]				
Benzaldehyde	95	955	961	77	105-106	56739	5.12	5.48	11.66	7.03	350	bitter almond[1]				
2(E)-Octenal	84	1052	1059	70	41-55	26811	7.38	8.39	10.19	7.77	3.0	green, nutty, fatty[2]				
_2(E)-Nonenal_*	88	1154	1157	70	41-69	18080	13.99	11.37	7.91	3.14	0.1	fatty, tallow[1,2]	6.80	5.10	6.90	3.30
Dec-(2E)-enal	82	1257	1265	70	41-43	19191	2.37	5.50	10.20	3.44	0.3	fatty, earthy[1]				
Alcohols																
1-Propanol, 2-methyl-	86	625	631	43	41-42	1050556	12.87	1.18	1.40	6.07	7000	ethereal, winey[1]				
1-Butanol, 3-methyl-	91	717	714	70	42-55	11819187	10.11	2.36	12.63	4.41	250	fruity, malty[1,2]				
1-Butanol, 2-methyl	93	719	721	57	41-56	3596635	12.70	4.07	12.98	3.72	300	malty, wine[2]				
1-Hexanol	95	858	867	56	39-55	258701	14.17	2.62	12.01	13.71	2500	green, flower[2]				
_1-Octen-3-ol_*	86	975	978	57	43-72	113325	18.85	1.41	1.89	3.67	1.0	mushroom, earthy[1,2]	3.50	2.10	3.67	8.10

1-Hexanol, 2-ethyl-	92	1025	1030	57	41-70	91541	21.13	14.11	14.06	11.90	270000	floral, green[1,2]				
1-Octanol	94	1068	1076	70	56-69	28204	5.63	2.91	16.05	14.16	110	waxy, green[1]				
Esters																
Isopentyl acetate	95	860	869	70	43-55	227224	14.12	5.61	13.53	0.26	2.0	sweet, fruity[1,2]				
Ethyl hexanoate	89	997	1003	88	70-99	554470	7.72	6.76	10.98	6.94	1.0					
Heterocycle compounds																
2-acetyl-pyrroline	90	916	918	83	68-111	29840	16.23	12.91	10.05	9.97	0.1	popcorn-like[1,2]			8.78	3.50
*2-pentyl- Furan**	85	986	991	81	82-138	207129	6.00	2.46	17.74	3.05	6.0	beany[1,2]	2.40	1.00	3.50	5.40

[1] www.thegoodscentscompany.com, [2] http://flavornet.org/, * Key-marker aroma compounds.

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Figure 1

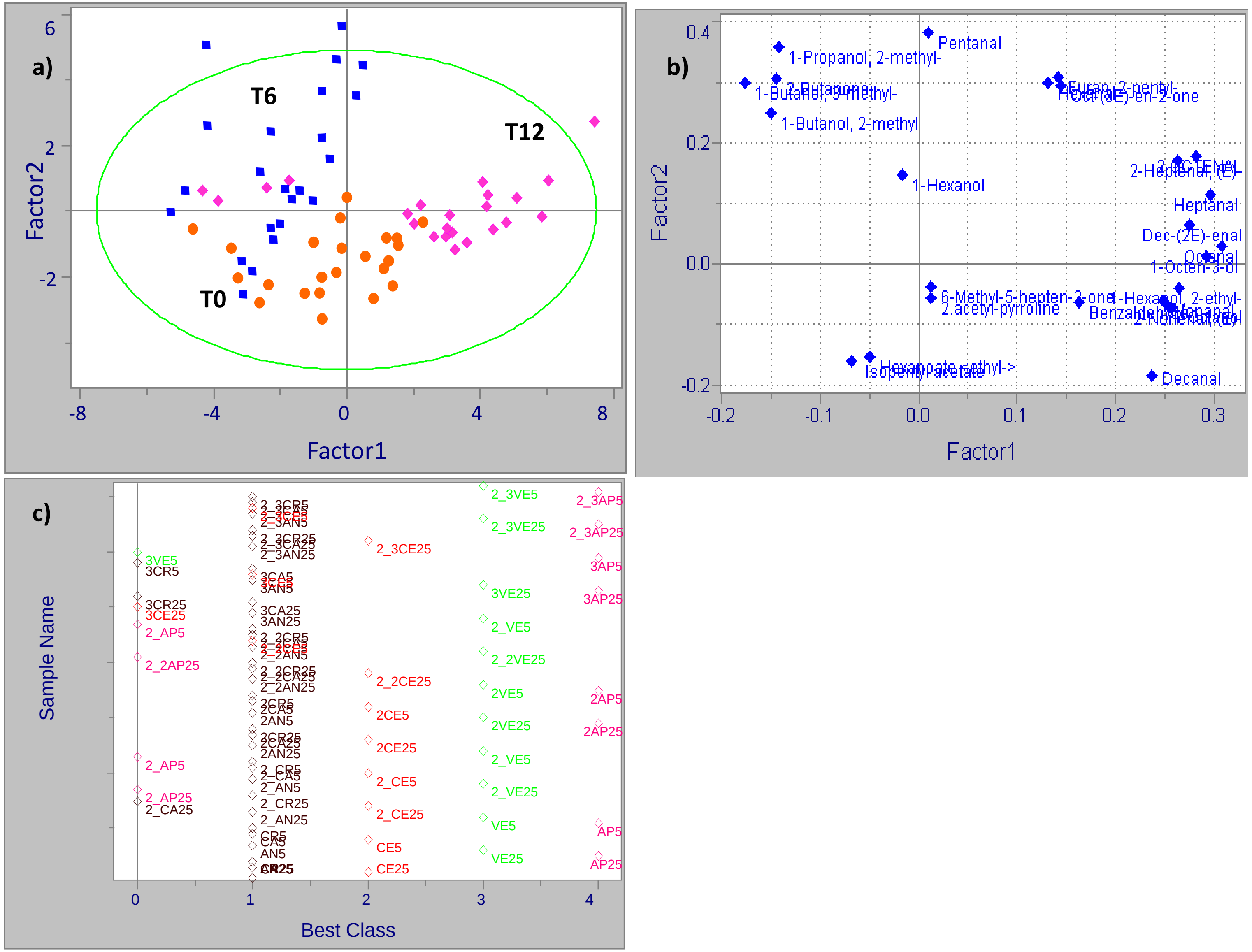


Figure 2

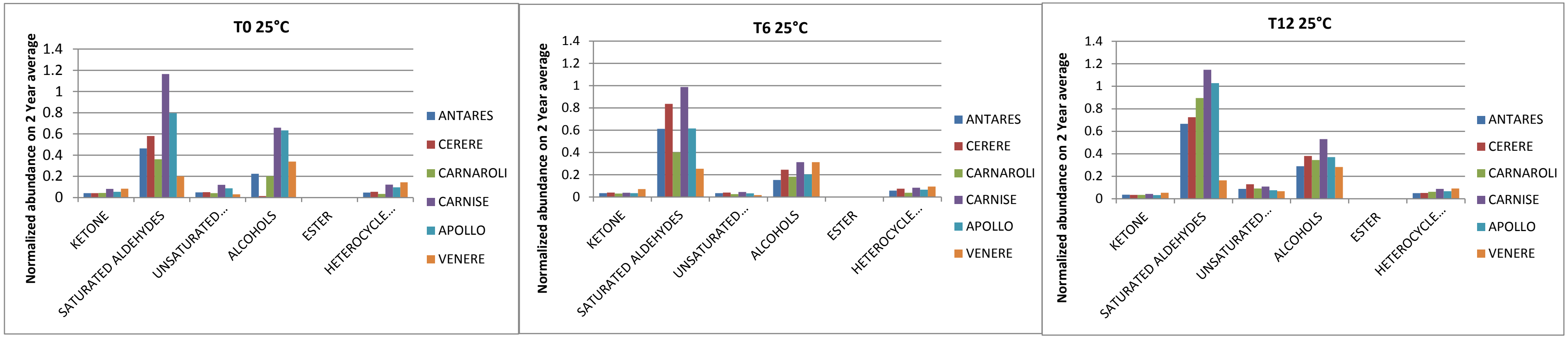

T0 25°C
Normalized abundance on 2 Year average
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KETONE
SATURATED ALDEHYDES
UNSATURATED...
ALCOHOLS
ESTER
HETEROCYCLE...
ANTARES
CERERE
CARNAROLI
CARNISE
APOLLO
VENERE
T6 25°C
Normalized abundance on 2 Year average
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KETONE
SATURATED ALDEHYDES
UNSATURATED...
ALCOHOLS
ESTER
HETEROCYCLE...
ANTARES
CERERE
CARNAROLI
CARNISE
APOLLO
VENERE
T12 25°C
Normalized abundance on 2 Year average
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KETONE
SATURATED ALDEHYDES
UNSATURATED...
ALCOHOLS
ESTER
HETEROCYCLE...
ANTARES
CERERE
CARNAROLI
CARNISE
APOLLO
VENERE

Figure 3

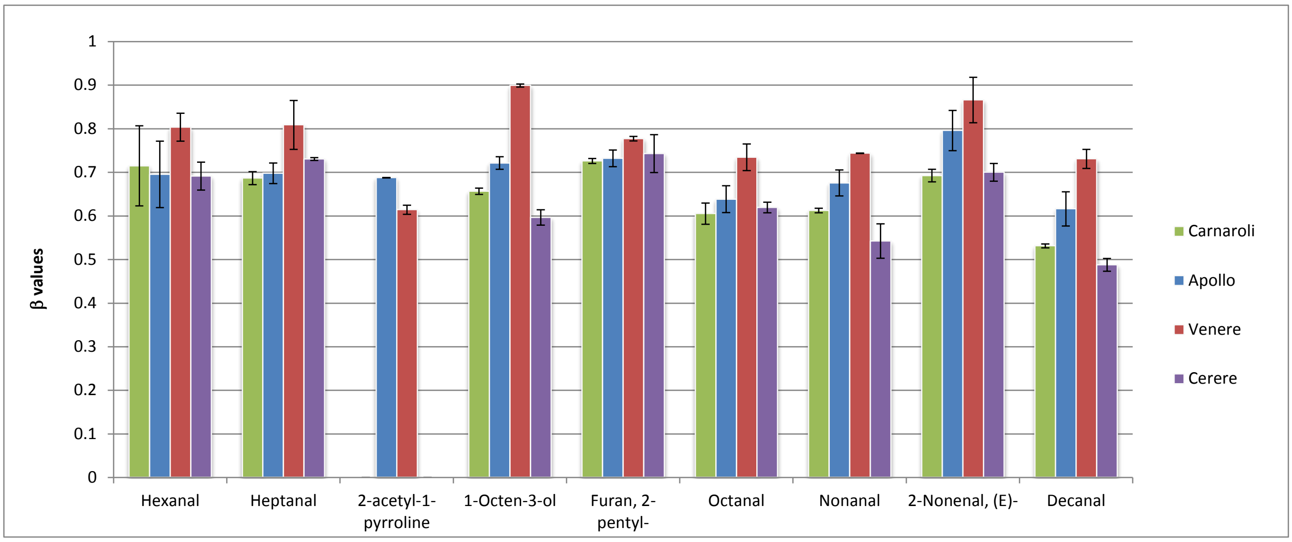
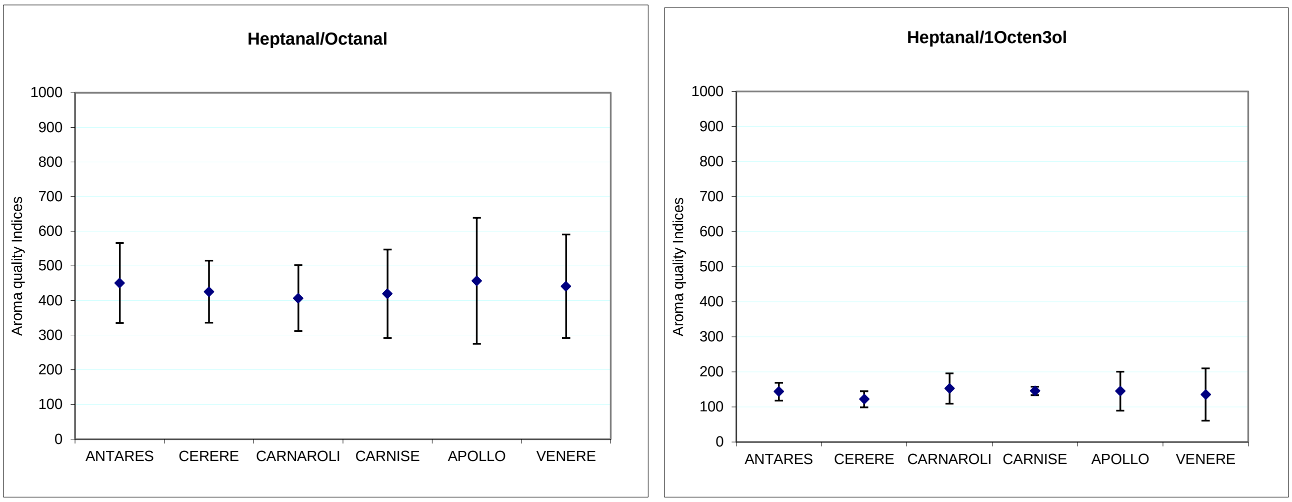

β values
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Hexanal
Heptanal
2-acetyl-1-pyrroline
1-Octen-3-ol
Furan, 2-pentyl-
Octanal
Nonanal
2-Nonenal, (E)-
Decanal
Carnaroli
Apollo
Venere
Cerere

Figure4


Heptanal/Octanal
Aroma quality Indices
1000
900
800
700
600
500
400
300
200
100
0
ANTARES
CERERE
CARNAROLI
CARNISE
APOLLO
VENERE
Heptanal/1Octen3ol
Aroma quality Indices
1000
900
800
700
600
500
400
300
200
100
0
ANTARES
CERERE
CARNAROLI
CARNISE
APOLLO
VENERE