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## PROFILING WINE CONSUMERS BY PRICE SEGMENT: A CASE STUDY IN BEIJING, CHINA

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### ABSTRACT

This study aimed to analyze and identify the purchase behavior and preferences of 976 Chinese wine consumers, particularly the differences in their demographics, purchase behaviors and recognition about wine based on a price segmentation categorized as High, Moderate, and Low Spenders. Price segmentation was defined as the price typically paid for a bottle (750 ml) of wine for daily drinking at home. Significant differences among the three segments were discovered based on income, educational background, job category, purchasing places, favorite brands, recognition of the importance of wine attributes and acceptable price for gift-oriented wine. Research on market segmentation based on price about Chinese wine market is limited. The current study provides insight into market segmentation and may serve as basis for future research.

*Keywords:* consumer behavior, consumer preferences, wine market, market segmentation

## 1. INTRODUCTION

The Chinese wine market has expanded in recent years, China produced 1.12 billion liters of wine and consumed 1.6 billion liters of wine in 2015, and China ranked ninth in terms of wine production and fifth in terms of wine consumption in the world (OIV, 2015), thus indicating that China has been a major player in the international market (VINEXPO, 2013). Trade reports show that the red wine sector is growing at a significant rate (EUROMONITOR INTERNATIONAL, 2013). The total volume of wine that China imported in 2015 reached 554 million liters, which is approximately 45% higher than that in 2014 (UN TRADE, 2015). The annual growth rate of the Chinese wine market is 25%-30% (CHINA DAILY, 2012). However, the per capita wine consumption in China is only 1.17 liters a year; therefore, the wine consumption of each Chinese consumer is not more than two bottles (750 ml/bottle) each year, whereas the world average is 3.32 liters (Wine Institute, 2015). The Chinese wine consumption per capita is far lower than that in western countries, and thus opportunities for growth are vast. Undoubtedly, as one of the emerging and new wine markets in the world, it can be forecasted that Chinese wine market will attract more and more domestic and international wine producers, marketers and researchers.

It is widely accepted that firms need to understand their consumers, which in turn requires analysis of all the dynamics and factors influencing consumers' buying behavior so as to improve their competitive advantages and business performance (GUNAY and BAKER, 2011). Understanding the purchase behavior of Chinese wine consumers is crucial because preferences and behaviors of these consumers significantly affect the development direction of wine markets (CHRISTODOULIDOU and JAMES, 2011). Consumer behavior is a cornerstone in marketing strategy and it involves conceptual dimensions such as decision-making, values, motivations, self-concept and personality, expectations, attitudes, perceptions, satisfaction, trust and loyalty (COHEN *et al.*, 2014). Consumers do not have homogeneous taste preferences (King *et al.*, 2012). Thus, segmenting the market is useful because different people have various needs (DOLNICAR, 2002). Consumers within a segment are relatively similar, and consumers between segments are comparatively different (BRUNNER and SIEGRIST, 2011). Thus, segmentation is a relevant tool in strategic marketing, which helps researchers understand consumers (DOLNICAR, 2002).

Market segmentation is defined as the process of dividing the total market into homogenous segments of consumers with similar needs and wants and the segmentation process addresses the needs of each subgroup efficiently (MARSHALL and JOHNSTON, 2010). Market segmentation is widely used in marketing products (RONDANCATALUÑA and ROSADIAZ, 2014). GUILLET and KUCUKUSTA (2016) provide a method for segmenting spa consumers according to their preferences in the selected spa attributes. Chen *et al.* (2013) use the market segmentation based on consumer motivation to analyze the bed and breakfast industry in Taiwan. PRAYAG *et al.* (2014) identify three clusters of travelers based on their service expectations, and determine that cultural differences are crucial for heterogeneity in travel behavior. CIRER COSTA (2013) investigates price formation and market segmentation in seaside accommodations. BRUWER and BULLER (2012) provide insights into the consumer behavior characteristics and consumption dynamics of Japanese wine consumers, explore the validity of existing generic consumer segmentation of the Japanese market, and consider its suitability to predict wine-purchasing behavior. VOORHEES *et al.* (2011) propose a novel perspective to improve segmentation in the hotel industry. Simpson and BRETHERTON (2004) utilize market segmentation based on lifestyle of customers to explore the wine tourism context.

Various studies on wine consumption have been conducted in different countries, but those on market segmentation in the Chinese wine market are scarce. OLSEN *et al.* (2015) divide wine consumers in the United States into three segments based on consumer variety-seeking behavior, namely, high variety-seeking, moderate variety-seeking and variety avoiders and find significant differences in high variety-seeking consumers compared with moderate variety-seeking and variety avoiders. MOLINA *et al.* (2015) identify the different segments of wine tourists who visit Spanish wineries. COHEN (2015) tests a segmentation of Hong Kong wine consumers based on the perception of wine labels and finds that approximately 95% of young Hong Kong Chinese wine consumers prefer “elegant contemporary” labels with red as the dominant color. GERGELY and DIETER (2014) test the segmentation of wine consumers based on the usage of sales channels and define six segments that are aggregated into two groups, namely, basic (discount, supermarket and food-retail consumers) and premium (cellar-door, wine-shop and multichannel consumers). TANG *et al.* (2011) use cluster analysis to divide wine consumers into six segments, namely, price-conscious wine consumers, involved consumers, knowledgeable consumers, image-oriented consumers, indifferent consumers, basic consumers, enjoyment-oriented consumers, and social consumers. BRUWER *et al.* (2002) identify nine different segmentation variables in wine market studies in Australia: quality, consumption, risk reduction, occasion based, cross-cultural, behavioral, involvement, geography and wine-related lifestyle. Wine knowledge is used in several segmentation studies on wine consumers, such as works of VIGARELLIS (2015), BRUWER and BULLER (2012), and JOHNSON and BASTIAN (2009).

The main segmentation variables used in existing studies are mainly related to consumers (interest in wine, knowledge of wine, motivations, sensation-seeking attitude and behavior, cultural variables, values and lifestyle) (MOLINA *et al.*, 2015). However, studies that conduct segmentation based on consumers’ perception of wine by price paid for a bottle (750 ml) of wine for daily drinking in Chinese wine market are non-existent. And according to the Wine Intelligence China Portraits (2015), Developing Drinkers who enjoy wine flavor, regard wine as a major part of their lives, and thereby often drink at home occupied an increasingly large proportion of Chinese wine consumers. Therefore, this topic is a new area for Chinese wine market research.

The current study aims to provide insight into the consumption characteristics and preferences of Chinese consumers in wine. Further the study categorizes consumers into three segments, namely, high, moderate, and low Spenders based on the preferred wine price for daily drinking, explores the difference in the three groups in Beijing and provides valuable information and marketing suggestions on the Chinese wine market for wine marketers, wine business owners and other stakeholders.

## 2. MATERIALS AND METHODS

### 2.1. Conceptual framework

Various studies focused on wine market segmentation (see literature review). Some studies highlighted consumer demographics, such as sex, age, income. Some studies focused on consumer consumption habits, such as consumption occasions, purchase places, channels of obtaining wine information. Other studies focused on consumer preferences about wine attributes, such as quality, brand, flavor and wine labels, and some examined consumer recognition of wine, such as knowledge in wine. The variables that can be used for segmentation can include consumption habits, cognitions, perceptions and acceptability. Therefore, a conceptual model was developed to show and examine the

potential segmentation variables that could be used for the segmentation of the wine market. The conceptual model is presented in Fig. 1.

In the current study, price segmentation is defined as the price typically paid for a bottle of wine for home consumption, provides a chance to understand differences among the segments.

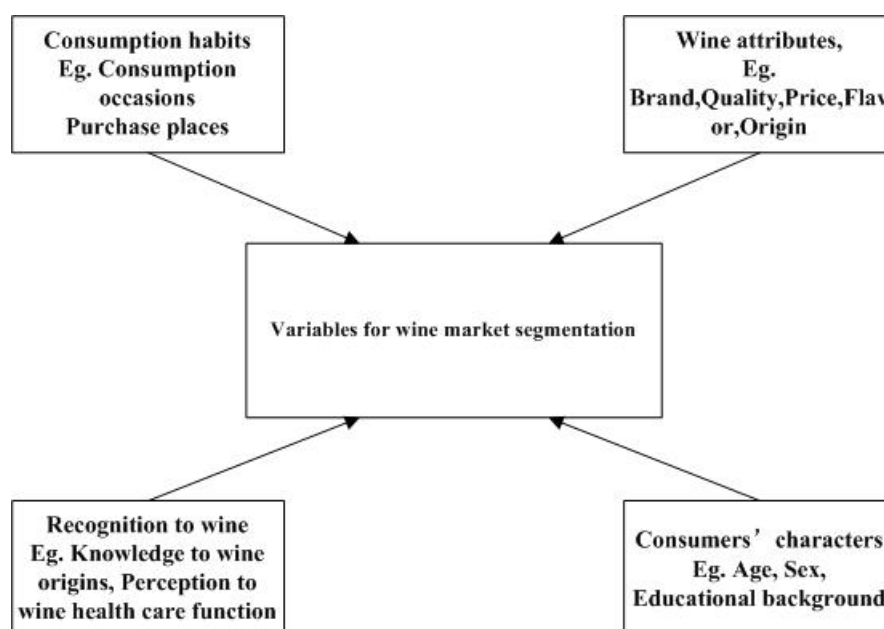


**Figure 1.** The conceptual model.

## 2.2. Questionnaire

The questionnaire on the consumption characteristics and preferences of consumers in wine was designed on the basis of the literature to collect information on consumer behavior. The questionnaire consisted of 20 questions, organized into four sections: consumer demographics (gender, age, level of wage, educational background and job category), consumers' purchase behavior (frequency of wine consumption, occasions for wine consumption and purchasing places), consumer awareness and preference in wine (consumers' recognition of wine attributes, cognition of the best or worst wine origins and awareness and preference in wine brands), consumers' acceptable price for wine (acceptable price for daily drinking and gift-oriented consumption).

## 2.3. Survey

In this research, Beijing was selected as the case because it is one of the most developed areas in China, and it is one of the cities with high wine consumption in China (WANG, 2006). Consumers' demand for wine in Beijing may indicate the future demand trends in China. A cross-sectional survey was conducted in Beijing, from September to October 2014. Consumers' characteristics were investigated on the basis of consumption behavior and wine preferences.

Questionnaire surveys are usually conducted via telephone, online, or face-to-face interviews. The face-to-face interview and online survey were employed in this study to

ensure sufficient sampling, given that respondents' cooperation is difficult to guarantee through telephone interviews. The process of the two research methods is as follows.

#### Face-to-face interview:

- ✓ First of all, six representative wine retailers in Beijing were selected to participate in the survey. Three supermarkets (Merry Mart, Carrefour and Wu Mart) and three wine stores (Guijie branch, Dongzhimen branch and Donglu branch of Cheers, which is a popular wine store in Beijing) were identified.
- ✓ Secondly, five undergraduate students from China Agricultural University were chosen as investigators. The students were recruited and trained before the survey.
- ✓ Thirdly, the face-to-face survey was conducted by randomly selecting the interviewees. The surveys were conducted between 9:00 and 17:00 and a typical respondent who purchased wine was given approximately 10 minutes to complete the questionnaire. After the five-day survey, 410 questionnaires were obtained. The questionnaire was reasonable and concise, and the survey was supervised by trained investigators. The completion of the questionnaires was preliminarily ideal. All the answered questionnaires were valid.

#### Online survey:

- ✓ Firstly, a famous professional questionnaire survey website in China called SOJUMP was used to ensure that random consumers in different areas could participate in the survey.
- ✓ Secondly, investigators released the corresponding website through different social media, such as WeChat and QQ to ensure the high respondent turnout of wine consumers in different regions in the survey. The online survey lasted one week and 604 questionnaires were obtained. After disregarding the incomplete or the logically paradoxical questionnaires, 566 valid questionnaires were acquired.

The questionnaire used in the online survey was same as that in the face-to-face survey. However, in the online survey, respondents could fill out the questionnaire at a convenient time and without a time limit. And the questionnaire survey website provided storage and downloading functions, so that analysts could examine the results at any time. A total of 1,014 questionnaires were distributed, and 976 valid ones were obtained. The recovery efficiency reached a high 96.3%.

## 2.4. Statistical methods

The collected information from the survey was transformed into statistical variables and processed by SPSS software 19.0. Descriptive statistical method and chi-square analysis testing were adopted to analyze the relation between consumers' demographics and purchase behavior which includes the frequency and occasion of wine purchase and the demographic differences among price segments. The mean responses with the standard deviation, frequencies and percentages of responses in each category were calculated and presented in tables and graphs.

### 3. RESULTS AND DISCUSSIONS

#### 3.1. Consumers' demographics

Table 1 shows the consumer demographics by gender, age, wage level, educational background and job category. The sample consisted of 455 women and 521 men. The most common age group was under 25, followed by 26-35. Per capita monthly income was nearly evenly distributed from 2,000 to 10,000. Well educated respondents (undergraduate and above) dominated the sample (74.9%). Staff members in enterprises (44.5%) represent the most common occupation in the job category, and they are followed by students, thus explaining the high educational level and young age of most of the respondents.

**Table 1.** Statistical characteristics of the respondents.

| Demographics                           | Category   | %,N=976 | Demographics                  | Category                     | %,N=976 |
|----------------------------------------|------------|---------|-------------------------------|------------------------------|---------|
| <b>Gender</b>                          | Male       | 53.4    | <b>Educational background</b> | Junior high school and below | 1.7     |
|                                        | Female     | 46.6    |                               | Senior high school           | 8.6     |
| <b>Age</b>                             | Under 25   | 45.9    |                               | Junior college               | 14.8    |
|                                        | 26-35      | 36.4    |                               | Undergraduate and above      | 74.9    |
|                                        | 36-45      | 11.8    | <b>Job category</b>           | Government official          |         |
|                                        | 46-55      | 4.9     |                               | Staff in enterprises         | 7.4     |
| <b>Per capita monthly income (CNY)</b> | Above 55   | 1.0     |                               | Liberal profession           | 44.5    |
|                                        | Under 2000 | 23.4    |                               | Farmers                      | 11.9    |
|                                        | 2001-3000  | 11.6    |                               | Education departments        | 0.3     |
|                                        | 3001-4000  | 15.7    |                               | Students                     | 4.4     |
|                                        | 4001-5000  | 17.7    |                               | Unemployed/retired           |         |
|                                        | 5001-7000  | 15.3    |                               | Else                         | 25.0    |
|                                        | 7001-10000 | 12.0    |                               |                              | 2.2     |
|                                        | Above10000 | 4.3     |                               |                              | 4.4     |

Notes: N, the total number of respondents.

#### 3.2. Purchasing behavior

Some studies considered wine as a female product (LI *et al.*, 2011). However, a qualitative study (LIU and MURPHY, 2007) determined that wine buying is a male interest in future Asian markets. Age was also found to be correlated with wine involvement (BRUWER, 2013). Therefore, a chi-square test was used to examine the difference of gender and age on the frequency of wine purchase. A significant difference caused by age was identified (Table 2).

Table 2 shows that approximately 18.0% of the men and 15.4% of the women often drank wine. While considering the difference of gender on wine drink, the chi-square test was insignificant, thus indicating that the frequency of wine consumption among men and women does not significantly differ. This result, which is consistent with that of FORBES (2012), suggests that further research could be conducted to explore the effect of gender on the frequency of wine purchases in China. The 26-35 age group dominated the cluster of often purchased wine. The under 25 age group mostly purchased wine occasionally, thereby indicating that young and middle-aged consumers preferred wine. Young people appeared to be the target of wine marketing in China (JENSTER and CHENG, 2008; LIU and MURPHY, 2007).

**Table 2.** Difference of gender and age on the frequency of wine purchase.

| Variables     | Wine purchase frequency (%) (N=976) |              |       | Chi-square Test |          |
|---------------|-------------------------------------|--------------|-------|-----------------|----------|
|               | often                               | occasionally | never | F               | Sig.     |
| <b>Gender</b> |                                     |              |       |                 |          |
| Male          | 18.0                                | 73.3         | 8.7   | 3.531           | 0.171    |
| Female        | 15.4                                | 72.7         | 11.9  |                 |          |
| <b>Age</b>    |                                     |              |       |                 |          |
| Under 25      | 7.8                                 | 75.9         | 16.3  | 81.735          | 0.000*** |
| 26-35         | 21.4                                | 72.4         | 6.2   |                 |          |
| 36-45         | 31.3                                | 67.8         | 0.9   |                 |          |
| 46-55         | 27.1                                | 66.7         | 6.2   |                 |          |
| Above 55      | 40.0                                | 60.0         | 0.0   |                 |          |

Notes: N, number of respondents, 3-never, \*\*\* Significant at 1% level respectively.

Wine is a social product and is consumed in various occasions. However, few Chinese consumers buy wine for home entertainment (LIU *et al.*, 2014). In China, wine consumption is mainly related to gift-giving, business banquets and special occasions such as the traditional holidays like the middle festival, and so on. Income and status-seeking are the main factors affecting wine consumption occasions (SUN *et al.*, 2009; RICHIE, 2007). In this research, a multi topic was designed to investigate the occasions on which consumers consume wine. Occasions on which wine is consumed vary (Table 3). The result showed that 50.8% respondents drank wine during holidays, 41.3% consumers drank wine daily at home, and 30.0% purchased wine as gifts. The results are consistent with those of previous studies (SUN *et al.*, 2009; JENSTER and CHENG, 2008; LIU and MURPHY, 2007).

The results of the chi-square analysis (Table 3) indicated that the occasions on which the respondents consumed wine were significantly different among consumers with various income levels. Respondents with an income of below 2000 (23.7%) mostly comprised those who consumed wine during holidays. This result is consistent with that of a previous research, which determined that consumers with low income tended to drink wine on special occasions such as holidays (Sun *et al.*, 2009). For business banquets, consumers whose income were CNY 7001- CNY 10000 (28.8%) seemed to have the money and opportunity to consume wine.

The survey determined that consumers most frequently purchased wine is supermarkets (61.5%), followed by wine stores (20.4%). The proportion of consumers who purchased wine online was relatively high (9.8%). The result is consistent with that of LIU *et al.*, (2014), who determined that Chinese wine consumers mostly purchased wine in supermarkets. This result is attributed to two reasons. First, supermarkets are convenient for consumers (LOCKSHIN *et al.*, 2012). Second, price discounts in supermarkets attract consumers who want to save (FLYNN *et al.*, 2010). The result also showed that online shopping for wine was still weak despite the prosperous electronic commerce in China. Many factors contribute to this result, such as the fragileness of wine bottles and false wine information.

**Table 3.** Influence of income on occasions on which wine is consumed.

| Variables                 | Consumption occasion <sup>a</sup> (%) (N=976) |          |          |       |        | Chi-square Test |          |
|---------------------------|-----------------------------------------------|----------|----------|-------|--------|-----------------|----------|
|                           | Business banquet                              | Holidays | As gifts | Daily | Others | F               | Sig.     |
| <b>Income<sup>b</sup></b> |                                               |          |          |       |        |                 |          |
| Below 2000                | 14                                            | 117      | 60       | 85    | 8      | 56.835          | 0.000*** |
| 2001-3000                 | 5                                             | 64       | 28       | 35    | 6      |                 |          |
| 3001-4000                 | 6                                             | 84       | 52       | 54    | 2      |                 |          |
| 4001-5000                 | 14                                            | 86       | 51       | 84    | 0      |                 |          |
| 5001-7000                 | 11                                            | 75       | 54       | 70    | 1      |                 |          |
| 7001-10000                | 21                                            | 46       | 33       | 51    | 1      |                 |          |
| Above10000                | 2                                             | 21       | 14       | 23    | 3      |                 |          |

Notes: <sup>a</sup>The question is multiple choice, <sup>b</sup> Average monthly income per capita. N, the total sample number of respondents, \*\*\* Significant at 1% level respectively.

### 3.3. Awareness and Preference in wine

#### 3.3.1. Consumers' cognition on the origins of wine

The cognition of consumers on the origin of wine is presented in Fig. 2. The result showed that consumers considered Xinjiang Province and the Ningxia Hui Autonomous Region as the best origins of wine, and Beijing City, Tianjin City and Hebei Province were deemed as the worst wine origins in China. The northwest areas are known to be famous wine origins in China (MU and FENG, 2010), this is consistent with the survey results. However, 15% of the consumers deemed Hebei Province as the best wine origin. More than 10% of the respondents determined Gansu, Shandong and Jilin Provinces as the best and worst wine origins. These results indicate that consumers' limited knowledge or lack of professional knowledge about wine origins.

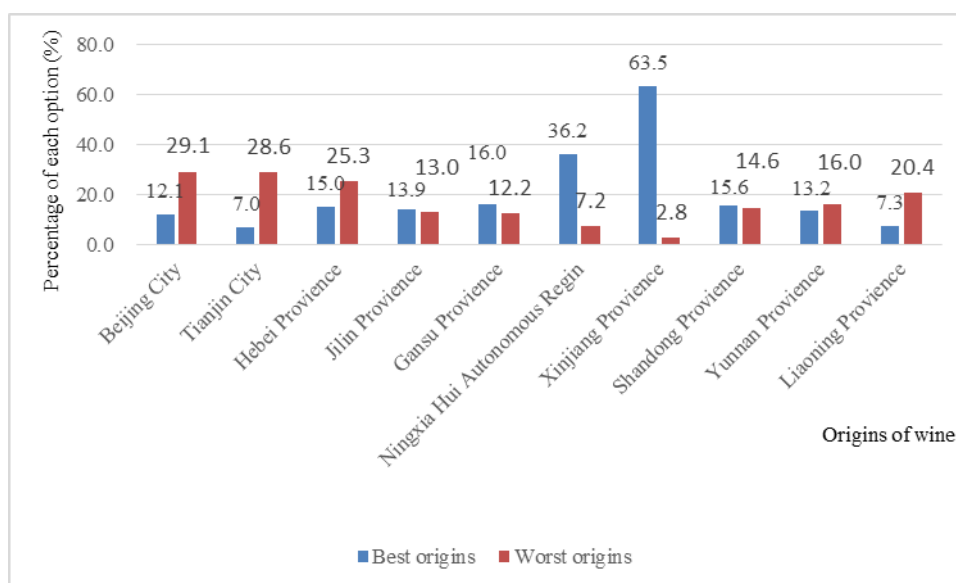


**Figure 2.** Consumers' cognition on wine origins in China.

### 3.4. Consumer awareness and preference in wine brands

Figure 3 shows consumers' familiarity with wine brands. Brand effect is very important to wine. Although the cognitive value of consumers in the existing brands of wine in China does not represent the actual value of the product, cognitive value is closely related to actual value. To some degree, cognitive value reflects the actual value of products (MU and FENG, 2010). Thus, 10 wine brands are considered in the current study and the respondents were requested to choose three brands that they were most familiar with and the three brands they were willing to buy. The results showed that Greatwall (71.6%), Changyu (70.8%), and Dybasty (35.7%) were the three highly cognitive wine brands for Chinese consumers. This result is related to brand history, advertising, and other factors (MU and FENG, 2010).

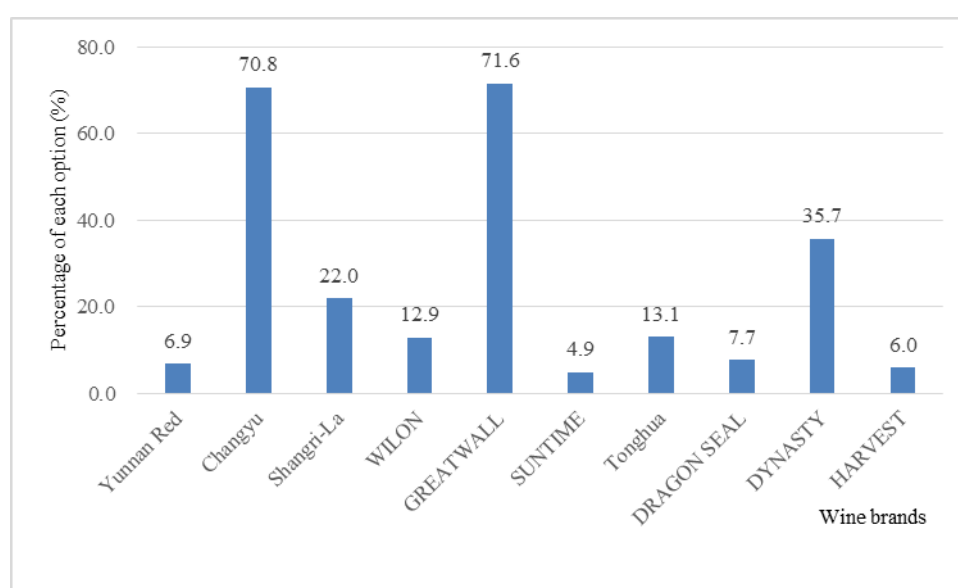


**Figure 3.** Consumers' familiarity with wine brands.

As shown in Fig. 4, in terms of buying, 39.8% of the respondents considered Changyu as their first choice, followed by Greatwall (26.3%). Forbes *et al.* (2014) verify that brand name, in the absence of other product information, influences consumer perception of quality and price, and purchase intentions, and that some categories of brand names perform better than others.

It is notable that foreign brands (21.1%) is also coming into consumers' eyes. These consumers consider foreign wine may be purer than that in China. The wine in China culture is underdeveloped, and consumers have limited knowledge about wine, including origins and brands. Most Chinese consumers are novice wine buyers, and tend to buy wines from the famous wine-producing regions they only knows. These consumers believe that wine imported from foreign countries is better than domestic wine (BRUWER, 2002), and they seek little information about other brands.

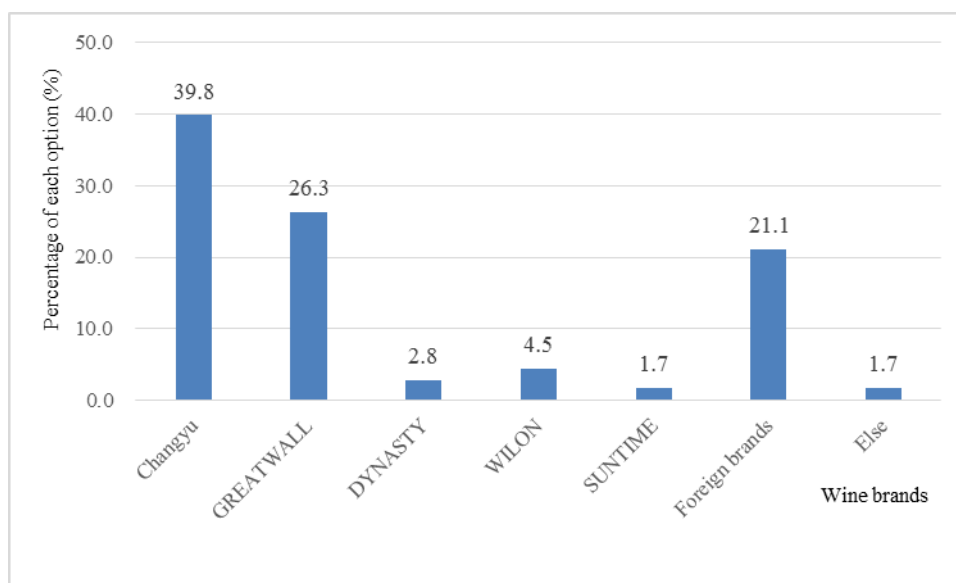


**Figure 4.** Consumers preferred wine brands when buying.

### 3.5. Consumers' recognition of wine attributes

Eight kinds of wine attributes, namely, price, quality, advertisements, recommendation of others, flavor, package, origins and productive years were selected in the current study. These scale items were based on the literature (HALL *et al.*, 2013; KING *et al.*, 2012; GOODMAN, 2009; GERAGHTY and TORRES, 2009). The respondents were asked to rank them according to importance. For the each attribute  $i$  ( $i = 1, 2, \dots, 8$ , representing the 8 kinds of wine attributes), its final score  $S_i$  can be mathematically formulized as follows:

$$S_i = \left( \sum_{j=1}^8 \text{value}_j * \text{frequency}_j \right) / n$$

Where  $\text{value}_i$  is the importance for attribute  $i$  and ranges from 1 to 8 (8-the most important, 7- the second important, 6- the third important, 5- the fourth important, 4- the fifth important, 3- the sixth important, 2- the seventh important, 1- the eighth important), and  $\text{frequency}_i$  is the times for  $\text{value}_i$  occurrence.  $n$  is the number of respondents.

The responses indicated that quality obtained the highest score of 5.78, followed by price (5.43), as shown in Fig. 5. The third highest score (5.05) pertained to taste, followed by source (2.72). The result indicated that consumers prioritized wine quality in purchasing wine. This result may indicate the need for the Chinese wine industry to improving wine quality.

### 3.6. Market segments

Before designing the questionnaire, the research staff surveyed two supermarkets and three wine shops in Beijing to acquire information on wine price. After analyzing the price data, the wine price ranges for daily drinking wine and wine for gifts were set in the questionnaire. However, the current study focused only on daily drinking of wine. In the analysis of market segmentation, 121 questionnaire were abandoned for missing the information about wine price for daily drinking. Fig. 6 shows that for daily drinking wine

(bottle/750 ml), most consumers accepted the price range of CNY 75-CNY 100, and few samples were willing to purchase wine higher than CNY 175. Based on consumers' preference in wine price for daily drinking, Chinese wine consumers in the current study were categorized into 3 groups (Table 4).

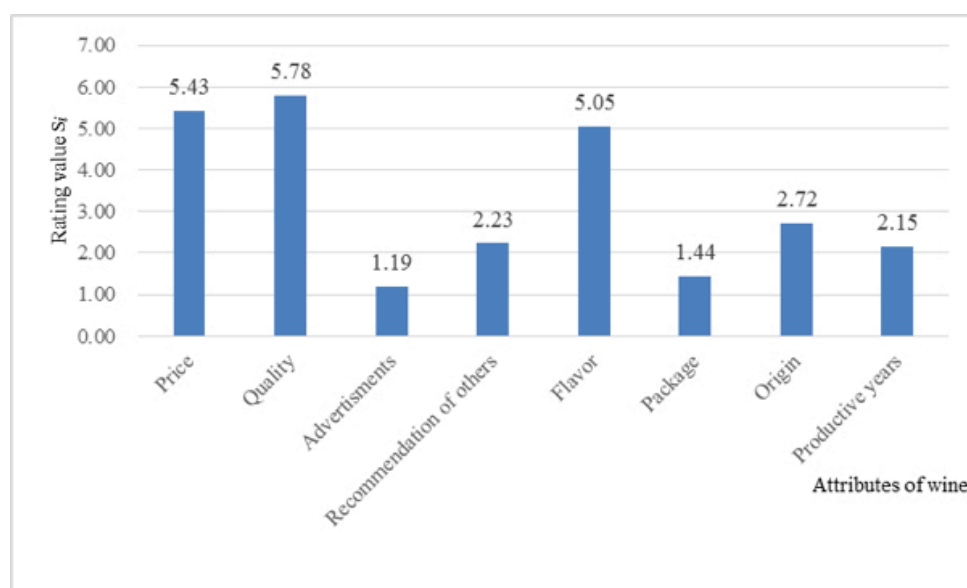


**Figure 5.** Consumers' recognition of wine attributes.

Notes: 8-most important, 7-second important, 6-third important, 5-fourth important, 4-fifth important, 3-sixth important, 2-seventh important, 1-eighth important.

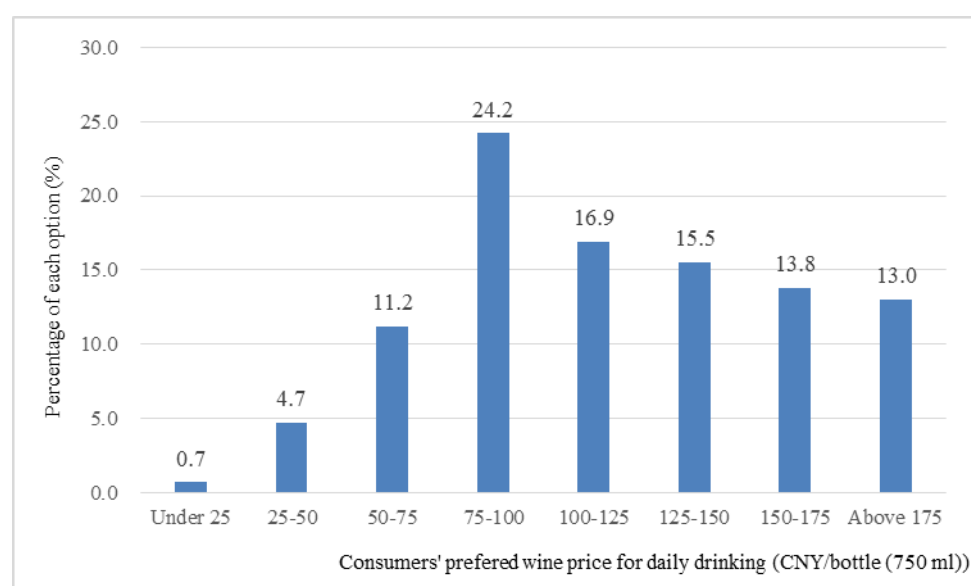


**Figure 6.** Consumers' acceptable price for wine for daily drinking.

These groups included low spenders, moderate spenders and high spenders. After the groups were identified, chi-square analysis was employed to identify the significant difference between the groups. The variables in the analyses were demographics, which include sex, age, income, level of education, and job category. The statistic results are shown in Table 5.

**Table 4.** Clustering Chinese wine consumers according to consumer preference in wine price for daily drinking

| Segments          | wine price for daily drinking |
|-------------------|-------------------------------|
| Low Spenders      | Below CNY 75 (16.6%)          |
| Moderate Spenders | Between CNY 75-100 (24.2%)    |
| High Spenders     | Above CNY 100 (59.2%)         |

**Table 5.** Demographic differences among price segments.

| Variables                     | Groups (N=825) |          |      | Chi-square Test |          |
|-------------------------------|----------------|----------|------|-----------------|----------|
|                               | Low            | Moderate | High | F               | Sig.     |
| <b>Gender</b>                 |                |          |      |                 |          |
| Male                          | 69             | 98       | 259  | 1.049           | 0.592    |
| Female                        | 68             | 102      | 229  |                 |          |
| <b>Age</b>                    |                |          |      |                 |          |
| Under 25                      | 70             | 87       | 211  | 6.492           | 0.592    |
| 26-35                         | 42             | 77       | 176  |                 |          |
| 36-45                         | 13             | 22       | 70   |                 |          |
| 46-55                         | 9              | 11       | 27   |                 |          |
| Above 55                      | 3              | 3        | 6    |                 |          |
| <b>Income<sup>a</sup></b>     |                |          |      |                 |          |
| Below 2000                    | 41             | 39       | 98   | 35.297          | 0.000*** |
| 2001-3000                     | 22             | 29       | 54   |                 |          |
| 3001-4000                     | 29             | 47       | 69   |                 |          |
| 4001-5000                     | 16             | 30       | 80   |                 |          |
| 5001-7000                     | 18             | 24       | 85   |                 |          |
| 7001-10000                    | 9              | 25       | 69   |                 |          |
| Above 10000                   | 2              | 6        | 35   |                 |          |
| <b>Educational background</b> |                |          |      |                 |          |
| Junior high school and below  | 3              | 1        | 13   | 28.469          | 0.000*** |
| Senior high school            | 23             | 29       | 29   |                 |          |
| Junior college                | 30             | 32       | 79   |                 |          |
| Undergraduate and above       | 81             | 138      | 369  |                 |          |
| <b>Job category</b>           |                |          |      |                 |          |
| Government official           | 5              | 12       | 39   | 114.014         | 0.000*** |
| Staff in enterprises          | 52             | 49       | 126  |                 |          |
| Liberal profession            | 18             | 32       | 147  |                 |          |
| Farmers                       | 1              | 1        | 2    |                 |          |
| Education departments         | 4              | 50       | 26   |                 |          |
| Students                      | 39             | 48       | 110  |                 |          |
| Unemployed/retired            | 6              | 5        | 10   |                 |          |
| Else                          | 12             | 3        | 10   |                 |          |

Notes: <sup>a</sup>Average monthly income per capita. N, the total sample number of respondents, \*\*\* Significant at 1% level respectively.

### 3.7. Demographic differences among the three segments

Chi-square Test was conducted to distinguish the significant consumer demographics, As Table 5 shows, three variables of income, educational background and job category are significant. Low spenders have a significantly lower income than the other two groups. Therefore, this may explain why more people prefer to middle-priced wine, after all, wine is also only a decoration in consumers' life. Wine is not like food, consumers are conscious about the price of wine. When wine price exceeds their psychological expectation, consumers are bound to reduce their wine consumption (MU and FENG, 2010). Income is an important predictor of wine involvement because consumers need to have the financial resources to support their interest in wine (BRUWER, 2013; Barber *et al.*, 2006).

The group composed of undergraduate and above dominated among the three groups. The education standard of high spenders was higher than that of the two other groups. The number of undergraduate and above respondents accounted for 75.3% of the high spenders, 59.1% of the moderate spenders and 69.0% of the low spenders. According to LIU *et al.* (2014), consumers are frequently exposed to information about wine because of the rapid development of the wine market in China, thus resulting in more mature consumers. THATCH (2008) argues that the actual tasting of wine helps consumers develop an educated palate.

Job category did significantly differ among the three groups. High spenders were likely to be liberal professions. Consumers who worked at education departments were likely to be moderate spenders, and staffs in enterprises mainly comprised the low spender group. Occupational difference reflects the status of consumers in society somehow. Researchers have verified that wine is considered as a symbol of social status and sophistication (FAN, 2007; LIU and MURPHY, 2007).

No significant difference was observed among the three groups in terms of gender and age.

Previous research indicated that wine is not a luxury goods for consumers with improved standard of living, and that people can significantly benefit from wine (PETTIGREW and CHARTERS, 2010). Thus, people prefer wine to other drinks, such as beer and liquor (ZHOU *et al.*, 2011).

Income, education background and job category may influence the three segments. To distinguish the actual factors that influence the three segments, a principal component analysis was conducted. Table 6 shows that the data passed the Kmo and Bartlett's test of sphericity. The eigenvalues of income and educational background were all above one, and the overall contribution rate of the three segments accounted for 82.829%. These findings indicate that income and educational background can be regarded as main factors affecting the preferred wine price of consumers for daily drinking.

**Table 6.** Principal component analysis of income, educational background and job category.

| Component              | Total | % of Variance | Cumulative<br>(%) | KMO and Bartlett's test of sphericity |          |
|------------------------|-------|---------------|-------------------|---------------------------------------|----------|
|                        |       |               |                   | F                                     | Sig.     |
| Income <sup>a</sup>    | 1.484 | 49.482        | 49.482            | 0.499                                 | 0.000*** |
| Educational background | 1.000 | 33.347        | 82.829            |                                       |          |
| Job category           | 0.515 | 17.171        | 100.000           |                                       |          |

Notes: <sup>a</sup>Average monthly income per capita, \*\*\*Significant at 1% level respectively.

### 3.8. Behavior and recognition differences in the three segments

For behavior, preferred purchase places and favorite brands were examined. First, the places where the three segments purchased wine were observed. Five common wine selling places were selected in the current study, and the respondents were asked to choose the places they usually purchase wine. The results were listed in Table 7.

**Table 7.** Behavior and preferences differences among the three segments.

| Variables              | Groups (N=825) |            |            | Chi-square Test |          |
|------------------------|----------------|------------|------------|-----------------|----------|
|                        | Low            | Moderate   | High       | F.              | Sig.     |
| <b>Purchase places</b> |                |            |            |                 |          |
| Supermarkets           | 94(68.6%)      | 135(67.5%) | 278(57.0%) | 20.287          | 0.009*** |
| Exclusive Shop         | 21(15.3%)      | 37(18.5%)  | 110(22.5%) |                 |          |
| Online shopping        | 17(12.4%)      | 15(7.5%)   | 49(10.0%)  |                 |          |
| Wine stores            | 1(0.7%)        | 11(5.5%)   | 39(8.0%)   |                 |          |
| Else                   | 4(2.9%)        | 2(1.0%)    | 12(2.5%)   |                 |          |
| <b>Favorite bands</b>  |                |            |            |                 |          |
| Changyu                | 53(38.7%)      | 76(38.0%)  | 194(39.8%) | 25.459          | 0.013**  |
| GREATWALL              | 41(29.9%)      | 70(35.0%)  | 112(23.0%) |                 |          |
| DYNASTY                | 6(4.4%)        | 3(1.5%)    | 9(1.8%)    |                 |          |
| WILON                  | 3(2.2%)        | 2(1.0%)    | 15(3.1%)   |                 |          |
| SUNTIME                | 4(2.9%)        | 3(1.5%)    | 7(1.4%)    |                 |          |
| Foreign brands         | 24(17.5%)      | 38(19.0%)  | 138(28.3%) |                 |          |
| Else                   | 6(4.4%)        | 8(4.0%)    | 13(2.7%)   |                 |          |

Notes: N, the total sample number of respondents, \*\*\*, \*\* Significant at 1% and 5% level respectively.

Consumers most frequently purchase wine in supermarkets among the three groups. The main factors affecting consumers to select the place to purchase wine are convenience and price (FENG *et al.*, 2012). As various supermarkets, which conveniently supply various and conveniently priced wines for consumers can be found in residential areas in China. Supermarkets are important in making wine accessible to the mainstream market (RICHIE, 2007).

Changyu, Greatwall, and foreign bands were preferred by the three segments. The most popular wine brand in China is Changyu, followed by Greatwall (Mu and Feng, 2010). And high spenders and low spenders also preferred Dynasty, Wilon, Sumtime and other brands.

The results of Chi-square Test indicated that consumer behavior and recognition of wine showed some statistical significantly difference. It was found that the number of high spenders was higher in other places where wine could be purchased, such as exclusive shops and wineries, than those of the other two groups. Low spenders were more likely to buy wine online shopping and other places, after all, products that sold online were cheaper than other places. For favorite brands, such as foreign brands, there was a great possibility that consumers were high spenders.

### 3.9. Knowledge and attitude differences among the three segments

To examine wine knowledge, consumers' cognition towards wine origins were analyzed. Table 8 shows that Xinjiang Province and the Ningxia Hui Autonomous Region were considered as the best wine origins by the three groups. High spenders considered Yunnan Province to be the best wine origin. To Hebei Province, which is one of the better wine origins, the number of low spenders is greater than those of the other two groups. Beijing City, Tianjin City and Hebei Province were considered as the worst wine origins in China by the three segments. Northwest areas are the best wine origins (MU and FENG, 2010), the survey result is consistent with this outcome. However, more than 5.0% of the respondents in each group considered Ganus, Shandong, and Jilin Provinces as the best and worst wine origins, thus indicating that consumers' limited knowledge about wine origins (Mu and Feng, 2010).

**Table 8.** Knowledge differences among the three segments.

|                                  | Groups (N=825) |          |      | Chi-square Test |       |
|----------------------------------|----------------|----------|------|-----------------|-------|
|                                  | Low            | Moderate | High | F               | Sig.  |
| <b>Best origins<sup>a</sup></b>  |                |          |      |                 |       |
| Beijing City                     | 19             | 26       | 55   | 22.076          | 0.229 |
| Tianjin City                     | 12             | 10       | 36   |                 |       |
| Hebei Province                   | 27             | 26       | 71   |                 |       |
| Jilin Province                   | 21             | 30       | 64   |                 |       |
| Gansu Province                   | 16             | 39       | 77   |                 |       |
| Ningxia                          | 49             | 85       | 165  |                 |       |
| Xinjiang Province                | 82             | 128      | 324  |                 |       |
| Shandong Province                | 24             | 24       | 81   |                 |       |
| Yunnan Province                  | 13             | 18       | 78   |                 |       |
| Liaoning Province                | 11             | 14       | 35   |                 |       |
| <b>Worst origins<sup>a</sup></b> |                |          |      |                 |       |
| Beijing City                     | 49             | 66       | 169  | 12.179          | 0.838 |
| Tianjin City                     | 45             | 61       | 172  |                 |       |
| Hebei Province                   | 39             | 59       | 149  |                 |       |
| Jilin Province                   | 21             | 32       | 74   |                 |       |
| Gansu Province                   | 15             | 32       | 72   |                 |       |
| Ningxia                          | 12             | 17       | 41   |                 |       |
| Xinjiang Province                | 5              | 3        | 19   |                 |       |
| Shandong Province                | 23             | 24       | 85   |                 |       |
| Yunnan Province                  | 25             | 40       | 91   |                 |       |
| Liaoning Province                | 39             | 56       | 104  |                 |       |

Notes: N, the total sample number of respondents, <sup>a</sup>The question is multiple choice.

For deeper statistical analysis of consumers' origins knowledge above findings, it is found that consumers presented no significant difference in wine origin knowledge. This finding is not surprising. According to Liu *et al.* (2014) confirmed that a wine culture is lacking in China and many Chinese wine consumers were novice wine buyers. Chinese even have low awareness of grape varieties or food matching (Jenster and Cheng, 2008).

Consumers' recognition of wine attributes was examined to determine the differences in attitude of the three segments. Table 9 shows that significant difference exists among the respondents in terms of the importance of wine attribute.

**Table 9.** Attitude differences among the three segments.

| Attributes             | Groups (N=825) |           |            | Chi-square Test |          |
|------------------------|----------------|-----------|------------|-----------------|----------|
|                        | Low            | Moderate  | High       | F.              | Sig.     |
| Most important         |                |           |            |                 |          |
| Price                  | 64(46.7%)      | 68(34.0%) | 134(27.5%) | 29.534          | 0.009*** |
| Quality                | 35(25.5%)      | 57(28.5%) | 140(28.7%) |                 |          |
| Advertisement          | 3(2.2%)        | 8(4.0%)   | 13(2.7%)   |                 |          |
| Recommended by friends | 6(4.4%)        | 5(2.5%)   | 36(7.4%)   |                 |          |
| Flavor                 | 16(11.7%)      | 38(19.0%) | 93(19.1%)  |                 |          |
| Package                | 2(1.5%)        | 5(2.5%)   | 6(1.2%)    |                 |          |
| Origins                | 6(4.4%)        | 12(6.0%)  | 42(8.6%)   |                 |          |
| Productive year        | 5(3.6%)        | 7(3.5%)   | 24(4.9%)   |                 |          |
| Second important       |                |           |            |                 |          |
| Price                  | 30(21.9%)      | 33(16.5%) | 52(10.7%)  | 30.602          | 0.006*** |
| Quality                | 47(34.3%)      | 68(34.0%) | 159(32.7%) |                 |          |
| Advertisement          | 13(9.5%)       | 7(3.5%)   | 23(4.7%)   |                 |          |
| Recommended by friends | 7(5.1%)        | 22(11.0%) | 46(9.5%)   |                 |          |
| Flavor                 | 30(21.9%)      | 38(19.0%) | 118(24.3%) |                 |          |
| Package                | 4(2.9%)        | 11(5.5%)  | 26(5.3%)   |                 |          |
| Origins                | 3(2.2%)        | 13(6.5%)  | 38(7.8%)   |                 |          |
| Productive year        | 3(2.2%)        | 8(4.0%)   | 24(4.9%)   |                 |          |
| Third important        |                |           |            |                 |          |
| Price                  | 19(13.9%)      | 36(18.0%) | 78(16.0%)  | 16.144          | 0.305    |
| Quality                | 18(13.1%)      | 23(11.5%) | 58(11.9%)  |                 |          |
| Advertisement          | 12(8.8%)       | 14(7.0%)  | 23(4.7%)   |                 |          |
| Recommended by friends | 20(14.6%)      | 25(12.5%) | 42(8.6%)   |                 |          |
| Flavor                 | 36(26.3%)      | 49(24.5%) | 121(24.8%) |                 |          |
| Package                | 9(6.6%)        | 15(7.5%)  | 42(8.6%)   |                 |          |
| Origins                | 16(11.7%)      | 26(13.0%) | 74(15.2%)  |                 |          |
| Productive year        | 7(5.1%)        | 12(6.0%)  | 50(10.2%)  |                 |          |
| Forth important        |                |           |            |                 |          |
| Price                  | 9(6.6%)        | 26(13.0%) | 82(16.8%)  | 25.874          | 0.027**  |
| Quality                | 6(4.4%)        | 14(7.0%)  | 33(6.8%)   |                 |          |
| Advertisement          | 7(5.1%)        | 11(5.5%)  | 24(4.9%)   |                 |          |
| Recommended by friends | 23(16.8%)      | 21(10.5%) | 39(8.0%)   |                 |          |
| Flavor                 | 14(10.2%)      | 22(11.0%) | 41(8.4%)   |                 |          |
| Package                | 21(15.3%)      | 18(9.0%)  | 44(9.0%)   |                 |          |
| Origins                | 24(17.5%)      | 48(24.0%) | 111(22.7%) |                 |          |
| Productive year        | 33(24.1%)      | 40(20.0%) | 114(23.4%) |                 |          |

Notes: N, the total sample number of respondents, \*\*\*, \*\* Significant at 1% and 5% level respectively.

Low spenders (46.7%) and moderate spenders (34.0%) considered price as the most important attribute, followed by quality, flavor, and origins. High spenders (28.7%) prioritized quality, followed by price, flavor, and source.

The results of the three groups were also inconsistent regarding the second most important wine attribute. High spenders focused on the intrinsic attributes (quality, flavor, and source).

The three segments did not significantly differ in the third most important attribute of wine, as flavor dominated all the three segments.

Low and high spenders chose productive year as forth most important attribute, and moderate spenders favored source. This segment result was similar to that of Liu *et al.* (2014), in which consumers were categorized to intrinsic and extrinsic cluster. The intrinsic cluster mainly sought information about wine quality, price, flavor, and brand name, which are considered as the intrinsic attributes of wine. The extrinsic cluster pertained to wine package, and foreign wine, which are regarded as the extrinsic attributes of wine. According to Liu *et al.* (2014), Chinese wine consumers with a high income were likely to belong to the intrinsic cluster, whereas consumers with a low income belonged to the extrinsic cluster. The previous analysis indicated that high spenders have a higher income than the other two groups. Therefore it was persuasive that why High Spenders prioritized quality and low spenders and moderate spenders considered price as the most important factor when purchase.

Attitude positively affects the identification of factors influencing the purchasing behaviors of consumers. The result indicates that quality, price, flavor and source are four most important wine attributes. Batt (2000) verifies that price is the most important factor affecting the purchasing behavior of consumers. Gunay and Baker (2011) demonstrate the importance of price, origin, and quality, and finds that quality and price are the most important factors affecting the purchase decision of consumers.

### **3.10. Gift-oriented acceptable price of wine difference among the three segments**

The previous analysis showed that a relatively high number of consumers purchased wine for daily drinking and gifts, which were quite different in terms of motivation, preferred price and brand preference (PAN, 2012). Consumers are willing to pay a premium for wine as gifts because wine is a status symbol (Liu and Murphy, 2007). Thus, the accepted price of wine as gifts may differ. Therefore, this study separately analyzed consumers' price acceptability of wine for daily drinking and that of wine for gifts. Before designing the questionnaire, the research staff surveyed two supermarkets and three wine shops in Beijing to acquire information about wine price. The price data and wine price ranges were set for wine as gifts in the questionnaire. Table 10 shows that the acceptable prices for gift-oriented wine differed among the three segments.

High spenders tend to buy high priced wine. Respondents with an income of CNY 100-CNY 150(30.7%) dominated the low spenders. Respondents with an income of CNY 150-CNY 200(26.0%) comprised the largest proportion in the moderate spenders group, followed by those with an income of CNY 200-CNY 250(21.0%). The price of the overall tendency of high spenders was higher than that of the other two groups. A consumer who buys a bottle (750 ml) of wine with a price above CNY 250 may be to be a high spender (77.7%).

A Chi-square Test was conducted to identity statistical significant. The results verify that there is great significant difference in preferred price of wine as gifts. Income is an important predictor of wine involvement because consumers need to have the financial resources to support their choice (BARBER *et al.*, 2006).

**Table 10.** Gift-oriented wine price preference difference among the three segments.

| Variables                                | Groups (N=825) |           |            | Chi-square Test |          |
|------------------------------------------|----------------|-----------|------------|-----------------|----------|
|                                          | Low            | Moderate  | High       | F.              | Sign.    |
| <b>Price for gift-oriented wine(CNY)</b> |                |           |            |                 |          |
| Under 50                                 | 3(2.2%)        | 0(0.0%)   | 2(0.4%)    |                 |          |
| 50-100                                   | 23(16.8%)      | 5(2.5%)   | 1(0.2%)    |                 |          |
| 100-150                                  | 42(30.7%)      | 28(14.0%) | 19(3.9%)   |                 |          |
| 150-200                                  | 30(21.9%)      | 52(26.0%) | 63(12.9%)  |                 |          |
| 200-250                                  | 16(11.7%)      | 42(21.0%) | 69(14.1%)  |                 |          |
| 250-300                                  | 3(2.2%)        | 17(8.5%)  | 55(11.3%)  | 262.813         | 0.000*** |
| 300-350                                  | 6(4.4%)        | 16(8.0%)  | 73(15.0%)  |                 |          |
| 350-400                                  | 4(2.9%)        | 18(9.0%)  | 45(9.2%)   |                 |          |
| 400-450                                  | 4(2.9%)        | 9(4.5%)   | 57(11.7%)  |                 |          |
| Above 450                                | 6(4.4%)        | 13(6.5%)  | 104(21.3%) |                 |          |

Notes: N, the total sample number of respondents, \*\*\*Significant at 1% and 5% level respectively.

The previous analysis shows that income and education background significantly differ among the three segments. High spenders are likely to have a high income and educational level. Sending expensive wine is an act of politeness and respect (SUN *et al.*, 2009). Therefore, high spenders had sufficient money and were more likely to choose expensive wine than other types of spenders.

### 3.11. Profiles of the three consumers segments

Creating profiles of the three different price segments based on the significant variables identified in this research is required. Table 11 illustrates the major differences among the segments.

**Table 11.** Profiles of Chinese consumers by price.

| Significant Variables                 | Groups                                         |                                                |                                                            |
|---------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------------------|
|                                       | Low                                            | Moderate                                       | High                                                       |
| <b>Demographics</b>                   |                                                |                                                |                                                            |
| Income                                | Below 2000                                     | 3001-4000                                      | 5001-7000                                                  |
| Educational background                | Undergraduate and above                        | Undergraduate and above                        | Undergraduate and above                                    |
| Job category                          | Staff enterprises                              | Education department                           | Liberal profession                                         |
| <b>Behaviors</b>                      |                                                |                                                |                                                            |
| Purchase places                       | supermarkets, exclusive shops, online shopping | supermarkets, exclusive shops, online shopping | Wine stores, supermarkets, exclusive shop, online shopping |
| Preferred brands                      | Changyu, GreatWall                             | Changyu, GreatWall                             | Changyu, foreign brands                                    |
| Attitude to wine attributes           | Price, quality, flavor, productive year        | Price, quality, flavor, origins                | Quality, price, flavor, productive year                    |
| Accepted price for gift-oriented wine | CNY 100-150                                    | CNY 150-200                                    | Above CNY 250                                              |

## 4. CONCLUSIONS

The frequency of wine consumption is affected by age. The 26-35 age group dominates the often purchase cluster, and under 25 age group is mostly in the purchase occasionally. These results show that more young are involved in wine consumption. Wine consumption occurs in many occasions, mainly at home, during holidays; most people also send wine as gifts. A significant difference is found between consumers' income and consumption occasion. Consumers with low income tend to consume wine on special occasions, such as the Chinese traditional holiday. And on occasions that require the consumption of wine such as business banquets, consumers whose income is CNY 7001-CNY 10000 (28.8%) seem to have much money and more chances to consume wine. Consumers are familiar with three brands, namely, Greatwall, Changyu, and Dynasty. This research introduces the Chinese wine consumer price segments, namely, low spenders, moderate spenders, high spenders, and explores their differences in demographics, behaviors and preferences, and attitudes. High spenders have incomes higher than those of the other two groups, and they may be liberal professions. Conversely, lower spenders may work in enterprises and moderate spenders can be in the education department.

All three groups choose supermarket as their first wine purchase place, and a considerable number of high spenders purchase wine in wine stores. Excluding Changyu, the favorite wine brands of high spenders are foreign brands, and the favorite wine of the other two groups is Greatwall.

Knowledge about wine origins of the three segments is limited, and their cognition of the importance of wine attributes has a significant difference. High spenders look for quality, price, flavor, productive year; moderate spenders look for price, quality, flavor, source; and low spenders look for price, quality, flavor, and productive year. A significant difference is observed in the acceptable wine price for gift-oriented. Low Spenders prefer wines priced at CNY 100-CNY 150, and moderate spenders are inclined to buy wine priced at CNY 150-CNY 200. High spenders prefer wines with the highest price at above CNY 250 among the three segments.

However, the investigation was only conducted in Beijing because of the limitation of time and energy and this is also the limitation of this paper. In order to better understand Chinese wine consumer consumption characteristics, survey in more provinces of China is the focus of the authors in the near future.

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## NUTRITIONAL EVALUATION OF FRESH AND DRIED GOJI BERRIES CULTIVATED IN ITALY

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### ABSTRACT

The nutritional profile of fresh and dried goji berries cultivated in Italy was investigated. The obtained data confirm goji berries as a source of nutritional and healthy components, such as vitamin E, minerals and fibre. Taking into account the Recommended Daily Allowance (RDA) for minerals and vitamins established by the Commission of the European Communities, Goji berries provide significant amounts of dietary fibre and zeaxanthin and can be declared on the label as a potential source of vitamins E and C. Moreover, dried goji berries can be declared as a source of K, P, Cu, Fe Mn, Zn.

*Keywords:* goji berries, *Lycium barbarum*, superfruit, wolfberries

## 1. INTRODUCTION

Fruits of *Lycium barbarum* L., belonging to Solanaceae family, commonly known as goji berries or wolfberries, have been used in Chinese traditional medicine for centuries. *Lycium barbarum* grows in China, Tibet and other parts of Asia and its fruits are 1-2 cm-long, bright orange-red ellipsoid berries. The native area of *Lycium* is not definitively established but it is likely found in the Mediterranean Basin (POTTERAT, 2010). Traditionally, goji berries are collected in summer and autumn. The fruits can be eaten fresh or dried, and they are also found in conventional food products, such as yoghurt, fruit juices, bakery foods, chocolate and others (MIKULIC-PETKOVSEK *et al.*, 2012a, 2012b). The drying process is intended to remove water from foodstuff in order to prevent microbial spoilage and chemical alterations, thus prolonging shelf life, while realizing space and weight saving (CINQUANTA *et al.*, 2010; CUCCURULLO *et al.*, 2012; FRATIANNI *et al.*, 2013). Traditionally, the berries are dried in the shade until the skin shrinks and then exposed to the sun until the outer skin becomes dry and hard but the pulp is still soft (AMAGASE and FARNSWORTH, 2011). The sun drying method is cheap, but there is a risk of damage due to dust and insect infestation. An alternative is hot air drying. Today the goji fruit market is significantly expanding because of an increased awareness of the possible health benefits, as fruits contain different nutrients, such as polysaccharide complexes, organic acids, phenolic compounds and antioxidants with high biological activity. Dietary fibre provides several health benefits, including the reduction of the risk of coronary heart disease, of diabetes, hypertension, obesity, stroke and some gastrointestinal disorders (EFSA, 2010). Recent studies indicate that polysaccharides from *Lycium barbarum* possess a range of biological activities, including antioxidant properties (AMAGASE and NANCE, 2008; CHANG and SO, 2008). Goji fruits constitute a variety of antioxidants such as ascorbic acid, different carotenoids (KULCZYŃSKI and GRAMZA-MICHAŁOWSKA, 2016) and high levels of phenolic compounds (ZHANG *et al.*, 2016). Carotenoids are a significant group of biologically-active constituents with health promoting properties (AMAGASE *et al.*, 2009; DONNO *et al.*, 2014) responsible for the colour of a wide variety of foods (FRATIANNI *et al.*, 2005).

The reddish-orange colour of *L. barbarum* fruits derives from a group of carotenoids, which make up only 0.03–0.5 % of the dried fruit. Zeaxanthin is the major carotenoid found in goji. This is a yellow pigment, an isomer of lutein and a derivative of  $\beta$ -carotene. When ingested, zeaxanthin accumulates in fatty tissues, but especially in the macula, a region of the retina, helping in protecting the macula from degeneration, which can be induced by excessive sun exposure (UV light) and by other oxidative processes. In goji, zeaxanthin is present as an ester of dipalmitate. Studies focusing on carotenoid goji berries are few and mainly aimed at the identification and quantification of ester-form carotenoids. INBARAJ *et al.* (2008) and ZHAO *et al.* (2013), in particular, identified free-forms and ester-forms of carotenoids. Beta-carotene, neoxanthin, and cryptoxanthin are also present at low concentrations (PENG *et al.*, 2005; WANG *et al.*, 2010). Regarding other antioxidants, studies made on *Lycium chinense* Miller reported high amounts of  $\alpha$ -tocopherol, together with other vitamin E compounds (ISABELLE *et al.*, 2010). Vitamin E is a generic term indicating structurally related compounds, namely tocopherols, comprising two groups of vitamers, i.e. tocopherols and tocotrienols, which occur in eight forms:  $\alpha$ -tocopherol ( $\alpha$ -T),  $\beta$ -tocopherol ( $\beta$ -T),  $\gamma$ -tocopherol ( $\gamma$ -T), and  $\delta$ -tocopherol ( $\delta$ -T) and  $\alpha$ -tocotrienol ( $\alpha$ -T3),  $\beta$ -tocotrienol ( $\beta$ -T3),  $\gamma$ -tocotrienol ( $\gamma$ -T3), and  $\delta$ -tocotrienol ( $\delta$ -T3). The potential health benefits of tocopherols have been the subject of several reviews (TIWARI and CUMMINS, 2009). Vegetable oils are the main tocopherol source; however, substantial amounts of these compounds are also reported in most cereal grains (FRATIANNI *et al.*, 2013; MIGNOGNA

*et al.*, 2015; PANFILI *et al.*, 2003). To our knowledge, no literature data are available on the composition and content of tocopherols in *L. barbarum* fruits. Goji is also an extremely rich source of many essential minerals, which are essential for many actions in the body, like muscle contraction, normal heart rhythm, nerve impulse conduction, oxygen transport, oxidative phosphorylation, enzyme activation, immune functions, antioxidant activity, bone health, and acid-base balance of the blood (WILLIAMS, 2005; SALDAML and SAĞLAM, 2007). An adequate daily amount of minerals is necessary for an optimal functioning of the body. For the above reported reasons, goji berries are often proposed as functional foods and have been included in the novel category of “superfruits” or “superfoods”.

Superfruits, a subcategory of superfoods, is a relatively recent word and is considered a new marketing approach to promoting common or rare fruits which can be consumed as foodstuffs or used as ingredients by manufacturers of functional foods, beverages and nutraceuticals. Superfruits have a high nutritional value due to their richness in nutrients, antioxidants, proven or potential health benefits and taste appeal (FELZENSZWALB, 2013). In the functional foods market, the products targeting health and mental well-being have prompted the food industry to increase the research and the development of these new foods, outlining a rapid expansion market in several countries (VICENTINI *et al.*, 2016). In the last years, goji berries have been cultivated in Italy and are available both as fresh and dried fruits. While several papers on the medical effect of goji berries have been published, little information is available on the nutritional composition of dried and, above all, fresh goji berries. The aim of the present study is therefore to determine the compositional and nutritional value of fresh and dried goji berries cultivated in Italy, with a particular focus on minerals and some antioxidant compounds, such as carotenoids, tocopherols and vitamin C, to increase the awareness about their nutritional profile.

## 2. MATERIAL AND METHODS

### 2.1. Sample collection and preparation

Fresh goji berries (*L. barbarum* L.) were provided by Favella Spa farm (Sibari, Southern Italy). The farm has 21000 plants in 5 Ha (2.5 m x 1 m), with a drip irrigation system. Goji berries were cultivated in two consecutively growing seasons (2014 and 2015) and were collected in July. All harvested fruits were randomly collected in the orchard from different plants and analysed fresh and air-dried. Fresh goji berries (about 1 cm size) were subjected to freeze-drying before analyses, as reported by FRATIANNI *et al.*, 2013 (fresh fruits). One-half of collected goji berries were air-dried in a convective dryer (B80FCV/E6L3, Termaks, Norway), at 60 °C, with an air velocity of 2.1 m/s, until a constant weight was reached (dried fruits). The drying time was about 21 h. Results are reported as the average of the two growing seasons (2014-2015).

### 2.3 Proximate composition analysis

Fresh and dried fruits were analysed for moisture, ash, fat, and protein (N×6.25) contents, according to standard methods of AOAC (2000). Dietary fibre content was determined according to AOAC method 991.43 (1995) and AACC method 32-07 (1995). Total dietary fibre content was the sum of insoluble and soluble dietary fibre content. Vitamin C was determined by using an enzymatic kit (Megazyme International, Ireland), following the manufacturer instructions.

## 2.4. Mineral analysis

Ultrapure nitric acid for trace analysis, sulfuric acid (96 %) and standard mono elements in nitric acid 2 % were purchased from Sigma-Aldrich (20151 Milan, Italy). The determination of metals (K, Ca, Co, Cu, Fe, Mg, Mn, Mo, Na, P, Se, Zn) in goji samples was carried out by using the technique of nitric mineralization and the analysis by spectrophotometry plasma emission (*Varian ICP 710*, OES Inductively Coupled Plasma-Optical Emission Spectrometers, Palo Alto, CA 94304-1038). Samples were ground and 0.5 g was digested with 10 ml of nitric acid with a mineralizer (SCP Science DIGIprep, Quebec H9X 4B6, Canada), with the following instrumental conditions: start at 40 °C for 15 minutes; heating at 60 °C for 15 minutes; stay at 60 °C for 15 minutes; heating to 90 °C for 20 minutes. The digested samples were cooled and brought to a volume of 50 ml with bidistilled water and analysed with the optical ICP. The precision was calculated as a mean deviation of three measurements.

## 2.5. Carotenoid extraction and determination

Carotenoid extraction was carried out using the direct solvent extraction method reported in FRATIANNI *et al.* (2013) with slight modifications due to the complex structure of goji berries. About 0.1 g of milled freeze-dried samples (fresh fruit) and air-dried samples (dried fruit) was weighed and placed in a screw-capped tube. Then, 5 ml of ethanolic pyrogallol (60 g/L) was added as an antioxidant. The sample was stirred for 10 minutes. After that, 2 ml of absolute ethanol was added and the sample was stirred again for a few minutes. The suspension was then extracted with 15 mL of n-hexane/ethyl acetate (9:1 v/v) and stirred; after that 15 mL of sodium chloride (10 g/L) was added. Further extractions with n-hexane/ethyl acetate (9:1 v/v) were made until the organic layer was colourless. Finally, the organic layer was collected and evaporated to dryness, and the dry residue was dissolved in methanol: MTBE 50:50 (v/v). This sample was used to determine the free carotenoids not esterified with the lipid components and carotenoids esterified with fatty acids (unsaponified). A volume of 2 ml of this extract was evaporated to dryness and subjected to alkaline hydrolysis under a nitrogen flux for 1 minute in a screw-capped tube with 1 ml of ethanolic pyrogallol (60 g/L), 10 ml of solvent HEAT (hexane: ethanol: acetone: toluene 10: 6: 7: 7 v/v/v/v), 2 ml of methanolic KOH (40 %) and glass balls. The tubes were placed in a 56 °C water bath and mixed every 5 to 10 min. After alkaline digestion at 56 °C for 20 minutes, the tubes were cooled in an ice bath, and 15 mL of sodium chloride (10 g/L) were added. The suspension was then extracted with 15 mL of n-hexane/ethyl acetate (9:1 v/v) until the organic layer was colourless. The organic layer was collected and evaporated to dryness, and the dry residue was dissolved in methanol: MTBE 50:50 (v/v). This sample was used to determine carotenoids esterified with lipid components (saponified). An aliquot of the carotenoid extract was separated, as in MOULY *et al.* (1999), by a reverse-phase HPLC system. An HPLC Dionex (Sunnyvale, CA) analytical system, consisting of U3000 pumps, and an injector loop (Rheodyne, Cotati) were used. Separation was performed as in FRATIANNI *et al.* (2013) by using a YMC (Hampstead, NC, USA) stainless steel column (250×4.6 mm i.d.) packed with 5 µm silica spheres that were chemically bonded with a C30 material at a flow rate of 1 mL/min. The mobile phase was methanol: MTBE (v/v). The eluted compounds were monitored by a photo-diode array detector (Dionex, Sunnyvale) set at 430 nm.

## 2.6. Carotenoid identification and quantification

Carotenoids were identified on the basis of their diode array spectral characteristics, retention times, and relative elution order, compared with known commercially available standards. All-trans- $\beta$ -carotene and lutein were from Sigma Chemicals (St. Luis, MO, USA); zeaxanthin and  $\beta$ -cryptoxanthin were obtained from Extrasynthese (Z.I. Lyon-Nord, Genay, France). Zeaxanthin dipalmitate was identified by means of its spectral characteristics found in literature (INBARAJ *et al.*, 2008). Compounds were identified by comparison of their retention times with those of known available standard solutions and quantified on the basis of the calibration curves of standard solutions. Zeaxanthin dipalmitate was quantified as zeaxanthin.

## 2.7. Tocol analysis

Tocols were determined after the saponification method of the extract described for carotenoids. An aliquot of the carotenoid extract was collected and evaporated to dryness, and the dry residue was dissolved in 2 ml of isopropyl alcohol (1 %) in n-hexane and was analysed by HPLC under normal phase conditions, using a 250 x 4.6 mm i.d., 5 mm particle size, and Kromasil Phenomenex Si column (Torrance, CA, USA) (PANFILI *et al.*, 2003). Fluorometric detection of all compounds was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm by means of an RF 2000 spectrofluorimeter (Dionex, Sunnyvale, USA). The mobile phase was n-hexane/ethyl acetate/acetic acid (97.3:1.8:0.9 v/v/v) at a flow rate of 1.6 mL/min (FRATIANNI *et al.*, 2002; PANFILI *et al.*, 2003). Compounds were identified by comparison of their retention times with those of known available standard solutions and quantified on the basis of the calibration curves of standard solutions. The concentration range was 5-25  $\mu$ g/ml for every tocol standard. Vitamin E activity was expressed as Tocopherol Equivalent (T.E.) (mg/100 g product), calculated as reported by SHEPPARD *et al.* (1993).

# 3. RESULTS AND DISCUSSION

## 3.1. Nutritional composition

The nutritional composition of fresh and dried goji berries is shown in Table 1. Fresh goji berries have 77.4 % moisture, 1.1 % fats, 2.5 % proteins, 15.3 % carbohydrates and 2.9 % fibre. In dried goji berries, 4.4 % fats, 10.2% proteins, 61.3 % carbohydrates and 11.4 % fibre were found. Similar results on dried goji were reported by ENDES *et al.* (2015). Our data suggest that dried fruits contain notable levels of dietary fibre, either as water-soluble form (2.6 %) or as insoluble form (8.8 %). The ratio between insoluble and soluble fibre is about 3:1. Dietary fibre intake recommendation for adults is 25 g/day (LARN, 2014). With the consumption of a portion of 30 g of dried fruits, dietary fibre intake for the adults is about 14 % of its daily recommended intake. Taking into account the European law (Regulation CE 1924/2006), dried goji can be declared in label with the claim "high fibre content", since it contains at least 6 g of fibre per 100 g. Finally, fresh and dried goji berries provide about 87 and 348 kcal/100 g, respectively.

**Table 1.** Nutritional composition of fresh and dried goji berries (g/100 g) (mean±standard deviation).

|              | Moisture | Fats     | Proteins  | Carbohydrates* | Soluble  | Fibre<br>Insoluble | Total | Ash       |
|--------------|----------|----------|-----------|----------------|----------|--------------------|-------|-----------|
| <b>Fresh</b> | 77.4±0.4 | 1.1±0.02 | 2.5±0.12  | 15.3           | 0.7±0.17 | 2.2±0.02           | 2.9   | 0.84±0.11 |
| <b>Dried</b> | 9.3±0.02 | 4.4±0.45 | 10.2±0.22 | 61.3           | 2.6±0.06 | 8.8±0.01           | 11.4  | 3.4±0.16  |

\* Calculated by difference.

### 3.2. Mineral composition

The content of both macro and microelements in goji berries is reported in Table 2. Potassium (K) is the predominant element (276.2 mg/100 g and 881.9 mg/100 g for fresh and dried fruits, respectively), followed by sodium (Na). Potassium and sodium play an important role in regulating blood pressure and the body's acid-base balance (CLAUSEN *et al.*, 2013). Goji could also be a good source of phosphorus (P) and calcium (Ca), with an appreciable concentration of magnesium (Mg), which is needed to prevent heart disease and growth retardation (CHATURVEDI *et al.*, 2004). A discrete amount of copper (Cu), iron (Fe) and manganese (Mn) were also found. BELLAIO *et al.* (2016), ENDES *et al.* (2015) and LLORENT-MARTÍNEZ *et al.* (2013) reported slightly different results. As in any other plant food, the mineral content of berries reflects the soil in which they are grown. It is important to highlight that essential and nonessential element concentration is dependent on the soil characteristics, the physiology of the plant, the water source composition, and fertilizers, insecticides, pesticides, and fungicides used in the plantations. Plants can absorb, carry, and accumulate chemical elements. Each species has its own requirements and differing levels of tolerance when absorbing and accumulating an element. The movement of the inorganic constituents is selectively controlled by the plant, with some being easily absorbed and others impeded to a different degree (NAOZUKA *et al.*, 2011).

**Table 2.** Average values of mineral elements in fresh and dried goji berries (mg/100 g) (mean±standard deviation).

|                     | Fresh       | Dried        |
|---------------------|-------------|--------------|
| <b>Ca</b>           | 26.6±4.90   | 101.3±22.60  |
| <b>K</b>            | 276.2±41.00 | 881.9±239.70 |
| <b>Mg</b>           | 12.7±2.80   | 45.9±9.20    |
| <b>Na</b>           | 57.3±8.70   | 209.8±72.30  |
| <b>P</b>            | 48.4±9.26   | 174.3±32.10  |
| <b>Co</b>           | 0.001       | 0.001        |
| <b>Cu</b>           | 0.3±0.04    | 0.8±0.25     |
| <b>Fe</b>           | 0.9±0.22    | 3.4±1.57     |
| <b>Mn</b>           | 0.2±0.03    | 0.5±0.18     |
| <b>Zn</b>           | 0.5±0.12    | 1.5±0.62     |
| <b>Se (µg/100g)</b> | 0.1±0.01    | 0.17±0.03    |
| <b>Mo (µg/100g)</b> | 0.00        | 0.00         |

Table 3 reports the percentage contribution to the RDA of 100 g of fresh and dried goji berries, according to Reg. EU 1169/2011. For dried goji berries, the percentage of RDA per portion (30 g) is also reported. From data, fresh goji berries can be declared on the label as a source of Cu; in fact, 100 g of fresh goji berries contributed to about 25% of the RDA. The contribution of other minerals from fresh goji is low. Dried goji berries can be declared as a source of K, P, Cu, Fe Mn, Zn. A consumption of 30 g of dried goji per day contributes to the RDA approximately of 25 % for Cu, 13 % for K and less than 10 % for other elements.

**Table 3.** Percentage contribution to the RDA of minerals in fresh and dried goji berries.

|                | <b>Reg. RDA<br/>mg/day</b> | <b>Fresh<br/>% RDA</b> | <b>Dried<br/>% RDA</b> | <b>Dried<br/>% RDA x 30g</b> |
|----------------|----------------------------|------------------------|------------------------|------------------------------|
| <b>Ca</b>      | 800                        | 3                      | 13                     | 4                            |
| <b>K</b>       | 2000                       | 14                     | 44                     | 13                           |
| <b>Mg</b>      | 375                        | 3                      | 12                     | 4                            |
| <b>P</b>       | 700                        | 7                      | 25                     | 7                            |
| <b>Cu</b>      | 1                          | 25                     | 84                     | 25                           |
| <b>Fe</b>      | 14                         | 6                      | 24                     | 7                            |
| <b>Mn</b>      | 2                          | 8                      | 26                     | 8                            |
| <b>Zn</b>      | 10                         | 5                      | 15                     | 5                            |
| <b>Se (µg)</b> | 55                         | 0                      | 0                      | 0                            |

### 3.3. Carotenoid, tocol and ascorbic acid amounts

Table 4 shows HPLC carotenoid analysis of fresh and dried fruits. Drying of fruits did not cause significant changes in carotenoid amounts (data not shown). Unsaponified carotenoids, determined after solvent extraction, and saponified carotenoids, determined after saponification of the extract, are reported.

**Table 4.** Average carotenoid amounts in fresh and dried goji berries (mg/100 g) (mean±standard deviation).

|                               | <b>Fresh</b>        |                   | <b>Dried</b>        |                   |
|-------------------------------|---------------------|-------------------|---------------------|-------------------|
|                               | <b>Unsaponified</b> | <b>Saponified</b> | <b>Unsaponified</b> | <b>Saponified</b> |
| <b>Lutein</b>                 | 0.0                 | 1.1±0.02          | 0.0                 | 5.7±0.44          |
| <b>Zeaxanthin</b>             | 0.0                 | 53.8±0.82         | 0.0                 | 186.0±3.80        |
| <b>β-cryptoxanthin</b>        | 0.0                 | 2.3±0.25          | 0.0                 | 6.1±0.14          |
| <b>Zeaxanthin dipalmitate</b> | 47.8±2.32           | 0.0               | 158.8±1.53          | 0.0               |
| <b>Esters</b>                 | 8.5±1.24            | 0.0               | 24.5±2.30           | 0.0               |
| <b>β-carotene</b>             | 0.2±0.01            | 0.1±0.01          | 0.9±0.09            | 1.0±0.20          |
| <b>Total carotenoids</b>      | 56.4±1.23           | 57.3±0.80         | 184.2±1.52          | 198.8±3.80        |

Unsaponified carotenoids are characterized by a significant peak, identified as zeaxanthin dipalmitate, the dominant ester of goji berries (WELLER and BREITHAUPT, 2003; INBARAJ *et al.*, 2008). Beta-carotene is also present. Before zeaxanthin dipalmitate peak, other unidentified peaks, probably carotenoid esters (INBARAJ *et al.*, 2008), were also

detected. The amount of zeaxanthin dipalmitate in dried fruits is about 159 mg/100 g and of  $\beta$ -carotene is about 1 mg/100 g. INBARAJ *et al.* (2008) found values of zeaxanthin dipalmitate and of  $\beta$ -carotene of 114.3 mg/100 g and 2.4 mg/100 g, respectively. This difference is probably due to the fact that carotenoid levels can be influenced by different harvest stage fruits, geographical origin, and seasonality (WEN-PING *et al.*, 2008). Saponification of the extract is necessary to convert esters to free-compounds and it is often used to remove chlorophylls, lipids and other analytical interferences (FRATIANNI *et al.*, 2015). The saponified extract of dried fruits shows high zeaxanthin contents (about 190 mg/100 g) and small lutein and  $\beta$ -cryptoxanthin amounts (about 6 mg/100). Small amounts of lutein were also found, after saponification, in a work of ZAO *et al.*, 2013. As a dietary supplement for eye health (CHENG *et al.*, 2005), a dose of 15 g per day was deemed beneficial in supplying adequate zeaxanthin (estimated at 3 mg/day). Thirty g of our goji samples provide a zeaxanthin amount of 14 mg/day (fresh fruit) and 48 mg/day (dried fruit). Table 5 shows the tocol amounts in fresh and dried goji berries. As for carotenoids, the drying treatment did not cause significant changes in tocol contents (data not shown).

**Table 5.** Average tocopherol amounts in fresh and dried goji berries (mg/100 g) (mean $\pm$ standard deviation).

|                                               | Fresh          | Dried          |
|-----------------------------------------------|----------------|----------------|
| <b><math>\alpha</math>-tocopherol</b>         | 1.4 $\pm$ 0.10 | 5.5 $\pm$ 0.48 |
| <b><math>\beta</math> -tocopherol</b>         | 1.0 $\pm$ 0.01 | 4.2 $\pm$ 0.04 |
| <b>Total tocopherols</b>                      | 2.4 $\pm$ 0.04 | 9.7 $\pm$ 0.20 |
| <b>Tocopherol Equivalent (TE)<sup>§</sup></b> | 2              | 8              |

<sup>§</sup> Calculated as in SHEPPARD *et al.*, 1993.

Goji berries were found as a source of  $\alpha$ - and  $\beta$ -tocopherol (about 1.4 and 1.0 mg/100 g, respectively, in fresh fruits, and 5.5 and 4.2 mg/100 g, respectively, in dried fruits). A paper by ISABELLE *et al.* (2015) reports, in *Lycium chinenses*, belonging to the same *Lycium* species, the presence of  $\alpha$ -tocopherol (3.9 mg/100 g), together with  $\gamma$ -tocopherol (0.46 mg/100 g),  $\delta$ -tocopherol (0.12 mg/100 g), and traces of  $\alpha$ - $\gamma$ - $\delta$  tocotrienol (< 0.1 mg/100 g). Table 5 also reports values of vitamin E activity provided by 100 g of product, expressed as Tocopherol Equivalent (TE) (mg/100 g product) (SHEPPARD *et al.*, 1993). Taking into account the Recommended Daily Allowance (RDA) for vitamin E, which is of 12 mg/day (Regulation EU 1169/2011), 100 g of fresh goji berries contribute approximately 16 % of the RDA, while 100 g of dried fruits contribute approximately 66 % of the RDA, so that to be declared in label as a source of vitamin E. A portion of dried goji berries (30 g) contributes approximately 20 % of the RDA. The concentration of vitamin C was about 40 mg/100 g in fresh fruits and 38 mg/100 g in dried fruits. DONNO *et al.* (2015) report an amount of about 42 mg/100 g in dried goji berries. Taking into account the Recommended Daily Allowance (RDA) for vitamin C of 80 mg/day (Regulation EU 1169/2011), 100 g of fresh or dried goji berries contribute approximately 50 % of the RDA, so that they can be declared on the label as a source of vitamin C. A portion of dried goji berries (30 g) contributes about 16 % of the RDA.

## 4. CONCLUSIONS

Goji berries cultivated in Italy were confirmed as an important source of healthy compounds, providing a significant contribution to the diet, in terms of some inorganic nutrients, and of dietary fibre, zeaxanthin, vitamins E and C. In particular, taking into account the Recommended Daily Allowance (RDA) for minerals and vitamins established by the Commission of the European Communities, dried goji berries can be declared as a source of K, P, Cu, Fe Mn, Zn. Moreover, both fresh and dried berries can be declared on the label as a potential source of vitamins E and C.

The presented results enhance the knowledge of the composition and the nutritional characteristics of fresh and dried goji berries cultivated in Italy and will help in verifying the information reported in the label.

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# OPTIMISATION OF ULTRASOUND AND MICROWAVE-ASSISTED EXTRACTION OF CAFFEYOYLQUINIC ACIDS AND CAFFEINE FROM COFFEE SILVERSKIN USING RESPONSE SURFACE METHODOLOGY

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## ABSTRACT

Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) of caffeoylquinic acids and caffeine from coffee silverskin (CS) at two particle size were investigated using response surface methodology and compared to a conventional solvent extraction (CSE). The impact of time and temperature on the extraction process was evaluated and extraction efficiency was optimised by measuring total phenolic content (TPC), radical scavenging capacity (RSC), caffeoylquinic acids (TotCQAs) and caffeine content. UAE allowed to obtain extracts with values of TPC, RSC and TotCQAs higher or similar to CSE; moreover UAE produced the highest caffeine content (14.24 g kg<sup>-1</sup> dw) with a significant reduction of extraction time.

*Keywords:* caffeine, coffee silverskin, microwave-assisted extraction (MAE), polyphenolic compounds; ultrasound-assisted extraction (UAE), response surface methodology (RSM)

## 1. INTRODUCTION

Coffee is the second largest traded commodity in the world after petroleum with a production of 8.8 million tons in 2015 (INTERNATIONAL COFFEE ORGANIZATION, 2015). Since approximately 90% of the coffee cherry is discarded during the conversion of the cherry into coffee brew (DEL CASTILLO *et al.*, 2016), million tons of by-products are generated. Coffee silverskin (CS) is a thin tegument of the outer layer of the two beans forming the green coffee seed obtained as a by-product of the roasting process. Since CS represents about 4.2 % (w/w) of coffee beans (DEL CASTILLO *et al.*, 2016), hundreds of thousands tons of waste are produced generating a disposal cost for industry. In the last years CS has attracted great attention as an abundant source of bioactive compounds such as caffeoylquinic acids and caffeine (BRESCIANI *et al.*, 2014). In particular caffeoylquinic acids are phenolic compounds that belong to the family of chlorogenic acids; different studies have evaluated antifungal, antibacterial, anti-inflammatory, antioxidant, anti-glycative, anti-carcinogenic and neuroprotective properties of these compounds (DEL CASTILLO *et al.*, 2016). Considering caffeine, a moderate amount of this alkaloid increases energy availability, cognitive performance and neuromuscular coordination (GLADE, 2010). In order to optimise the extraction of bioactive compounds from CS, in the last years several extraction methods with different yield, complexity and cost, such as Soxhlet extraction, solid-state fermentation (SSF), subcritical water and solid-liquid extraction were applied (MURTHY and NAIDU, 2010; MACHADO *et al.*, 2012; NARITA and INOUE, 2012; BALLESTEROS *et al.*, 2014; COSTA *et al.*, 2014).

Among the extraction techniques, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are considered efficient for extracting analytes reducing extraction time and energy consumption. Regarding UAE, the responsive of ultrasonic effect are the cavitation bubbles that grow during rarefaction phases and decrease in size during compression cycles; when the size of the bubbles reaches a critical point, they collapse during a compression cycle and release large amounts of energy that destroys the cell walls of the plant matrix producing the discharge of cell content into the medium (CHEMAT *et al.*, 2011). UAE applied on spent coffee ground (SCG), the solid waste obtained after coffee brewing and having similar composition to CS, has been reported to be an efficient method to improve the extraction of antioxidant compounds (SEVERINI *et al.*, 2016).

MAE is an efficient method that has garnered increasing interest in various fields mainly due to its particular heating mechanism and its moderate capital cost (CHAN *et al.*, 2011); microwaves can penetrate into certain materials and interact with the polar components of the matrix to generate heat. MAE has been found to be an optimal extraction technique to recover caffeine from a plant matrix such as tea (WANG *et al.*, 2011).

An important role in the extraction of caffeoylquinic acids and caffeine from a plant matrix is represented by the particle size of the sample (PINELO *et al.*, 2007; ASTILL *et al.*, 2001). In literature no studies are reported on the use of ultrasounds and microwaves for the extraction of bioactive compounds from CS and on the influence of CS particle size on the extraction process.

Response surface methodology (RSM) is a multivariate statistic technique used to optimise extraction processes of bioactive compounds from plant matrices; in particular different studies reported the application of RSM to optimise the extraction of caffeine and chlorogenic acid from a plant matrix (D'ARCHIVIO *et al.*, 2016; BAE *et al.*, 2015).

This study aimed to optimise UAE and MAE of the three most abundant caffeoylquinic acids and caffeine from CS using RSM; this statistical approach was used to evaluate the impact of time and temperature on total phenolic content, radical scavenging capacity, caffeoylquinic acids and caffeine content of the extracts. Coffee silverskin was used at 80-

and 250  $\mu\text{m}$  particle size in order to test a possible effect of the grain size on the extraction process. Finally extraction efficiency of UAE and MAE methods was compared to a conventional solvent extraction (CSE).

## 2. MATERIALS AND METHODS

### 2.1. Plant material and chemical reagents

A CS blend produced by roasting Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*) coffee beans was provided by Torrefazione della Piazza (Sant'Antonino di Susa, Turin, Italy).

Caffeine, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, ethanol, methanol, formic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, Folin-Ciocalteu reagent and sodium carbonate were purchased from Sigma-Aldrich (Milano, Italy). Ultrapure water was produced using a Milli-Q System (Millipore, Milan, Italy).

### 2.2. Sample preparation

CS was ground to obtain a powder with 80- and 250- $\mu\text{m}$  particle size using an ultracentrifugal mill Retsch ZM 200 (Retsch GmbH, Haan, Germany) and stored in vacuum bags at +4°C until use. The moisture content of CS ( $6.9 \pm 0.3\%$ ) was determined using an electronic moisture balance (Eurotherm, Gibertini Elettronica, Milan, Italy) with 5 g of sample in order to express the results on dry weight basis (dw).

### 2.3. Extraction procedure

According to the optimization study performed on CS by BALLESTEROS *et al.* (2014), 60% (v/v) ethanol and solvent/solid ratio of 35 mL g<sup>-1</sup> were used as fixed parameters for UAE, MAE and CSE. After the extractions, extracts were cooled in an ice bath to stop the extraction process and centrifuged at 16,800 g for 10 min; supernatants were then filtered (0.45  $\mu\text{m}$ ) and immediately analysed.

#### 2.3.1. Ultrasound-assisted extraction (UAE)

UAE was carried out using an ultrasonic bath (Sonica® 3300EP S3; Soltec, Milan, Italy) at a 40 kHz frequency with 300 W of power. The flasks containing 1 g of CS and 35 mL of 60% (v/v) ethanol were immersed into the ultrasonic bath, where the water level was fixed at 2 cm above the liquid surface in the flask. For each experimental condition of the Central Composite Design (CCD) reported in Table 1, water bath temperature was set and maintained constant ( $\pm 1$  °C) using an external chiller.

#### 2.3.2. Microwave-assisted extraction (MAE)

MAE was carried out using a Start D microwave digestion system (Milestone, Bergamo, Italy) with an SK Rotor equipped with digestion vessels composed of high-purity PTFE. CS (1 g) and 35 mL of 60% (v/v) ethanol were put into the digestion vessels and a power of 280 W was then applied. For each experimental condition of CCD (Table 1), temperature was measured using the internal temperature probe of the apparatus.

**Table 1.** CCD experimental matrix.

| Run  | Extraction time<br>(coded value) ( $x_1$ ) | Extraction temperature<br>(coded value) ( $x_2$ ) | Extraction time<br>(real value) ( $X_1$ , min) | Extraction temperature<br>(real value) ( $X_2$ , °C) |
|------|--------------------------------------------|---------------------------------------------------|------------------------------------------------|------------------------------------------------------|
| 1    | -1                                         | -1                                                | 19                                             | 37                                                   |
| 2    | 1                                          | -1                                                | 41                                             | 37                                                   |
| 3    | 1                                          | 1                                                 | 41                                             | 73                                                   |
| 4    | -1                                         | 1                                                 | 19                                             | 73                                                   |
| 5    | -1.4141                                    | 0                                                 | 15                                             | 55                                                   |
| 6    | 1.4141                                     | 0                                                 | 45                                             | 55                                                   |
| 7    | 0                                          | -1.4141                                           | 30                                             | 30                                                   |
| 8    | 0                                          | 1.4141                                            | 30                                             | 80                                                   |
| 9-13 | 0                                          | 0                                                 | 30                                             | 55                                                   |

### 2.3.3. Conventional solvent extraction (CSE)

CSE was carried out mixing 1 g of CS and 35 mL of 60% (v/v) ethanol into a 40 mL amber vial inserted into a Digital Pulse Mixer (Glas-Col, U.S.A.); stirring was carried out at 200 rpm, 70% duty cycle and 70 pulses per minute.

### 2.4. Total phenolic content (TPC)

Total phenolic content (TPC) was measured following Folin-Ciocalteu's method (SINGLETON and ROSSI, 1965) with modifications as described by ZHOU and YU (2006). Briefly, 0.05 mL of phenolic extract was mixed with 0.250 mL of Folin-Ciocalteu's reagent and 3 mL of ultrapure water. The mixture was incubated at room temperature for 3 minutes; then 0.75 mL of 20% (w/v) sodium carbonate was added and the obtained mixture was incubated in the dark at room temperature for 2 h. A mixture of the solvent and reagents was used as blank. The specific absorbance at 765 nm was measured using a UV-visible spectrophotometer (UV-1800 PharmaSpec, Shimadzu, Milan, Italy). Gallic acid was used to construct the calibration curve (linearity range 0-500 mg/L,  $R^2=0.998$ ). TPC was expressed as g gallic acid equivalents (GAE)  $\text{kg}^{-1}$  of CS on dry weight basis (dw).

### 2.5. Radical scavenging capacity (RSC)

Radical scavenging capacity (RSC) of the extracts was determined according to GADOW *et al.* (1997). Phenolic extract (75  $\mu\text{L}$ ) was mixed with 3 mL of  $6.1 \times 10^{-5}$  M DPPH $\cdot$  solution in methanol and incubated for 1 h at room temperature in the dark (SHARMA and BHAT, 2009). Discolouration of the purple DPPH $\cdot$  solution was measured at 515 nm. Methanol was used as a control, and a methanol solution of DPPH was used as a blank. The inhibition percentage (IP) of the DPPH $\cdot$  by the phenolic extract was calculated using the following equation:

### 2.6. HPLC-PDA analysis

The three caffeoylquinic acids considered (3-O-caffeoylquinic acid, 3-CQA; 4-O-caffeoylquinic acid, 4-CQA; 5-O-caffeoylquinic acid, 5-CQA) and caffeine were quantified using an HPLC-PDA Thermo-Finnigan Spectra System (Thermo-Finnigan, Waltham, USA) equipped with a Finnigan Surveyor PDA Plus detector. ChromQuest software (version

5.0) was used for instrument control and data processing. Compounds were separated using a Kinetex Phenyl-Hexyl C18 column 5  $\mu\text{m}$ , 150  $\times$  4.6 mm (Phenomenex, Castel Maggiore, Italy). Flow rate was set at 1.0 mL/min, temperature at 35°C and injection volume at 10  $\mu\text{L}$ . A gradient elution with 0.1% formic acid (A) and methanol (B) as the solvents was applied as follows: 0.0–3.0 min, 10–30% of B; 3.0–8.0 min, 30–35% of B; 8.0–11.0 min, 35–40% of B; 11.0–30.0 min, 40–80% of B; 30.0–32.0 min, 80–10% of B. PDA spectra were recorded using a full scan modality over the wavelength ( $\lambda$ ) range 200 to 400 nm, and data were quantified using the external standard method with six-point calibration curves. In particular, the caffeoylquinic acids were quantified at 325 nm while caffeine at 273 nm with the respective standards ( $R^2 = 0.9987$ , LOD = 0.20  $\mu\text{g/mL}$ , LOQ = 0.62  $\mu\text{g/mL}$  for 3-*O*-caffeoylquinic acid,  $R^2 = 0.9893$ , LOD = 0.25  $\mu\text{g/mL}$ , LOQ = 0.66  $\mu\text{g/mL}$  for 4-*O*-caffeoylquinic acid,  $R^2 = 0.9993$  for 5-*O*-caffeoylquinic acid, LOD = 0.16  $\mu\text{g/mL}$ , LOQ = 0.50  $\mu\text{g/mL}$  and  $R^2 = 0.9837$ , LOD = 0.014  $\mu\text{g/mL}$ , LOQ = 0.046  $\mu\text{g/mL}$  for caffeine). Data were expressed as g  $\text{kg}^{-1}$  of CS on dw.

## 2.7. Experimental design and statistical analysis

A two factorial  $2^2$  central composite design (CCD) was employed to evaluate the effects of time ( $X_1$ ) and temperature ( $X_2$ ) on TPC, RSC, caffeine and TotCQAs (as the sum of 3-CQA, 4-CQA and 5-CQA) values of the extracts obtained using UAE, MAE and CSE methods. Extraction experiments were performed in triplicate for each experimental condition of the Central Composite Design (Table 1).

As reported in Table 1, variables were codified such that their values ranged between  $\pm 1.414$  and the central point was repeated five times.

The values of the independent variables were coded using following equation:

$$x_i = (X_i - X_m) / \Delta X$$

where  $x_i$  is the coded value of an independent variable,  $X_i$  is the real value of an independent variable,  $X_m$  is the mean of the real values of an independent variable at the central point, and  $\Delta X$  is the step change value.

According to PAVLIĆ *et al.* (2016), response surface regressions were used to analyse TPC, RSC, TotCQAs and caffeine of obtained extracts and were fitted to the following second-order polynomial model:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

where  $Y$  are the predicted responses (TPC, RSC, TotCQAs and caffeine),  $X_1$  and  $X_2$  correspond to independent variables time and temperature,  $b_0$  is the constant coefficient,  $b_1$  and  $b_2$  represent the linear coefficients,  $b_{11}$  and  $b_{22}$  the quadratic effects and  $b_{12}$  the cross-product coefficient.

The 24 second-degree polynomial equations, describing the four predicted responses obtained for each of the extraction method applied on the two CS particle sizes, were calculated using STATISTICA software (version 7.0, StatSoft Inc., Tulsa, OK, USA). Also the respective surface plots were developed using STATISTICA software. The values of TPC, RSC, TotCQAs and caffeine registered in the 13 experiments of CCD and observed at optimal conditions were compared among the extraction methods by analysis of variance (ANOVA) in order to evaluate the extraction efficiency of UAE and MAE compared to CSE; the means of the triplicates were separated at a 95% confidence interval using Duncan's test.

### 3. RESULTS AND DISCUSSIONS

#### 3.1. Model fitting

Second order polynomial model is the empirical model most commonly used for optimization methodology. Least-squares regression analysis of variables was used to determine the corresponding coefficients within the quadratic models and their ability to predict the responses (Table 2).

The quality of the generated models was evaluated by analysis of variance (ANOVA) and R square of the models; as shown in Table 2, ANOVA results showed that all the models had very low p values ( $\leq 0.0001$ ). In addition, high R square values suggest that the proposed models were generally adequate to explain most of the variability (Table 2).

#### 3.2. Effects of the process variables on total phenolic content (TPC)

Experimental data of TPC obtained using UAE, CSE and MAE on CS at 80- and 250  $\mu\text{m}$  particle size (p.s.) are reported in Table 3.

TPC value ranged from 5.23 GAE  $\text{kg}^{-1}$  dw (MAE, 30 min, 80°C, 250  $\mu\text{m}$  p.s.) to 10.58 GAE  $\text{kg}^{-1}$  dw (CSE, 30 min, 80°C, 80  $\mu\text{m}$  p.s.). As shown in the ANOVA test reported in Table 2, TPC value was affected by the linear and quadratic terms of time for UAE applied on CS at 80- and 250  $\mu\text{m}$  and for MAE applied on CS at 80  $\mu\text{m}$ ; the linear and quadratic terms of temperature affected TPC value for all the extraction methods applied on CS at 80  $\mu\text{m}$  and for CSE and MAE applied on CS at 250  $\mu\text{m}$ . According to BALLESTEROS *et al.* (2014), 30 min may be considered an enough time to be used in the three extraction processes to obtain a high phenolic content. Temperature exerted a higher effect on TPC than extraction time, especially for CSE and MAE; an increase in temperature favors the extraction of phenolics by enhancing the diffusion coefficient of solvent, solubility of solutes, diffusion rate of analytes, and reducing solvent viscosity and surface tension (JU and HOWARD, 2003).

Response surface plots representing the effect of time and temperature on total phenolic content (TPC) of the coffee silverskin at 80  $\mu\text{m}$  particle size extracts obtained using conventional solvent extraction (CSE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have been reported in Figs. 1, 2 and 3 respectively. Since no significant differences were observed in the extraction efficiency using the two CS particle sizes, data related to 250  $\mu\text{m}$  particle size have been reported in the text without graphical representations.

Considering MAE, above 51.5°C temperature exerted a negative effect on TPC value (Fig. 3).

Other studies have shown that chlorogenic acids, phenolic compounds to whom the selected caffeoylquinic acids belong, decreased at temperatures higher than 50°C using MAE (UPADHYAY *et al.*, 2012). Phenolic compounds with several hydroxyl groups in their aromatic rings such as caffeoylquinic acids are unstable in a solvent and can easily degrade under microwave radiation combined with high temperatures (INCE *et al.*, 2014). Finally the increase of TPC value at high temperatures using CSE and UAE could confirm this hypothesis, since temperature without microwaves did not produce a degradation of the phenolic compounds (Figs. 1 and 2).

**Table 2.** Regression coefficients of the predicted quadratic polynomial models of TPC, RSC, TotCQAs and caffeine obtained using CSE, UAE, and MAE extraction methods on coffee silverskin at 80- and 250  $\mu\text{m}$  particle sizes.

| CSE <sup>a</sup>    | Coffee silverskin (80- $\mu\text{m}$ particle size) |                        |                         |                         | CSE <sup>a</sup>    | Coffee silverskin (250- $\mu\text{m}$ particle size) |                        |                         |                         |
|---------------------|-----------------------------------------------------|------------------------|-------------------------|-------------------------|---------------------|------------------------------------------------------|------------------------|-------------------------|-------------------------|
|                     | TPC                                                 | RSC                    | TotCQAs                 | Caffeine                |                     | TPC                                                  | RSC                    | TotCQAs                 | Caffeine                |
| $b_0$               | 7.9106 <sup>***</sup>                               | 33.9350 <sup>***</sup> | 180.0969 <sup>***</sup> | 35.97 <sup>ns</sup>     | $b_0$               | 5.6448 <sup>***</sup>                                | 22.0388 <sup>***</sup> | 156.2306 <sup>***</sup> | 192.9056 <sup>**</sup>  |
| $b_1$ , time        | 0.0239 <sup>ns</sup>                                | 0.0358 <sup>*</sup>    | 2.0294 <sup>***</sup>   | 28.13 <sup>***</sup>    | $b_1$ , time        | 0.0441 <sup>*</sup>                                  | 0.1896 <sup>***</sup>  | 3.3476 <sup>***</sup>   | 21.4610 <sup>***</sup>  |
| $b_2$ , temperature | -0.0064 <sup>ns</sup>                               | 0.1387 <sup>***</sup>  | 0.5358 <sup>ns</sup>    | 21.63 <sup>***</sup>    | $b_2$ , temperature | 0.0597 <sup>***</sup>                                | 0.4139 <sup>***</sup>  | 1.2105 <sup>***</sup>   | 19.2199 <sup>***</sup>  |
| $b_{11}$            | -0.0003 <sup>ns</sup>                               | -0.0001 <sup>ns</sup>  | -0.0039 <sup>ns</sup>   | -0.24 <sup>***</sup>    | $b_{11}$            | -0.0006 <sup>ns</sup>                                | -0.0015 <sup>**</sup>  | -0.0427 <sup>***</sup>  | -0.1459 <sup>***</sup>  |
| $b_{22}$            | 0.0003 <sup>***</sup>                               | -0.0009 <sup>**</sup>  | 0.0069 <sup>**</sup>    | -0.11 <sup>***</sup>    | $b_{22}$            | -0.0003 <sup>**</sup>                                | -0.0027 <sup>***</sup> | -0.0080 <sup>***</sup>  | -0.1030 <sup>***</sup>  |
| $b_{12}$            | 0.0002 <sup>ns</sup>                                | -0.0004 <sup>***</sup> | -0.0230 <sup>***</sup>  | -0.19 <sup>***</sup>    | $b_{12}$            | 0.0002 <sup>ns</sup>                                 | -0.0014 <sup>***</sup> | -0.0036 <sup>ns</sup>   | -0.1533 <sup>***</sup>  |
| $R^2$               | 0.96                                                | 0.96                   | 0.91                    | 0.92                    | $R^2$               | 0.92                                                 | 0.97                   | 0.90                    | 0.94                    |
| p value             | < 0.0001                                            | < 0.0001               | < 0.0001                | < 0.0001                | p value             | < 0.0001                                             | < 0.0001               | < 0.0001                | < 0.0001                |
| UAE <sup>b</sup>    | TPC                                                 | RSC                    | TotCQAs                 | Caffeine                | UAE <sup>b</sup>    | TPC                                                  | RSC                    | TotCQAs                 | Caffeine                |
| $b_0$               | 4.1174 <sup>***</sup>                               | -5.0522 <sup>*</sup>   | -30.0050 <sup>ns</sup>  | 573.9909 <sup>***</sup> | $b_0$               | 4.6483 <sup>**</sup>                                 | 16.8912 <sup>***</sup> | -62.2672 <sup>*</sup>   | 691.4779 <sup>***</sup> |
| $b_1$ , time        | 0.1676 <sup>***</sup>                               | 0.7384 <sup>***</sup>  | 4.9254 <sup>***</sup>   | -2.6802 <sup>ns</sup>   | $b_1$ , time        | 0.2595 <sup>***</sup>                                | 0.8034 <sup>***</sup>  | 7.8861 <sup>***</sup>   | 7.7372 <sup>**</sup>    |
| $b_2$ , temperature | 0.0714 <sup>***</sup>                               | 1.0476 <sup>***</sup>  | 6.0426 <sup>***</sup>   | 27.6387 <sup>***</sup>  | $b_2$ , temperature | -0.0321 <sup>ns</sup>                                | 0.2369 <sup>***</sup>  | 5.9860 <sup>***</sup>   | 16.4356 <sup>***</sup>  |
| $b_{11}$            | -0.0018 <sup>***</sup>                              | -0.0022 <sup>ns</sup>  | -0.0201 <sup>ns</sup>   | 0.0956 <sup>*</sup>     | $b_{11}$            | -0.0029 <sup>***</sup>                               | -0.0075 <sup>***</sup> | -0.0648 <sup>***</sup>  | -0.0045 <sup>ns</sup>   |
| $b_{22}$            | -0.0002 <sup>*</sup>                                | -0.0057 <sup>***</sup> | -0.0277 <sup>***</sup>  | -0.2021 <sup>***</sup>  | $b_{22}$            | 0.0009 <sup>**</sup>                                 | -0.0005 <sup>ns</sup>  | -0.0279 <sup>***</sup>  | -0.0866 <sup>***</sup>  |
| $b_{12}$            | -0.0011 <sup>***</sup>                              | -0.0098 <sup>***</sup> | -0.0703 <sup>***</sup>  | -0.0704 <sup>*</sup>    | $b_{12}$            | -0.0011 <sup>*</sup>                                 | -0.0042 <sup>***</sup> | -0.0679 <sup>***</sup>  | -0.1212 <sup>***</sup>  |
| $R^2$               | 0.92                                                | 0.94                   | 0.86                    | 0.94                    | $R^2$               | 0.74                                                 | 0.90                   | 0.86                    | 0.89                    |
| p value             | < 0.0001                                            | < 0.0001               | < 0.0001                | < 0.0001                | p value             | < 0.0001                                             | < 0.0001               | < 0.0001                | < 0.0001                |
| MAE <sup>c</sup>    | TPC                                                 | RSC                    | TotCQAs                 | Caffeine                | MAE <sup>c</sup>    | TPC                                                  | RSC                    | TotCQAs                 | Caffeine                |
| $b_0$               | 1.9917 <sup>***</sup>                               | 26.2296 <sup>***</sup> | 125.0379 <sup>***</sup> | 108.9807 <sup>ns</sup>  | $b_0$               | 3.6921 <sup>***</sup>                                | 25.2296 <sup>***</sup> | 110.1193 <sup>***</sup> | 119.3925 <sup>ns</sup>  |
| $b_1$ , time        | 0.1011 <sup>***</sup>                               | -0.0880 <sup>**</sup>  | -1.0855 <sup>ns</sup>   | 33.1830 <sup>***</sup>  | $b_1$ , time        | 0.0543 <sup>ns</sup>                                 | -0.0880 <sup>**</sup>  | -0.1901 <sup>ns</sup>   | 39.3521 <sup>***</sup>  |
| $b_2$ , temperature | 0.1680 <sup>***</sup>                               | 0.2242 <sup>***</sup>  | 4.5193 <sup>***</sup>   | 18.0169 <sup>***</sup>  | $b_2$ , temperature | 0.1100 <sup>***</sup>                                | 0.2242 <sup>***</sup>  | 4.6963 <sup>***</sup>   | 14.0377 <sup>***</sup>  |
| $b_{11}$            | -0.0015 <sup>***</sup>                              | 0.0011 <sup>**</sup>   | -0.0009 <sup>ns</sup>   | -0.3733 <sup>***</sup>  | $b_{11}$            | -0.0015 <sup>**</sup>                                | 0.0011 <sup>**</sup>   | -0.0136 <sup>ns</sup>   | -0.3500 <sup>***</sup>  |
| $b_{22}$            | -0.0016 <sup>***</sup>                              | -0.0024 <sup>***</sup> | -0.0627 <sup>***</sup>  | -0.0791 <sup>***</sup>  | $b_{22}$            | -0.0015 <sup>***</sup>                               | -0.0024 <sup>***</sup> | -0.0630 <sup>***</sup>  | -0.0254 <sup>ns</sup>   |
| $b_{12}$            | -0.0001 <sup>ns</sup>                               | 0.0005 <sup>ns</sup>   | 0.0281 <sup>*</sup>     | -0.1912 <sup>***</sup>  | $b_{12}$            | 0.0008 <sup>*</sup>                                  | 0.0005 <sup>ns</sup>   | 0.0241 <sup>*</sup>     | -0.2891 <sup>***</sup>  |
| $R^2$               | 0.92                                                | 0.94                   | 0.92                    | 0.84                    | $R^2$               | 0.86                                                 | 0.94                   | 0.93                    | 0.89                    |
| p value             | < 0.0001                                            | < 0.0001               | < 0.0001                | < 0.0001                | p value             | < 0.0001                                             | < 0.0001               | < 0.0001                | < 0.0001                |

<sup>a</sup>Conventional solvent extraction; <sup>b</sup>Ultrasound-assisted extraction; <sup>c</sup>Microwave-assisted extraction.

P value, probability of F value for the model.

\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, ns= Not significant.

**Table 3.** Central composite design with the observed TPC, RSC, TotCQAs and caffeine values of coffee silverskin (80- and 250  $\mu\text{m}$  particle sizes) extracts obtained using CSE, UAE, and MAE.

| Coffe silverskin at 80- $\mu\text{m}$ particle size  |                                 |                               |                              |          |                                     |                               |                               |          |
|------------------------------------------------------|---------------------------------|-------------------------------|------------------------------|----------|-------------------------------------|-------------------------------|-------------------------------|----------|
| Run                                                  | TPC (g GAE $\text{kg}^{-1}$ dw) |                               |                              |          | RSC ( $\mu\text{mol TE g}^{-1}$ dw) |                               |                               |          |
|                                                      | CSE <sup>a</sup>                | UAE <sup>b</sup>              | MAE <sup>c</sup>             | <i>p</i> | CSE <sup>a</sup>                    | UAE <sup>b</sup>              | MAE <sup>c</sup>              | <i>p</i> |
| 1                                                    | 8.73 $\pm$ 0.02 <sup>A</sup>    | 8.35 $\pm$ 0.10 <sup>B</sup>  | 7.51 $\pm$ 0.02 <sup>C</sup> | ***      | 38.28 $\pm$ 0.21 <sup>A</sup>       | 32.05 $\pm$ 0.72 <sup>B</sup> | 30.47 $\pm$ 0.02 <sup>C</sup> | ***      |
| 2                                                    | 8.94 $\pm$ 0.06 <sup>A</sup>    | 8.58 $\pm$ 0.01 <sup>B</sup>  | 7.55 $\pm$ 0.02 <sup>C</sup> | ***      | 38.63 $\pm$ 0.04 <sup>A</sup>       | 38.41 $\pm$ 0.12 <sup>B</sup> | 30.01 $\pm$ 0.04 <sup>C</sup> | ***      |
| 3                                                    | 10.15 $\pm$ 0.06 <sup>A</sup>   | 8.76 $\pm$ 0.02 <sup>B</sup>  | 7.14 $\pm$ 0.04 <sup>C</sup> | ***      | 39.40 $\pm$ 0.04 <sup>A</sup>       | 38.47 $\pm$ 0.16 <sup>B</sup> | 29.45 $\pm$ 0.04 <sup>C</sup> | ***      |
| 4                                                    | 9.77 $\pm$ 0.04 <sup>A</sup>    | 9.35 $\pm$ 0.05 <sup>B</sup>  | 7.17 $\pm$ 0.03 <sup>C</sup> | ***      | 39.38 $\pm$ 0.02 <sup>B</sup>       | 39.47 $\pm$ 0.06 <sup>A</sup> | 29.55 $\pm$ 0.04 <sup>C</sup> | ***      |
| 5                                                    | 8.98 $\pm$ 0.08 <sup>A</sup>    | 8.60 $\pm$ 0.02 <sup>B</sup>  | 7.55 $\pm$ 0.10 <sup>C</sup> | ***      | 39.06 $\pm$ 0.08 <sup>A</sup>       | 38.67 $\pm$ 0.27 <sup>B</sup> | 30.57 $\pm$ 0.09 <sup>C</sup> | ***      |
| 6                                                    | 9.57 $\pm$ 0.14 <sup>A</sup>    | 8.67 $\pm$ 0.02 <sup>B</sup>  | 7.89 $\pm$ 0.01 <sup>C</sup> | ***      | 39.16 $\pm$ 0.08 <sup>A</sup>       | 39.02 $\pm$ 0.33 <sup>A</sup> | 31.09 $\pm$ 0.10 <sup>B</sup> | ***      |
| 7                                                    | 8.53 $\pm$ 0.06 <sup>A</sup>    | 8.46 $\pm$ 0.04 <sup>A</sup>  | 7.15 $\pm$ 0.04 <sup>B</sup> | ***      | 37.80 $\pm$ 0.11 <sup>A</sup>       | 32.27 $\pm$ 0.28 <sup>B</sup> | 29.62 $\pm$ 0.06 <sup>C</sup> | ***      |
| 8                                                    | 10.58 $\pm$ 0.10 <sup>A</sup>   | 9.41 $\pm$ 0.06 <sup>B</sup>  | 7.01 $\pm$ 0.07 <sup>C</sup> | ***      | 39.37 $\pm$ 0.04 <sup>A</sup>       | 39.22 $\pm$ 0.38 <sup>A</sup> | 28.62 $\pm$ 0.08 <sup>B</sup> | ***      |
| 9                                                    | 9.31 $\pm$ 0.07 <sup>A</sup>    | 8.92 $\pm$ 0.05 <sup>B</sup>  | 7.90 $\pm$ 0.08 <sup>C</sup> | ***      | 39.30 $\pm$ 0.05 <sup>A</sup>       | 39.16 $\pm$ 0.22 <sup>A</sup> | 30.75 $\pm$ 0.03 <sup>B</sup> | ***      |
| 10                                                   | 9.33 $\pm$ 0.13 <sup>A</sup>    | 9.19 $\pm$ 0.08 <sup>A</sup>  | 8.01 $\pm$ 0.06 <sup>B</sup> | ***      | 39.10 $\pm$ 0.02 <sup>B</sup>       | 39.29 $\pm$ 0.12 <sup>A</sup> | 30.63 $\pm$ 0.07 <sup>C</sup> | ***      |
| 11                                                   | 9.43 $\pm$ 0.02 <sup>A</sup>    | 9.03 $\pm$ 0.11 <sup>B</sup>  | 7.90 $\pm$ 0.04 <sup>C</sup> | ***      | 39.17 $\pm$ 0.10 <sup>B</sup>       | 39.34 $\pm$ 0.05 <sup>A</sup> | 30.44 $\pm$ 0.06 <sup>C</sup> | ***      |
| 12                                                   | 9.26 $\pm$ 0.09 <sup>A</sup>    | 8.93 $\pm$ 0.03 <sup>B</sup>  | 8.16 $\pm$ 0.11 <sup>C</sup> | ***      | 39.16 $\pm$ 0.07 <sup>A</sup>       | 38.97 $\pm$ 0.35 <sup>A</sup> | 30.44 $\pm$ 0.02 <sup>B</sup> | ***      |
| 13                                                   | 9.38 $\pm$ 0.06 <sup>A</sup>    | 9.13 $\pm$ 0.03 <sup>B</sup>  | 8.17 $\pm$ 0.06 <sup>C</sup> | ***      | 39.12 $\pm$ 0.09 <sup>B</sup>       | 39.41 $\pm$ 0.10 <sup>A</sup> | 30.44 $\pm$ 0.16 <sup>C</sup> | ***      |
| Run                                                  | TotCQAs (g $\text{kg}^{-1}$ dw) |                               |                              |          | Caffeine (g $\text{kg}^{-1}$ dw)    |                               |                               |          |
|                                                      | CSE <sup>a</sup>                | UAE <sup>b</sup>              | MAE <sup>c</sup>             | <i>p</i> | CSE <sup>a</sup>                    | UAE <sup>b</sup>              | MAE <sup>c</sup>              | <i>p</i> |
| 1                                                    | 2.34 $\pm$ 0.00 <sup>A</sup>    | 2.04 $\pm$ 0.01 <sup>B</sup>  | 2.00 $\pm$ 0.07 <sup>B</sup> | ***      | 9.81.00 $\pm$ 0.03 <sup>C</sup>     | 12.86 $\pm$ 0.05 <sup>A</sup> | 9.93 $\pm$ 0.04 <sup>B</sup>  | ***      |
| 2                                                    | 2.51 $\pm$ 0.01 <sup>A</sup>    | 2.20 $\pm$ 0.09 <sup>B</sup>  | 2.00 $\pm$ 0.03 <sup>C</sup> | ***      | 11.31 $\pm$ 0.18 <sup>B</sup>       | 12.89 $\pm$ 0.12 <sup>A</sup> | 11.01 $\pm$ 0.10 <sup>C</sup> | ***      |
| 3                                                    | 2.63 $\pm$ 0.03 <sup>A</sup>    | 2.18 $\pm$ 0.06 <sup>B</sup>  | 1.71 $\pm$ 0.01 <sup>C</sup> | ***      | 12.03 $\pm$ 0.02 <sup>B</sup>       | 13.49 $\pm$ 0.06 <sup>A</sup> | 11.93 $\pm$ 0.06 <sup>B</sup> | ***      |
| 4                                                    | 2.63 $\pm$ 0.02 <sup>A</sup>    | 2.54 $\pm$ 0.02 <sup>B</sup>  | 1.50 $\pm$ 0.03 <sup>C</sup> | ***      | 11.91 $\pm$ 0.04 <sup>C</sup>       | 13.98 $\pm$ 0.03 <sup>A</sup> | 12.29 $\pm$ 0.07 <sup>B</sup> | ***      |
| 5                                                    | 2.38 $\pm$ 0.01 <sup>A</sup>    | 2.21 $\pm$ 0.02 <sup>B</sup>  | 1.89 $\pm$ 0.06 <sup>C</sup> | ***      | 11.10 $\pm$ 0.09 <sup>C</sup>       | 13.91 $\pm$ 0.17 <sup>A</sup> | 11.47 $\pm$ 0.06 <sup>B</sup> | ***      |
| 6                                                    | 2.58 $\pm$ 0.03 <sup>A</sup>    | 2.26 $\pm$ 0.01 <sup>B</sup>  | 1.99 $\pm$ 0.01 <sup>C</sup> | ***      | 11.99 $\pm$ 0.09 <sup>B</sup>       | 13.75 $\pm$ 0.15 <sup>A</sup> | 11.12 $\pm$ 0.08 <sup>C</sup> | ***      |
| 7                                                    | 2.37 $\pm$ 0.02 <sup>A</sup>    | 1.84 $\pm$ 0.01 <sup>C</sup>  | 2.04 $\pm$ 0.02 <sup>B</sup> | ***      | 10.63 $\pm$ 0.07 <sup>C</sup>       | 11.31 $\pm$ 0.05 <sup>A</sup> | 11.01 $\pm$ 0.04 <sup>B</sup> | ***      |
| 8                                                    | 2.68 $\pm$ 0.02 <sup>A</sup>    | 2.38 $\pm$ 0.07 <sup>B</sup>  | 1.06 $\pm$ 0.03 <sup>C</sup> | ***      | 12.12 $\pm$ 0.15 <sup>B</sup>       | 13.39 $\pm$ 0.13 <sup>A</sup> | 12.27 $\pm$ 0.08 <sup>B</sup> | ***      |
| 9                                                    | 2.53 $\pm$ 0.01 <sup>A</sup>    | 2.31 $\pm$ 0.02 <sup>B</sup>  | 1.98 $\pm$ 0.07 <sup>C</sup> | ***      | 11.81 $\pm$ 0.17 <sup>B</sup>       | 13.70 $\pm$ 0.10 <sup>A</sup> | 11.70 $\pm$ 0.35 <sup>B</sup> | ***      |
| 10                                                   | 2.49 $\pm$ 0.01 <sup>A</sup>    | 2.31 $\pm$ 0.04 <sup>B</sup>  | 1.96 $\pm$ 0.02 <sup>C</sup> | ***      | 11.86 $\pm$ 0.21 <sup>B</sup>       | 13.74 $\pm$ 0.05 <sup>A</sup> | 11.94 $\pm$ 0.33 <sup>B</sup> | ***      |
| 11                                                   | 2.53 $\pm$ 0.04 <sup>A</sup>    | 2.37 $\pm$ 0.02 <sup>B</sup>  | 1.87 $\pm$ 0.03 <sup>C</sup> | ***      | 12.05 $\pm$ 0.19 <sup>B</sup>       | 13.75 $\pm$ 0.09 <sup>A</sup> | 12.08 $\pm$ 0.08 <sup>B</sup> | ***      |
| 12                                                   | 2.49 $\pm$ 0.05 <sup>A</sup>    | 2.32 $\pm$ 0.06 <sup>B</sup>  | 2.02 $\pm$ 0.07 <sup>C</sup> | ***      | 12.08 $\pm$ 0.38 <sup>B</sup>       | 13.73 $\pm$ 0.13 <sup>A</sup> | 12.28 $\pm$ 0.11 <sup>B</sup> | ***      |
| 13                                                   | 2.46 $\pm$ 0.03 <sup>A</sup>    | 2.28 $\pm$ 0.07 <sup>B</sup>  | 2.03 $\pm$ 0.04 <sup>C</sup> | ***      | 12.17 $\pm$ 0.28 <sup>B</sup>       | 13.69 $\pm$ 0.07 <sup>A</sup> | 12.23 $\pm$ 0.22 <sup>B</sup> | ***      |
| Coffe silverskin at 250- $\mu\text{m}$ particle size |                                 |                               |                              |          |                                     |                               |                               |          |
| Run                                                  | TPC (g GAE $\text{kg}^{-1}$ dw) |                               |                              |          | RSC ( $\mu\text{mol TE g}^{-1}$ dw) |                               |                               |          |
|                                                      | CSE <sup>a</sup>                | UAE <sup>b</sup>              | MAE <sup>c</sup>             | <i>p</i> | CSE <sup>a</sup>                    | UAE <sup>b</sup>              | MAE <sup>c</sup>              | <i>p</i> |
| 1                                                    | 8.31 $\pm$ 0.05 <sup>A</sup>    | 8.14 $\pm$ 0.02 <sup>A</sup>  | 6.67 $\pm$ 0.25 <sup>B</sup> | ***      | 36.13 $\pm$ 0.09 <sup>A</sup>       | 34.42 $\pm$ 0.31 <sup>B</sup> | 29.47 $\pm$ 0.02 <sup>C</sup> | ***      |
| 2                                                    | 8.75 $\pm$ 0.02 <sup>A</sup>    | 8.59 $\pm$ 0.09 <sup>B</sup>  | 6.61 $\pm$ 0.05 <sup>C</sup> | ***      | 37.13 $\pm$ 0.17 <sup>B</sup>       | 38.41 $\pm$ 0.25 <sup>A</sup> | 29.01 $\pm$ 0.04 <sup>C</sup> | ***      |
| 3                                                    | 9.87 $\pm$ 0.04 <sup>A</sup>    | 8.87 $\pm$ 0.01 <sup>B</sup>  | 5.88 $\pm$ 0.29 <sup>C</sup> | ***      | 39.00 $\pm$ 0.09 <sup>B</sup>       | 39.38 $\pm$ 0.07 <sup>A</sup> | 28.45 $\pm$ 0.04 <sup>C</sup> | ***      |
| 4                                                    | 9.25 $\pm$ 0.18 <sup>A</sup>    | 9.28 $\pm$ 0.01 <sup>A</sup>  | 5.35 $\pm$ 0.16 <sup>B</sup> | ***      | 39.05 $\pm$ 0.14 <sup>A</sup>       | 38.56 $\pm$ 0.67 <sup>A</sup> | 28.55 $\pm$ 0.04 <sup>B</sup> | ***      |
| 5                                                    | 8.59 $\pm$ 0.02 <sup>A</sup>    | 7.56 $\pm$ 0.13 <sup>B</sup>  | 6.52 $\pm$ 0.35 <sup>C</sup> | ***      | 38.07 $\pm$ 0.31 <sup>A</sup>       | 35.18 $\pm$ 0.85 <sup>B</sup> | 29.57 $\pm$ 0.09 <sup>C</sup> | ***      |
| 6                                                    | 9.24 $\pm$ 0.12 <sup>A</sup>    | 9.00 $\pm$ 0.08 <sup>A</sup>  | 6.63 $\pm$ 0.18 <sup>B</sup> | ***      | 38.62 $\pm$ 0.07 <sup>B</sup>       | 39.20 $\pm$ 0.07 <sup>A</sup> | 30.09 $\pm$ 0.10 <sup>C</sup> | ***      |
| 7                                                    | 8.05 $\pm$ 0.21 <sup>A</sup>    | 8.49 $\pm$ 0.34 <sup>A</sup>  | 6.73 $\pm$ 0.14 <sup>B</sup> | ***      | 34.74 $\pm$ 0.19 <sup>B</sup>       | 37.68 $\pm$ 0.39 <sup>A</sup> | 28.62 $\pm$ 0.06 <sup>C</sup> | ***      |
| 8                                                    | 9.63 $\pm$ 0.04 <sup>B</sup>    | 10.46 $\pm$ 0.07 <sup>A</sup> | 5.23 $\pm$ 0.13 <sup>C</sup> | ***      | 39.31 $\pm$ 0.06 <sup>A</sup>       | 39.40 $\pm$ 0.50 <sup>A</sup> | 27.62 $\pm$ 0.08 <sup>B</sup> | ***      |
| 9                                                    | 9.29 $\pm$ 0.05 <sup>A</sup>    | 8.88 $\pm$ 0.51 <sup>A</sup>  | 6.95 $\pm$ 0.16 <sup>B</sup> | ***      | 38.84 $\pm$ 0.13 <sup>A</sup>       | 38.53 $\pm$ 0.19 <sup>B</sup> | 29.75 $\pm$ 0.03 <sup>C</sup> | ***      |
| 10                                                   | 9.25 $\pm$ 0.09 <sup>A</sup>    | 9.35 $\pm$ 0.05 <sup>A</sup>  | 6.92 $\pm$ 0.21 <sup>B</sup> | ***      | 38.83 $\pm$ 0.19 <sup>B</sup>       | 39.13 $\pm$ 0.04 <sup>A</sup> | 29.63 $\pm$ 0.07 <sup>C</sup> | ***      |
| 11                                                   | 8.98 $\pm$ 0.16 <sup>A</sup>    | 8.53 $\pm$ 0.08 <sup>A</sup>  | 6.80 $\pm$ 0.37 <sup>B</sup> | ***      | 38.65 $\pm$ 0.21 <sup>A</sup>       | 38.81 $\pm$ 0.31 <sup>A</sup> | 29.44 $\pm$ 0.06 <sup>B</sup> | ***      |
| 12                                                   | 9.06 $\pm$ 0.20 <sup>A</sup>    | 8.73 $\pm$ 0.30 <sup>A</sup>  | 6.53 $\pm$ 0.25 <sup>B</sup> | ***      | 38.66 $\pm$ 0.20 <sup>A</sup>       | 38.61 $\pm$ 0.52 <sup>A</sup> | 29.44 $\pm$ 0.02 <sup>B</sup> | ***      |
| 13                                                   | 9.08 $\pm$ 0.09 <sup>A</sup>    | 8.74 $\pm$ 0.27 <sup>A</sup>  | 6.97 $\pm$ 0.10 <sup>B</sup> | ***      | 38.81 $\pm$ 0.19 <sup>A</sup>       | 38.80 $\pm$ 0.83 <sup>A</sup> | 29.44 $\pm$ 0.16 <sup>B</sup> | ***      |
| Run                                                  | TotCQAs (g $\text{kg}^{-1}$ dw) |                               |                              |          | Caffeine (g $\text{kg}^{-1}$ dw)    |                               |                               |          |
|                                                      | CSE <sup>a</sup>                | UAE <sup>b</sup>              | MAE <sup>c</sup>             | <i>p</i> | CSE <sup>a</sup>                    | UAE <sup>b</sup>              | MAE <sup>c</sup>              | <i>p</i> |
| 1                                                    | 2.38 $\pm$ 0.01 <sup>A</sup>    | 2.09 $\pm$ 0.01 <sup>A</sup>  | 2.03 $\pm$ 0.05 <sup>B</sup> | ***      | 9.88 $\pm$ 0.07 <sup>B</sup>        | 12.64 $\pm$ 0.04 <sup>A</sup> | 9.93 $\pm$ 0.03 <sup>B</sup>  | ***      |
| 2                                                    | 2.52 $\pm$ 0.02 <sup>A</sup>    | 2.38 $\pm$ 0.02 <sup>B</sup>  | 1.99 $\pm$ 0.05 <sup>C</sup> | ***      | 11.69 $\pm$ 0.09 <sup>C</sup>       | 13.41 $\pm$ 0.07 <sup>A</sup> | 11.85 $\pm$ 0.05 <sup>B</sup> | ***      |
| 3                                                    | 2.58 $\pm$ 0.02 <sup>A</sup>    | 2.43 $\pm$ 0.01 <sup>A</sup>  | 1.60 $\pm$ 0.15 <sup>B</sup> | ***      | 12.40 $\pm$ 0.02 <sup>B</sup>       | 13.80 $\pm$ 0.08 <sup>A</sup> | 12.02 $\pm$ 0.02 <sup>C</sup> | ***      |
| 4                                                    | 2.47 $\pm$ 0.03 <sup>B</sup>    | 2.64 $\pm$ 0.02 <sup>A</sup>  | 1.47 $\pm$ 0.08 <sup>C</sup> | ***      | 11.73 $\pm$ 0.05 <sup>C</sup>       | 13.93 $\pm$ 0.03 <sup>A</sup> | 12.27 $\pm$ 0.08 <sup>B</sup> | ***      |
| 5                                                    | 2.35 $\pm$ 0.01 <sup>A</sup>    | 2.23 $\pm$ 0.03 <sup>B</sup>  | 1.91 $\pm$ 0.07 <sup>C</sup> | ***      | 11.31 $\pm$ 0.18 <sup>B</sup>       | 13.48 $\pm$ 0.10 <sup>A</sup> | 11.14 $\pm$ 0.03 <sup>B</sup> | ***      |
| 6                                                    | 2.52 $\pm$ 0.004 <sup>A</sup>   | 2.34 $\pm$ 0.03 <sup>B</sup>  | 2.04 $\pm$ 0.01 <sup>C</sup> | ***      | 12.12 $\pm$ 0.04 <sup>B</sup>       | 13.51 $\pm$ 0.02 <sup>A</sup> | 11.43 $\pm$ 0.13 <sup>C</sup> | ***      |
| 7                                                    | 2.42 $\pm$ 0.02 <sup>A</sup>    | 2.03 $\pm$ 0.03 <sup>B</sup>  | 2.03 $\pm$ 0.03 <sup>B</sup> | ***      | 10.66 $\pm$ 0.06 <sup>C</sup>       | 11.92 $\pm$ 0.06 <sup>A</sup> | 11.52 $\pm$ 0.03 <sup>B</sup> | ***      |

|    |                        |                        |                        |     |                         |                         |                         |     |
|----|------------------------|------------------------|------------------------|-----|-------------------------|-------------------------|-------------------------|-----|
| 8  | 2.54±0.02 <sup>A</sup> | 2.48±0.04 <sup>A</sup> | 1.19±0.05 <sup>B</sup> | *** | 12.14±0.07 <sup>C</sup> | 14.01±0.03 <sup>A</sup> | 12.31±0.08 <sup>B</sup> | *** |
| 9  | 2.57±0.02 <sup>A</sup> | 2.55±0.03 <sup>A</sup> | 2.02±0.04 <sup>B</sup> | *** | 11.97±0.09 <sup>B</sup> | 13.67±0.15 <sup>A</sup> | 11.99±0.07 <sup>B</sup> | *** |
| 10 | 2.55±0.03 <sup>A</sup> | 2.53±0.03 <sup>A</sup> | 2.02±0.01 <sup>B</sup> | *** | 11.94±0.03 <sup>C</sup> | 13.54±0.12 <sup>A</sup> | 12.10±0.05 <sup>B</sup> | *** |
| 11 | 2.54±0.04 <sup>A</sup> | 2.53±0.05 <sup>A</sup> | 1.94±0.07 <sup>B</sup> | *** | 11.99±0.03 <sup>B</sup> | 13.54±0.15 <sup>A</sup> | 11.94±0.17 <sup>B</sup> | *** |
| 12 | 2.55±0.02 <sup>A</sup> | 2.43±0.06 <sup>A</sup> | 1.93±0.09 <sup>B</sup> | *** | 12.06±0.13 <sup>B</sup> | 13.61±0.09 <sup>A</sup> | 12.02±0.24 <sup>B</sup> | *** |
| 13 | 2.52±0.02 <sup>A</sup> | 2.39±0.05 <sup>B</sup> | 2.06±0.04 <sup>C</sup> | *** | 11.93±0.12 <sup>B</sup> | 13.70±0.06 <sup>A</sup> | 12.11±0.09 <sup>B</sup> | *** |

Data are expressed as the means±standard deviation (n = 3).

Values in each row having different capitals letters indicate significant differences at  $P < 0.05$  (Duncan's test).

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , ns= Not significant

dw: dry weight; TotCQAs: total caffeoylquinic acids.

<sup>a</sup>Conventional solvent extraction; <sup>b</sup>Ultrasound-assisted extraction, <sup>c</sup>Microwave-assisted extraction.

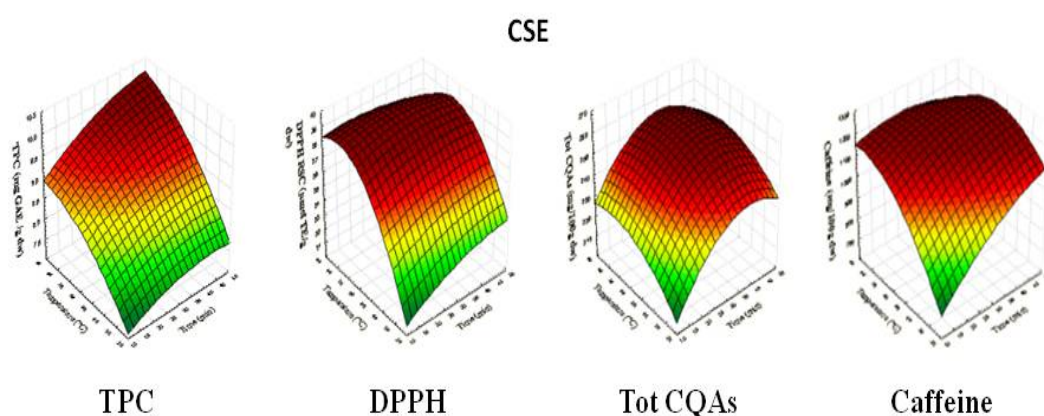


**Figure 1.** Response surface plots representing the effect of time and temperature on total phenolic content (TPC), radical scavenging capacity (RSC), total caffeoylquinic acids (TotCQAs) and caffeine content of the coffee silverskin (80  $\mu\text{m}$  particle size) extracts obtained using conventional solvent extraction (CSE).

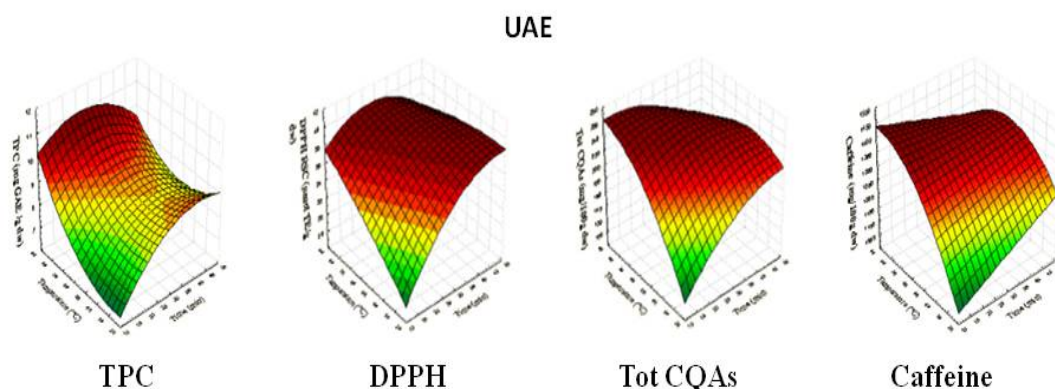


**Figure 2.** Response surface plots representing the effect of time and temperature on total phenolic content (TPC), radical scavenging capacity (RSC), total caffeoylquinic acids (TotCQAs) and caffeine content of the coffee silverskin (80  $\mu\text{m}$  particle size) extracts obtained using ultrasound-assisted extraction (UAE).

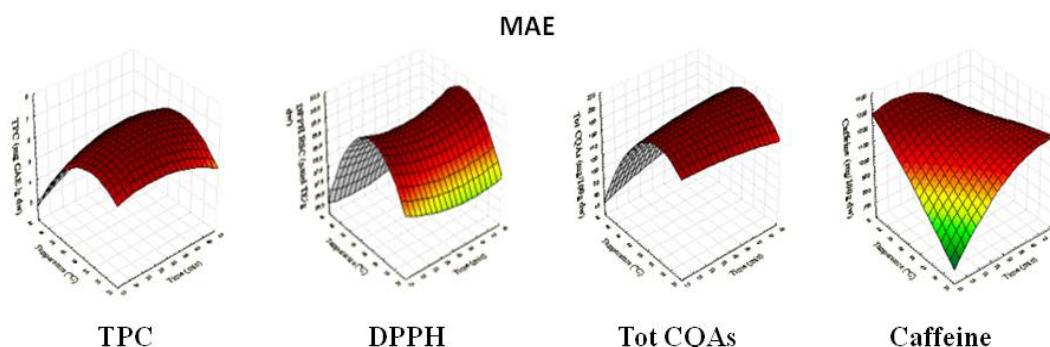


**Figure 3.** Response surface plots representing the effect of time and temperature on total phenolic content (TPC), radical scavenging capacity (RSC), total caffeoylquinic acids (TotCQAs) and caffeine content of the coffee silverskin (80  $\mu\text{m}$  particle size) extracts obtained using microwave-assisted extraction (MAE).

### 3.3. Effects of the process variables on the DPPH radical scavenging capacity (RSC)

As shown in Table 3, RSC values ranged from 27.62  $\mu\text{mol TE/g dw}$  (MAE, 30 min, 80°C, 250  $\mu\text{m p.s.}$ ) to 39.47  $\mu\text{mol TE/g dw}$  (UAE, 19 min, 73°C, 80  $\mu\text{m p.s.}$ ). Table 2 shows how RSC value was affected by linear and quadratic terms of temperature for all the extraction methods applied on CS at 80  $\mu\text{m}$  and for CSE and MAE applied on CS at 250  $\mu\text{m}$  particle size. Moreover RSC value was affected by both linear and quadratic terms of extraction time for MAE applied on CS at 80  $\mu\text{m}$ . As seen for TPC, also RSC was generally more affected by temperature than time, especially for CSE and MAE methods.

Considering the surface plots reported in Figs. 1, 2 and 3, a positive effect of extraction time was observed for UAE while a temperature increase caused a rapid increase of RSC value for all the extraction methods, except MAE applied at temperatures higher than 50 °C, as seen for TPC.

According to NARITA and INOUE (2012), a correlation between TPC and RSC trends has been observed meaning the high contribute of phenolic compounds in antioxidant activity of CS extracts.

### 3.4. Effects of the process variables on total caffeoylquinic acids (TotCQAs)

Table 3 shows how TotCQAs ranged from 1.06  $\text{g kg}^{-1} \text{ dw}$  (MAE, 30 min, 80°C, 80  $\mu\text{m p.s.}$ ) to 2.68  $\text{g kg}^{-1}$  (CSE, 30 min, 80°C, 80  $\mu\text{m p.s.}$ ). Considering the ANOVA test on CS extracts at 80- and 250  $\mu\text{m}$  particle sizes (Table 2), TotCQAs value was affected by linear terms of extraction time and temperature for UAE and by linear and quadratic terms of temperature for MAE. As seen for TPC and RSC, TotCQAs was more affected by temperature than time for all the extraction methods. Also surface plots of TotCQAs were generally comparable, for each extraction method, to that observed for TPC and RSC (Figs. 1, 2 and 3); these similar trends could confirm that 3-CQA, 4-CQA and 5-CQA are the main compounds in phenolic content of CS and the main responsible of CS antioxidant activity, as reported by BRESCIANI *et al.* (2014).

According to UPADHYAY *et al.* (2012), caffeoylquinic acids content decreased at temperatures above 50°C using MAE, confirming a possible degradation of the selected compounds caused by the combination of microwaves and high temperatures (Fig. 3).

### 3.5. Effects of the process variables on caffeine content

As reported in Table 3, caffeine content ranged from 9.81 g kg<sup>-1</sup> dw (CSE, 19 min, 37°C, 80 µm p.s.) to 14.01 g kg<sup>-1</sup> dw (UAE, 30 min, 80°C, 250 µm p.s.).

Table 2 shows how the caffeine content was affected by linear and quadratic terms of time and temperature for CSE and MAE applied on CS at the two particle sizes while UAE was especially affected by extraction temperature. As seen for TPC, RSC and TotCQAs, also in this case temperature was the variable that produced the most relevant effects on the extraction processes.

In contrast with TotCQAs values, caffeine content obtained by MAE was rapidly increased also at high temperatures (Fig. 3); caffeine stability in these conditions has been reported by LIU *et al.* (2012) that optimised microwave-assisted extraction of caffeine from coffee at 120°C.

### 3.6. Optimization and verification of the models

Optimization of CSE, UAE and MAE methods was carried out to obtain extracts with the highest predicted TPC, RSC, TotCQAs and caffeine values (Table 4).

Following the model proposed by ZIELINSKI *et al.* (2015), the errors in relation to the predicted models of the optimization variables were verified. An external validation was performed using the optimal conditions of time and temperature obtained by the predict models; all the observed values were within the predicted interval to a level of 95 % (Table 4), meaning that experimental data matched well the predicted values and interval ranges generated by RSM. Comparing the observed values at optimal conditions with the ones reported in literature, TPC values of 10.01 g GAE kg<sup>-1</sup> dw (CSE, 45 min, 80°C, 80 µm p.s.) and 9.91 g GAE kg<sup>-1</sup> dw (UAE, 29.5 min, 80°C, 250 µm p.s.) were higher than the one obtained during CS extraction with water at 80°C during 60 min (7.00 g GAE kg<sup>-1</sup> dw) performed by NARITA and INOUE (2012); moreover UAE allowed to split in half the extraction time. Observed TotCQAs values of 2.61 g kg<sup>-1</sup> dw (CSE, 24.5 min, 80°C, 80 µm p.s.) and 2.57 g kg<sup>-1</sup> dw (UAE, 15 min, 80°C, 80 µm p.s.) can be compared with the sum of 3-CQA, 4-CQA and 5-CQA (4.31 g kg<sup>-1</sup> dw) obtained by BRESCIANI *et al.* (2014) using two times a sonic bath for 30 min and then a Dubnoff bath for 1 h at 70°C; CSE and UAE optimized in our study allowed to obtain a slightly lower TotCQAs value balanced by a significant time reduction (86.4 % for CSE and 91.7 % for UAE). Finally the highest caffeine content of 14.24 g kg<sup>-1</sup> dw (UAE, 15 min, 66°C, 80 µm p.s.) obtained in our study was higher than the one reported by NARITA and INOUE (2012) using subcritical water at 210°C (4.1 g kg<sup>-1</sup> dw). Considering UAE, highest predicted and observed values were generally obtained at high temperatures according to other studies that reported a maximum UAE extraction of several bioactive compounds at 80°C (TOMŠIK *et al.*, 2016; ZHANG *et al.*, 2011; ZHU *et al.*, 2017).

### 3.7. Comparison among the combinations of extraction method and CS particle size

The observed values of TPC, RSC, TotCQAs and caffeine obtained at optimal time and temperature conditions by each combination of extraction method and CS particle size were compared in order to evaluate the most efficient combination for each response (Fig. 4).

**Table 4.** Predicted and observed values of TPC, RSC, TotCQAs and caffeine at optimal time and temperature conditions obtained using CSE, UAE, and MAE extraction methods on CS at 80- and 250- $\mu$ m particle sizes.

| <b>CS 80 <math>\mu</math>m</b>         |                           |                                   | <b>CSE</b>            |       |      |                        |                   |                   |
|----------------------------------------|---------------------------|-----------------------------------|-----------------------|-------|------|------------------------|-------------------|-------------------|
| <b>Response variables</b>              | <b>Optimal conditions</b> |                                   | <b>Observed value</b> |       |      | <b>Predicted value</b> | <b>-95 % Pred</b> | <b>+95 % Pred</b> |
|                                        | <b>T (min)</b>            | <b>T (<math>^{\circ}</math>C)</b> |                       |       |      |                        |                   |                   |
| TPC (g GAE kg <sup>-1</sup> dw)        | 45                        | 80                                | 10.01                 | $\pm$ | 0.21 | 10.51                  | 9.97              | 10.75             |
| RSC ( $\mu$ mol TE g <sup>-1</sup> dw) | 45                        | 67                                | 39.01                 | $\pm$ | 0.46 | 39.41                  | 38.96             | 39.86             |
| TotCQAs (g kg <sup>-1</sup> dw)        | 24.5                      | 80                                | 2.61                  | $\pm$ | 0.05 | 2.69                   | 2.61              | 2.78              |
| Caffeine (g kg <sup>-1</sup> dw)       | 31.5                      | 69                                | 12.11                 | $\pm$ | 0.12 | 12.26                  | 11.80             | 12.72             |
| <b>CS 250 <math>\mu</math>m</b>        |                           |                                   | <b>CSE</b>            |       |      |                        |                   |                   |
| <b>Response variables</b>              | <b>Optimal conditions</b> |                                   | <b>Observed value</b> |       |      | <b>Predicted value</b> | <b>-95 % Pred</b> | <b>+95 % Pred</b> |
|                                        | <b>T (min)</b>            | <b>T (<math>^{\circ}</math>C)</b> |                       |       |      |                        |                   |                   |
| TPC (g GAE kg <sup>-1</sup> dw)        | 45                        | 80                                | 9.83                  | $\pm$ | 0.15 | 9.99                   | 9.59              | 10.39             |
| RSC ( $\mu$ mol TE g <sup>-1</sup> dw) | 31                        | 68.5                              | 39.01                 | $\pm$ | 0.50 | 39.19                  | 38.66             | 39.72             |
| TotCQAs (g kg <sup>-1</sup> dw)        | 36.5                      | 67.5                              | 2.54                  | $\pm$ | 0.05 | 2.58                   | 2.53              | 2.63              |
| Caffeine (g kg <sup>-1</sup> dw)       | 40                        | 63.5                              | 12.13                 | $\pm$ | 0.11 | 12.34                  | 11.94             | 12.73             |
| <b>CS 80 <math>\mu</math>m</b>         |                           |                                   | <b>UAE</b>            |       |      |                        |                   |                   |
| <b>Response variables</b>              | <b>Optimal conditions</b> |                                   | <b>Observed value</b> |       |      | <b>Predicted value</b> | <b>-95 % Pred</b> | <b>+95 % Pred</b> |
|                                        | <b>T (min)</b>            | <b>T (<math>^{\circ}</math>C)</b> |                       |       |      |                        |                   |                   |
| TPC (g GAE kg <sup>-1</sup> dw)        | 22                        | 80                                | 9.25                  | $\pm$ | 0.16 | 9.43                   | 9.25              | 9.61              |
| RSC ( $\mu$ mol TE g <sup>-1</sup> dw) | 15                        | 79                                | 40.81                 | $\pm$ | 0.76 | 41.1                   | 39.39             | 42.81             |
| TotCQAs (g kg <sup>-1</sup> dw)        | 15                        | 80                                | 2.57                  | $\pm$ | 0.05 | 2.61                   | 2.42              | 2.80              |
| Caffeine (g kg <sup>-1</sup> dw)       | 15                        | 66                                | 14.24                 | $\pm$ | 0.15 | 14.29                  | 13.86             | 14.72             |
| <b>CS 250 <math>\mu</math>m</b>        |                           |                                   | <b>UAE</b>            |       |      |                        |                   |                   |
| <b>Response variables</b>              | <b>Optimal conditions</b> |                                   | <b>Observed value</b> |       |      | <b>Predicted value</b> | <b>-95 % Pred</b> | <b>+95 % Pred</b> |
|                                        | <b>T (min)</b>            | <b>T (<math>^{\circ}</math>C)</b> |                       |       |      |                        |                   |                   |
| TPC (g GAE kg <sup>-1</sup> dw)        | 29.5                      | 80                                | 9.91                  | $\pm$ | 0.24 | 10.38                  | 9.54              | 11.22             |
| RSC ( $\mu$ mol TE g <sup>-1</sup> dw) | 31                        | 80                                | 39.21                 | $\pm$ | 1.80 | 39.93                  | 38.73             | 41.13             |
| TotCQAs (g kg <sup>-1</sup> dw)        | 19                        | 80                                | 2.54                  | $\pm$ | 0.05 | 2.61                   | 2.44              | 2.79              |
| Caffeine (g kg <sup>-1</sup> dw)       | 15                        | 80                                | 14.02                 | $\pm$ | 0.21 | 14.22                  | 13.69             | 14.74             |
| <b>CS 80 <math>\mu</math>m</b>         |                           |                                   | <b>MAE</b>            |       |      |                        |                   |                   |
| <b>Response variables</b>              | <b>Optimal conditions</b> |                                   | <b>Observed value</b> |       |      | <b>Predicted value</b> | <b>-95 % Pred</b> | <b>+95 % Pred</b> |
|                                        | <b>T (min)</b>            | <b>T (<math>^{\circ}</math>C)</b> |                       |       |      |                        |                   |                   |
| TPC (g GAE kg <sup>-1</sup> dw)        | 32                        | 51.5                              | 7.34                  | $\pm$ | 0.23 | 7.78                   | 7.02              | 8.55              |
| RSC ( $\mu$ mol TE g <sup>-1</sup> dw) | 45                        | 51.5                              | 26.44                 | $\pm$ | 0.72 | 26.61                  | 26.20             | 27.01             |
| TotCQAs (g kg <sup>-1</sup> dw)        | 45                        | 46                                | 1.98                  | $\pm$ | 0.06 | 2.02                   | 1.82              | 2.22              |
| Caffeine (g kg <sup>-1</sup> dw)       | 24                        | 80                                | 11.58                 | $\pm$ | 0.21 | 11.96                  | 11.28             | 12.64             |
| <b>CS 250 <math>\mu</math>m</b>        |                           |                                   | <b>MAE</b>            |       |      |                        |                   |                   |
| <b>Response variables</b>              | <b>Optimal conditions</b> |                                   | <b>Observed value</b> |       |      | <b>Predicted value</b> | <b>-95 % Pred</b> | <b>+95 % Pred</b> |
|                                        | <b>T (min)</b>            | <b>T (<math>^{\circ}</math>C)</b> |                       |       |      |                        |                   |                   |
| TPC (g GAE kg <sup>-1</sup> dw)        | 30                        | 44.5                              | 6.76                  | $\pm$ | 0.20 | 6.85                   | 6.35              | 7.35              |
| RSC ( $\mu$ mol TE g <sup>-1</sup> dw) | 45                        | 51.5                              | 28.44                 | $\pm$ | 0.26 | 28.68                  | 28.28             | 29.09             |
| TotCQAs (g kg <sup>-1</sup> dw)        | 31.5                      | 43.5                              | 2.08                  | $\pm$ | 0.02 | 2.08                   | 1.93              | 2.24              |
| Caffeine (g kg <sup>-1</sup> dw)       | 23                        | 80                                | 7.18                  | $\pm$ | 0.02 | 7.86                   | 7.16              | 8.57              |

The data are expressed as the means $\pm$ standard deviation (n = 3).

<sup>a</sup>Conventional solvent extraction; <sup>b</sup>Ultrasound-assisted extraction, <sup>c</sup>Microwave-assisted extraction.

Generally lower values were obtained using MAE than CSE or UAE, regardless of CS particle size. Other studies showed a better capacity of UAE compared to MAE to extract chlorogenic acids (ROUTRAY and ORSAT, 2014); as shown in Fig. 4, UAE and CSE exhibited comparable maximum observed values of TPC and TotCQAs, while RSC was higher using UAE than CSE, regardless of the particle size; considering TPC, RSC and TotCQAs values, UAE allowed to obtain higher or similar values to CSE with a significant reduction of extraction time, especially at 80  $\mu\text{m}$  particle size (Table 4). According to CHOUNG *et al.* (2014), caffeine content was significantly ( $p \leq 0.05$ ) higher using UAE than CSE or MAE (Fig. 4). Generally maximum observed values were significantly affected by extraction method while no significant differences were observed between the two particle sizes of CS, with the only exception of caffeine content for MAE (Fig. 4). Nevertheless a lower CS particle size allowed to reduce extraction time up to 32.9% for TotCQAs obtained with CSE and up to 51.6% for RSC obtained with UAE (Table 4).

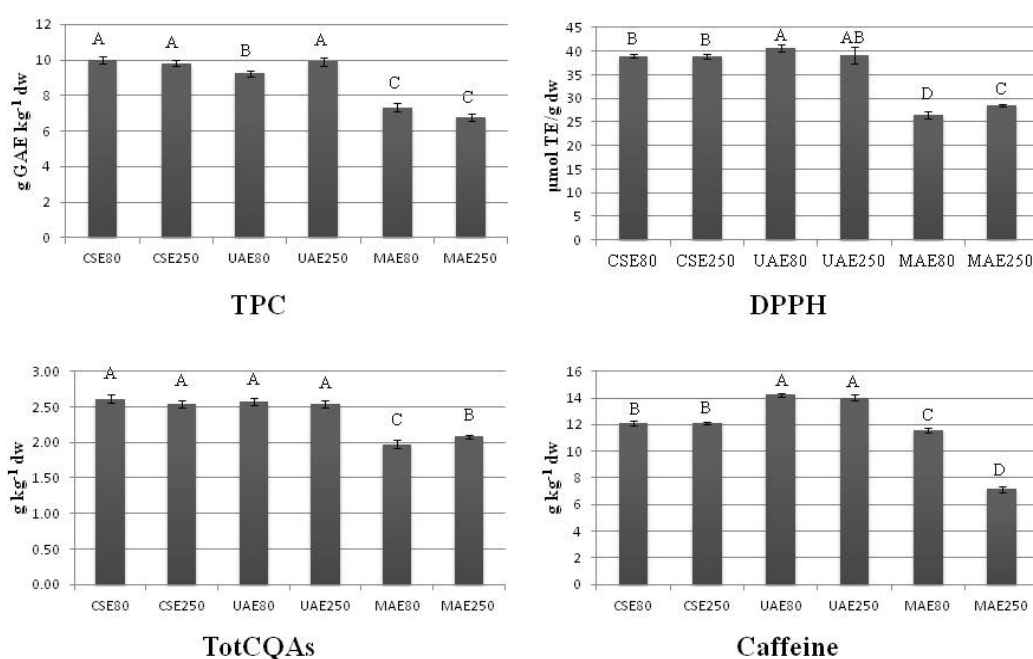


**Figure 4.** Comparison of the observed values (mean $\pm$ standard deviation) of total phenolic content (TPC), radical scavenging capacity (RSC), total caffeoylquinic acids (TotCQAs) and caffeine content obtained at optimal time and temperature conditions by each combination of extraction method and CS particle size. The standard deviation bars with different letters are significant different ( $p \leq 0.05$ ).

#### 4. CONCLUSIONS

CSE, UAE and MAE of phenolic compounds and caffeine from coffee silverskin at 80- and 250  $\mu\text{m}$  particle sizes were optimised using RSM approach. All the quadratic polynomial models were able to predict and optimise CSE, UAE and MAE processes.

ANOVA showed that temperature was the process variable that most affected the extraction processes. In particular a positive correlation was observed between an increase of temperature and TPC, RSC, TotCQAs and caffeine values for CSE and UAE; using MAE above 50°C, a negative effect on TPC, RSC and TotCQAs was observed meaning a possible degradation of the selected caffeoylquinic acids due to a combination of high temperatures

and microwaves. Comparing extraction methods, lowest values of TPC, RSC, TotCQAs and caffeine were obtained using MAE; UAE allowed to obtain extracts with values of TPC, RSC and TotCQAs higher or similar to CSE with a significant reduction of extraction time, especially at 80  $\mu\text{m}$  particle size. Moreover, UAE produced a significant higher content of caffeine compared to CSE in the half of the extraction time. Generally maximum observed values were not affected by the particle size of CS. Nevertheless, using the lower particle size, a reduction of extraction time was observed. In conclusion, UAE applied at high temperatures represented a fast and efficient substitute for conventional solvent extraction of bioactive compounds from coffee silverskin.

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# EFFECT OF HIGH FREQUENCY ULTRASOUNDS ON LYCOPENE AND TOTAL PHENOLIC CONCENTRATION, ANTIOXIDANT PROPERTIES AND $\alpha$ -GLUCOSIDASE INHIBITORY ACTIVITY OF TOMATO JUICE

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## ABSTRACT

Tomato juice was subjected to high frequency ultrasounds (378 and 583 kHz) at increasing energy densities (up to 250 MJ/m<sup>3</sup>). Results relevant to the treatments at high frequency providing an energy density of 250 MJ/m<sup>3</sup> were compared with those obtained at 24 kHz delivering the same energy density. Lycopene and total phenolic concentration, as well as the  $\alpha$ -glucosidase inhibitory activity of tomato juice, were not affected by ultrasound regardless the frequency and energy density. However, the antioxidant properties were negatively affected by high frequency ultrasounds.

*Keywords:* antioxidant properties,  $\alpha$ -glucosidase inhibitory activity, high frequency ultrasounds, tomato juice, total phenolic concentration

## 1. INTRODUCTION

The consumer's interest towards functional food which claims to have health-promoting or disease-preventing properties is increasing. This has led to the study and development of processes able to preserve the food nutritional and sensory quality as well as to enhance the bioactivity of certain constituents (KNORR *et al.*, 2004).

Amongst these, ultrasound (US) processing, which is a non-thermal technology characterized by low energy consumption, has been found to be suitable for different applications in the food industry. Ultrasound technology is widely used to emulsify, encapsulate ingredients, extract bioactive molecules and inactivate microorganisms (PIYASENA *et al.*, 2003; CANSÉLIER *et al.*, 2002; SORIA and VILLAMIEL, 2010; KENTISH and ASHOKKUMAR, 2011; CHEMAT *et al.*, 2011; MANE *et al.*, 2015).

Ultrasonics can be considered as waves with a frequency range higher than 18 kHz. During the wave propagation into a liquid medium, cavitation phenomenon occurs. In particular, cavitation involves the formation and violent collapse of small bubbles, generating shock waves associated to high local temperatures (1000-5000 K) and pressures (100-50000 bar) inside the collapsing bubbles (LEIGHTON, 1994). These extreme conditions lead to the occurrence of physical phenomena (microjet, turbulence, shear forces) and formation of free radicals (GOGATE *et al.*, 2003).

High frequency (100 kHz to 1000 kHz) low power ultrasounds is used for food quality monitoring and diagnostic purposes. However, recent studies have found that free radicals generated by high frequency ultrasounds can react with bioactive compounds and enhance their functionality. In particular, hydroxyl radicals generated during ultrasonication can enhance the hydroxylation degree of food materials and consequently modify their antioxidant activity (ASHOKKUMAR *et al.*, 2008).

Tomatoes, an important agricultural commodity worldwide, are well known for their nutritional properties. In fact, they are a good source of bioactive compounds such as carotenoids and phenolic compounds (GIOVANELLI *et al.*, 1999; LENUCCI *et al.*, 2012; ZANFINI *et al.*, 2017). Lycopene is the most abundant carotenoid in tomatoes and its chemical structure confers important properties, such as oxygen-radical scavenging and quenching capacity (DI MASCO *et al.*, 1989; SHI and LE MAGUER 2000; RAO and RAO, 2007). Moreover, phenolic compounds, although present in lower amount, may also contribute to beneficial effects of tomato products, because of their high antioxidant activity and ability to inhibit carbohydrate hydrolysing enzymes, such as  $\alpha$ -glucosidase (HANHINEVA *et al.*, 2010; NAIR *et al.*, 2013; TOMAS *et al.*, 2017). The latter, located in the brush-border surface of intestinal cells, catalyses the hydrolysis of complex carbohydrates and disaccharides to absorbable monosaccharides (KIM *et al.*, 2010). As a consequence, the  $\alpha$ -glucosidase inhibition can suppress the influx of glucose from the intestinal tract to blood vessels resulting in an important strategy in the management of postprandial hyperglycemia (KWON *et al.*, 2008).

Nevertheless, the nutritional value due to carotenoids and phenolic concentration might be modified during processing. In fact, there are several studies dealing with the effect of thermal and non-thermal processing (high pressure homogenization, pulsed electric field and ultrasounds) on bioactive molecules naturally occurring in tomato derivatives in order to obtain fruit and vegetables derivatives able to accomplish desired nutritional functions (COLLE *et al.*, 2010; ODRIOZOLA-SERRANO *et al.*, 2009; ANESE *et al.*, 2015). However, the effect of high frequency ultrasound treatment on tomato properties has been less studied (GOLMOHAMADI *et al.*, 2013). Because of this, the aim of the present study was to evaluate whether the high frequency ultrasounds might modify nutritional functions of the main bioactive compounds in tomato extract. To achieve this aim, the objectives of this work were to investigate the effect of high frequency (378 and 584 kHz)

ultrasounds on bioactives present in tomato (lycopene and total phenolic compounds) and the effects of extracts on the antioxidant properties and  $\alpha$ -glucosidase inhibitory activity. Moreover, the treatment was performed for increasing length of time (up to 60 min), thus providing increasing energy densities (up to 250 MJ/m<sup>3</sup>). The results obtained by providing the maximum energy density value were compared with those obtained for samples subjected to low frequency ultrasounds (24 kHz). Such investigation represents a preliminary study aimed to understand whether high frequency US could represent a suitable technological tool for steering tomato juice functionality. No industrial application was considered at this stage of the research.

## 2. MATERIALS AND METHODS

### 2.1. Sample preparation

Commercial pasteurized tomato juice (7.5 °Brix) was sieved (20 mesh) to separate seeds and coarse particles, and submitted to ultrasound treatment. Tomato juice not subjected to ultrasound treatment was used as a control.

### 2.2. Sonication

Ultrasound treatments were conducted using two ultrasonic processors operating at either 378 kHz or 583 kHz, and 24 kHz.

#### 2.2.1. Ultrasound treatment at 378 kHz and 583 kHz

An ultrasonic processor (Meinhardt Ultraschlltechnik Leipzig, Germany) equipped with two transducers (operating at 378 kHz and 584 kHz) was used. Aliquots of 250 mL of tomato juice were introduced into a glass reaction vessel (63 mm internal diameter) with a cooling jacket (wall thickness 5 mm) connected to a cryostatic bath (Fisher Scientific, ISOTEMP Thermostatic bath). Samples were subjected to sonication for increasing length of time up to 60 min. During the ultrasound treatment, the temperature never exceeded 20 °C.

#### 2.2.2. Ultrasound treatment at 24 kHz

An ultrasonic processor (Hieschler Ultrasonics GmbH, mod. UP400S, Teltow, Germany) with a titanium horn tip diameter of 22 mm operating at 24 kHz was used. Aliquots of 100 mL of tomato juice were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass vessels. The tip of the sonicator horn was placed in the centre of the tomato juice, with an immersion depth in the fluid of 50 mm. Sample were subjected to sonication up to 3 min. During the ultrasound treatment the temperature never exceeded 40 °C.

### 2.3. Determinations

#### 2.3.1. Power and energy density computation

The power density ( $P_s$ , W/m<sup>3</sup>) transferred from the ultrasounds probe to the sample was determined calorimetrically during preliminary trials by recording the temperature (T, K) increase during the treatment, following eq. (1) (RASO *et al.*, 1999).

$$P_v(T) = dc_p(\partial T/\partial t) \quad (1)$$

where  $d$  is the sample density ( $\text{kg}/\text{m}^3$ ),  $c_p$  is the water heat capacity ( $4186 \text{ J}/\text{kg K}$ ). The energy density ( $\text{MJ}/\text{m}^3$ ) was calculated by multiplying the power density value by the duration of the treatment (HULSMANS *et al.*, 2010).

### 2.3.2. Lycopene concentration

The lycopene extraction was performed following the procedure of SADLER *et al.*, (1990), with minor modification, under subdued light to prevent carotenoid degradation and isomerisation. Aliquots of 25 mL of extraction solution (hexane:acetone:ethanol, 2:1:1 v/v/v) were added to 1 g of tomato juice. The mixture was stirred at room temperature for 20 min. Reagent grade water (7.5 mL) was added and stirring was continued for 10 min. The hexane phase, containing lycopene, was separated from the polar phase using a separation funnel. Immediately after extraction, the absorbance of lycopene was measured at 472 nm with a spectrophotometer (Thermo Fischer Scientific, Genesys 10S UV/VIS Spectrophotometer) using hexane as reference. The total lycopene concentration was calculated using the Beer-Lambert law, considering the extinction coefficient of lycopene in hexane equal to  $1.8 \times 10^5 \text{ L}/\text{molcm}$ .

### 2.3.3. Total phenolic concentration

The total quantity of phenolic components was measured by the Folin-Ciocalteu method (SINGLETON and ROSSI, 1965). Aliquots of 10 mL of methanol:water (1:1 v/v) were added to 1 g of tomato juice and the mixture was stirred for 5 min. The solution was filtered (QL10, size 150 mm) and 100  $\mu\text{L}$  was added to 5 mL of a 1:10 dilution of Folin-Ciocalteu reagents and 0.9 mL of distilled water. After 5 min, aliquots of 3.5 mL of  $\text{Na}_2\text{CO}_3$  (115 g/L) were added and the mixture left in the dark, at room temperature for 2 hours. The absorbance of the solution was measured at 765 nm. The optical density was compared to a standard curve prepared with 0 to 500 mg/L of gallic acid and the results were expressed as mg GAE (gallic acid equivalents)/100 g.

### 2.3.4. Optical microscopy

Tomato juice microstructure was analysed by using an optical microscope (Leica DM 2000, Leica Microsystems, Heerburg, Switzerland). The pictures were taken by a digital camera (Leica EC3, Leica Microsystems, Heerburg, Switzerland), using the Leica Suite LAS EZ software (Leica Microsystems, Heerburg, Switzerland).

### 2.3.5. Antioxidant activity

The antioxidant activity was determined following the procedure of BENZIE and STRAIN (1996) determined using ferric reducing antioxidant potential (FRAP). Aliquots of 20 mL of acetone:water (80:20 v/v) mixture were added to 1 g of tomato juice and the mixture was stirred for 5 min. Aliquots of 90  $\mu\text{L}$  of this mixture were added to 3 mL of FRAP solution and incubated in a water bath at  $37^\circ\text{C}$  for 4 min followed by measurement of absorbance at 593 nm against a blank. The optical density was compared to the standard curve for ferrous sulphate ( $\text{FeSO}_4$ ) solution, with concentrations between 0 and 1 mM. Results were expressed as FeII (mM) produced/100 g sample.

### 2.3.6. $\alpha$ -glucosidase inhibitory activity

The  $\alpha$ -glucosidase inhibitory activity of tomato juice was determined spectrophotometrically (UV-2501PC, UV-Vis recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) following the method of SING *et al.*, (2014) with some modifications. The tomato juice was first centrifuged at 2200 g for 3 min at 20 °C. Aliquots of 30  $\mu$ L of 1 U/mL  $\alpha$ -glucosidase in phosphate buffer (100 mM, pH 7.0) were introduced into 1 mL capacity cuvette in the presence of 100  $\mu$ L of tomato extract and a volume of 100 mM phosphate buffer (pH 7.0), giving a final volume of 900  $\mu$ L in the cuvette, and mixed thoroughly. After 10 min of incubation at 37 °C, the reaction was started by the addition of 100  $\mu$ L of 5 mM 4-nitrophenyl-  $\alpha$ -D-glucopyranoside (pNGP) solution (Sigma-Aldrich, Milano, Italy) in 100 mM phosphate buffer (pH 7.0) as substrate. The release of p-nitrophenol from pNGP was monitored at 405 nm for 15 min at 37 °C. The changes in absorbance per min were calculated by linear regression, applying the pseudo zero order kinetic model. The eventual stationary phase was excluded from the regression of data. Control samples were run in the absence of tomato extract. The inhibitory activity (IA%) of samples on  $\alpha$ -glucosidase was calculated according to the following equation:

$$IA\% = 100 - \left( \frac{k_s}{k_c} \cdot 100 \right) \quad (2)$$

where  $k_s$  and  $k_c$  are the constant rates (Abs/min) of the enzymatic activity in the presence or in the absence of the inhibitor.

### 2.4. Data analysis

The results reported here are the averages of at least two measurements carried out on two replicated experiments ( $n \geq 4$ ). Data are reported as mean value  $\pm$  standard deviation. Statistical analysis was performed using R v. 2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ( $p < 0.05$ ).

## 3. RESULTS AND DISCUSSIONS

### 3.1. Effect of ultrasounds on lycopene and total phenolic concentration of tomato juice

Table 1 shows lycopene and phenolic concentration of tomato juice subjected to high frequency ultrasounds (378 and 584 kHz) with increasing energy density. No differences in lycopene concentration were found among the untreated and ultrasonically treated samples, regardless of the frequency and energy density. The total phenolic concentration was slightly affected by the application of high frequency ultrasounds. A significant increase of the phenolic concentration was observed for the tomato juice subjected to ultrasounds at 378 kHz, providing an energy density of 250 MJ/m<sup>3</sup>. However, from a practical point of view, this result does not appear to be relevant in terms of total phenolics recovery. To our knowledge, only GOLMOHAMADI *et al.* (2013) have investigated the effect of high frequency ultrasounds (490 kHz) on the total phenolics in red raspberry puree. They found that ultrasonication did not affect the total phenolic concentration. However, results of GOLMOHAMADI *et al.* (2013) cannot be directly

compared with those obtained in this study, due to discrepancies in the frequencies as well as the lack of information concerning the energy density applied.

Levels of lycopene and total phenolics presented in the tomato juice after treatment at 378 and 583 kHz (250 MJ/m<sup>3</sup>) were compared with those obtained at 24 kHz with the same energy density (Table 1). In fact, although the application of low frequency ultrasounds is commonly used in food processing, no data are present in the literature comparing the effect of high and low frequency ultrasounds at the same energy density. It can also be observed that the ultrasound treatment at 24 kHz did not cause any significant change in lycopene concentration. This result is in agreement with previously published data (ANESE *et al.*, 2013) relating to tomato juice ultrasonically treated at 24 kHz with an energy density of 731 MJ/m<sup>3</sup>. Moreover, the low frequency ultrasound treatment did not significantly modify the total phenolic concentration of tomato juice. This result is, however, contrary to that of CHEMAT *et al.* (2011) and the discrepancy may be attributed to differences in the process parameters.

**Table 1.** Lycopene and total phenolic concentrations, antioxidant activity and  $\alpha$ -glucosidase inhibitory activity of untreated and ultrasonically treated tomato juice. Data are referred to increasing energy density provided at 24 kHz, 378 kHz and 583 kHz.

| Frequency (kHz) | Time (min) | Energy density (MJ/m <sup>3</sup> ) | Lycopene (mg/g)        | Total phenolic (GAE mg/100 g) | Antioxidant activity (Fell mM/100 g) | $\alpha$ -glucosidase inhibitory activity (%) |
|-----------------|------------|-------------------------------------|------------------------|-------------------------------|--------------------------------------|-----------------------------------------------|
| Untreated       | 0          | 0                                   | 0.35±0.01 <sup>a</sup> | 192.1±4.0 <sup>bc</sup>       | 25.0±2.7 <sup>a</sup>                | 72±4 <sup>a</sup>                             |
| 24              | 3          | 250                                 | 0.36±0.01 <sup>a</sup> | 193.9±1.0 <sup>bc</sup>       | n.d.                                 | 74±2 <sup>a</sup>                             |
| 378             | 15         | 15                                  | 0.35±0.01 <sup>a</sup> | 192.9±1.0 <sup>bc</sup>       | 17.8±0.5 <sup>b</sup>                | n.d.                                          |
|                 | 60         | 59                                  | 0.35±0.02 <sup>a</sup> | 191.4±1.0 <sup>bc</sup>       | 15.7±0.3 <sup>b</sup>                | 78±2 <sup>a</sup>                             |
|                 | 15         | 62                                  | 0.36±0.01 <sup>a</sup> | 205.0±2.0 <sup>ab</sup>       | 19.2±0.5 <sup>b</sup>                | n.d.                                          |
|                 | 60         | 250                                 | 0.37±0.02 <sup>a</sup> | 212.2±6.0 <sup>a</sup>        | 18.8±1.1 <sup>b</sup>                | 78±2 <sup>a</sup>                             |
| 583             | 15         | 8                                   | 0.35±0.00 <sup>a</sup> | 197.1±3.0 <sup>bc</sup>       | 18.7±0.6 <sup>b</sup>                | n.d.                                          |
|                 | 60         | 31                                  | 0.35±0.01 <sup>a</sup> | 195.0±2.0 <sup>bc</sup>       | 17.8±2.2 <sup>b</sup>                | 76±1 <sup>a</sup>                             |
|                 | 15         | 62                                  | 0.33±0.00 <sup>a</sup> | 194.3±3.0 <sup>bc</sup>       | 19.3±1.8 <sup>b</sup>                | n.d.                                          |
|                 | 60         | 250                                 | 0.35±0.01 <sup>a</sup> | 187.1±5.0 <sup>c</sup>        | 18.7±0.3 <sup>b</sup>                | 79±3 <sup>a</sup>                             |

Means with different letters within the same column are significantly different (p<0.05).

n.d.: not determined.

Although the application of high and low frequency ultrasounds did not affect the levels of bioactive components, slight differences in the tomato microstructure were observed. Fig. 1 shows the micrographs of tomato juice subjected to ultrasounds at 24 kHz, 378 kHz, 583 kHz at an energy density of 250 MJ/m<sup>3</sup>. When compared to the untreated tomato juice, the low frequency ultrasonically processed samples showed a partial disruption of cell membranes with carotenoids distributed into the matrix. However, no differences between the untreated sample and those processed at 387 and 583 kHz were observed. The differences in the microstructure of samples treated at low and high frequency can be attributed to cavitation phenomena occurring during ultrasound treatment. In fact, during low frequency (24 kHz) ultrasounds, transient cavitation phenomena are responsible for the rapid change in fluid pressure and temperature, which might cause cell wall disruption and carotenoids released. By contrast, microstreaming phenomena are

generated by stable cavitation at high frequency (378 kHz and 583 kHz) ultrasounds; these are reported not to cause dramatic structure changes (MCCLEMENTS, 1995).

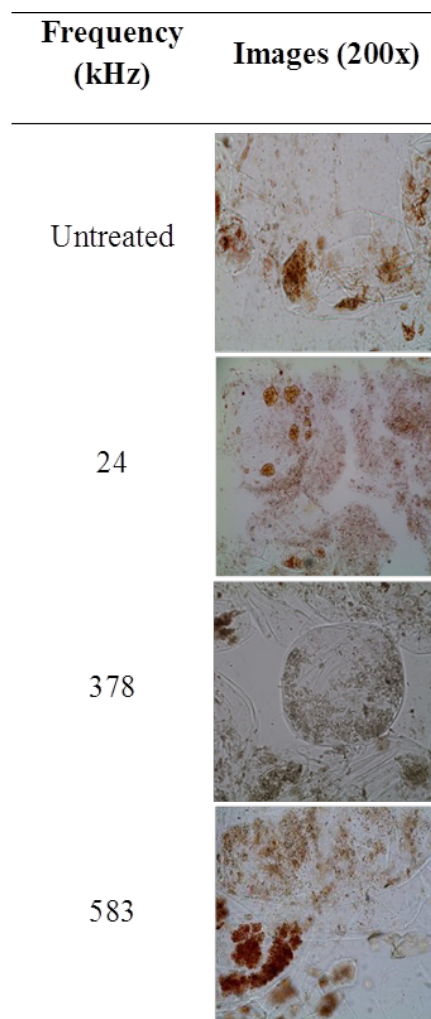


**Figure 1.** Images of tomato juice subjected to ultrasounds at 24 kHz, 378 kHz and 583 kHz (at 250 MJ/m<sup>2</sup> corresponding to 3 min for ultrasonically treated sample at 24 kHz and 60 min for 378 and 583 kHz ultrasonically treated sample) along with an untreated sample.

### 3.2. Effect of ultrasounds on antioxidant properties and $\alpha$ -glucosidase inhibitory activity of tomato juice

Table 1 shows the effect of high frequency ultrasounds at 378 kHz and 583 kHz on the antioxidant activity of tomato juice. As compared with the untreated sample, the application of the high frequency ultrasounds caused a significant reduction in the antioxidant activity. This seems to be in contradiction with the levels of lycopene and total phenolic compounds which were affected by ultrasounds. It should be kept in mind that these are not the only compounds with antioxidant properties present in tomato product. For instance, ascorbic acid is a well-known antioxidant and this vitamin has been destroyed by high frequency ultrasounds (GOLMOHAMADI *et al.*, 2013; PORTENLÄNGER and HEUNSIGER, 1992). This result is in contrast with data reported

by ASHOKKUMAR *et al.* (2008) for a phenol model aqueous solution subjected to 358 kHz. The authors attributed the increase in antioxidant properties of the phenolic component in the model solution to the generation of OH· radicals due to the homolysis of water molecules and subsequent phenol hydroxylation. The decrease in antioxidant capacity observed under our experimental conditions may be attributed to the addition of the hydroxyl radicals in a non-preferred position of the aromatic ring. This has previously been observed for a cyanidin 3-glucoside model system (ASHOKKUMAR *et al.*, 2008). These results suggest that controlled hydroxylation is needed to promote an increase in the antioxidant properties of phenolic compounds, which is indeed difficult to achieve. Increasing attention has been recently paid towards the capability of phenolic compounds to control blood glucose levels related to type 2 diabetes (HANHINEVA *et al.*, 2010; LORDAN *et al.*, 2013). In particular, phenolics were found to inhibit digestive enzymes involved in starch breakdown, such as  $\alpha$ -glucosidase. Table 1 shows the  $\alpha$ -glucosidase inhibitory activity of tomato juice subjected (or not) to ultrasounds at 24 kHz, 378 kHz and 583 kHz at an energy density of 250 MJ/m<sup>3</sup>. It can be observed that in our experimental conditions for the untreated sample  $\alpha$ -glucosidase inhibition was about 72%. Moreover, no significant changes in  $\alpha$ -glucosidase inhibition were found when considering the ultrasound treated samples.

#### 4. CONCLUSIONS

The results of this study showed that ultrasound treatments at 24, 378 and 583 kHz had no effect on major bioactive compounds (i.e. lycopene and total phenolics) as well as on  $\alpha$ -glucosidase inhibitory activity of tomato juice regardless the frequency and the energy density. On the other hand, the antioxidant properties appear to be reduced by high frequency ultrasounds. Thus, we conclude that high frequency ultrasound treatments do not seem to effectively alter the bioactive concentration or improve the functionality of tomato juice.

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## GREY RELATION ANALYSIS OF SOLAR DRYING PROCESS PARAMETER ON COPRA

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### ABSTRACT

The methodology for the optimization of the drying parameters on solar drying of copra was investigated and studied in this paper. This paper investigates the influence of the process parameters like initial mass, inclination angle and time period on the output parameters such as weight reduction rate and moisture content. Based on the analysis, optimal levels of parameters were determined and the same was validated through the confirmation test. The confirmation results reveal that, there is considerable improvement in the weight reduction rate, moisture content and grey relational grade and they improved by 37.36%, 32.28% and 32.94 % respectively. It is observed that the drying performance can be effectively improved with respect to the initial parametric setting.

*Keywords:* Weight Reduction rate (WRR), moisture content, Taguchi, grey relational analysis & design of experiment

## 1. INTRODUCTION

The use of solar dryers in the drying of agricultural products can significantly reduce or eliminate product wastage, food poisoning and so on, thereby, enhancing productivity of the farmers in obtaining higher revenue. Drying using solar radiation, that is, drying under direct sunlight, is one of the oldest techniques used by man to preserve agriculture based food and non-food products (CHANDRAKUMAR and JIWANLAL, 2013). This form of energy is free, renewable and abundant in any part of the world, especially for countries situated in the tropics. However, in order to maximize its advantage and optimize the efficiency of drying using solar radiation, appropriate measures in terms of technology need to be taken in order to make this technique a sustainable one. Such technology is known as solar drying and it is fast becoming a popular option to replace mechanical thermal dryers, owing to the high cost of fossil fuels which is growing in demand, but dwindling in supply.

SRINIVASAN and BALUSAMY (2015) have studied the performance of forced convection solar dryer, integrated with heat storage materials for processing copra. PARDHI and BHAGORIA (2013) have carried out preliminary investigations and under controlled condition of drying experiments constructed a mixed-mode solar dryer with forced convection, using smooth and rough plate solar collector. DHARMALINGAM *et al.* (2015) have used  $L_{27}$  Orthogonal array to determine the Signal-to-noise ratio (S/N ratio) and Analysis of variance (ANOVA) was conducted. Based on the experiments, they concluded that pulse on-time and electrolyte concentration are the most significant parameters for Material Removal Rate (MRR). Gap current and electrolyte concentration which are the influencing parameters for lesser overcut were experimentally investigated. DHARMALINGAM *et al.* (2014a) have found that the influence of the process parameters is through the response surface methodology and grey relational analysis. DHANUSHKODI *et al.* (2014) reported that a directly forced convection solar drier, integrated with recirculation of air has been developed and its performance is tested for drying grapes under the meteorological conditions of Coimbatore, India. The specific moisture extraction rate was estimated to be 0.87 kg/kWh. DHARMALINGAM *et al.* (2015) also stated that ANOVA was used for identifying the significant parameters affecting the responses.

## 2. MATERIALS AND METHODS

### 2.1 Description of the solar dryer

The developed solar dryer (Fig. 2.1) consists of a glass sheet-covered flat plate solar collector, used to simplify construction and reduce costs. The solar collector is connected directly to the drying chamber without any additional air ducts. The top surface of the insulator in the collector is painted black to absorb solar radiation. The collector is covered with a transparent Ultra Violet (U.V)-stabilized glass sheet that is fixed to the collector frame using reinforced plastic clamps in the drying chamber, and a wire mesh is placed on top of the insulators. A glass is placed on top of the black V groove sheet, on which the product to be dried are spread. This arrangement allows drying air to flow around the whole surface of the product being dried. One side of this sheet is fixed to the drying chamber frame and the other side is fixed to a metal tube, allowing the sheet to be rolled up and down for loading and unloading the dryer. This fixing method is designed to facilitate the replacement of the sheets. In general, the transparent sheet can be used for 1-2 years and the air could last for 3-5 years. The glass is installed at the back of the collector

to suck ambient air into the collector. The blowers are intentionally installed below to constantly reduce its temperature, thus, maintaining its efficiency. Both the collector and the drying chamber are installed on mild steel block substructures. All parts of the dryer, including the back insulator and metal frames were designed using a modular concept, which facilitates the transport and installation of the dryer. This solar dryer uses solar energy both in the thermal form of the drying process and in the electrical form for driving the blower, by means of the solar collector and solar module, respectively. Therefore, the dryer could be used in rural areas where there is no supply of electricity. The dryer is a passive system in the sense that it has no moving parts. The sun rays entering through the collector glazing energizes it. The absorption of the rays is enhanced by the inside surface of the collector painted black and the absorbed energy heats the air inside the collector. The greenhouse effect achieved within the collector drives the air current through the drying chamber. If the vents are open, the hot air rises and escapes through the upper vent in the drying chamber, while cooler air at ambient temperature enters through the lower vent in the collector. The Fig. 2.2 shows the copra drying process in the solar dryer.



**Figure 2.1.** Arrangement of solar dryer.



**Figure 2.2.** Copra in solar dryer.

## 2.2. Optimization Methodology

The optimization of process parameters is the key step in the Taguchi method (DHARMALINGAM *et al.* 2014b). Twenty seven experimental runs ( $L_{27}$ ) based on the Orthogonal Array (OA) of Taguchi methods have been carried out (DHARMALINGAM *et al.* 2014c). The multi-response optimization (Grey Relational Analysis) of the process parameters has been performed for drying, using weight reduction rate and moisture content. The drying time, weight reduction rate and moisture content are noted twice for every trial.

## 3. RESULTS AND CONCLUSIONS

### 3.1. Major results and inferences

The assignment of factors with their levels identified in this investigation are given in Table 3.1.

**Table 3.1.** Drying process parameters and their corresponding levels.

| Symbol | Factors                         | Level 1       | Level 2 | Level 3 |
|--------|---------------------------------|---------------|---------|---------|
| A      | Initial mass $M_i$ (g)          | 1000          | 750     | 500     |
| B      | Inclination Angle( $^{\circ}$ ) | 30            | 45      | 60      |
| C      | Time period (Hr)                | 11AM -12 Noon | 1-2 PM  | 3-4 PM  |

Based on the Taguchi's  $L_{27}$  OA, drying experiments were conducted on solar dryer for copra. The experimental results were gathered for each trial. The S/N ratios were calculated for all the responses since the objective of this work was to maximize the weight reduction rate and the minimization of moisture content. Therefore, for Weight Reduction Rate (WRR), the larger-is-better type was considered and for moisture content, smaller-is-better type was considered for the analysis. The S/N ratio was computed for each of the twenty-seven trial conditions for the weight reduction rate and moisture content and is shown in Table 3.2 below. The weight reduction rate (%) in the Table 3.2 was calculated by measuring the weight of Copra after drying between different time intervals. The optimal setting levels for each factor resulting from the S/N ratios are shown in Table 3.3.

**Table 3.2.** Experimental results for L<sub>27</sub> OA of copra.

| Trial No. | Initial Mass (Mi) g | Inclination Angle (°) | Time period | Weight reduction rate (%) | Moisture content | S/N Ratio for WRR | S/N ratio for Moisture Content | Grey Relational Weight reduction rate (%) | Grey Relational Moisture content | Grey Grade    |
|-----------|---------------------|-----------------------|-------------|---------------------------|------------------|-------------------|--------------------------------|-------------------------------------------|----------------------------------|---------------|
| 1         | 1000                | 30                    | 11-12       | 23.25                     | 69.38            | 27.328            | -36.825                        | 0.5052                                    | 0.3358                           | 0.4205        |
| 2         | 1000                | 30                    | 1-2         | 36.37                     | 42.18            | 31.215            | -32.502                        | 0.8871                                    | 0.4159                           | 0.6515        |
| 3         | 1000                | 30                    | 3-4         | 15.12                     | 21.08            | 23.591            | -26.477                        | 0.3940                                    | 0.6233                           | 0.5086        |
| 4         | 1000                | 45                    | 11-12       | 27.12                     | 33.09            | 28.666            | -30.394                        | 0.5878                                    | 0.4707                           | 0.5293        |
| 5         | 1000                | 45                    | 1-2         | 34.32                     | 45.60            | 30.711            | -33.179                        | 0.8144                                    | 0.4010                           | 0.6077        |
| 6         | 1000                | 45                    | 3-4         | 14.46                     | 22.36            | 23.203            | -26.989                        | 0.3831                                    | 0.5980                           | 0.4905        |
| 7         | 1000                | 60                    | 11-12       | 26.32                     | 35.12            | 28.406            | -30.911                        | 0.6160                                    | 0.4560                           | 0.5360        |
| 8         | 1000                | 60                    | 1-2         | 33.35                     | 44.06            | 30.462            | -32.881                        | 0.7827                                    | 0.4074                           | 0.5951        |
| 9         | 1000                | 60                    | 3-4         | 12.21                     | 24.06            | 21.734            | -27.626                        | 0.3468                                    | 0.5692                           | 0.4580        |
| 10        | 750                 | 30                    | 11-12       | 25.32                     | 37.06            | 28.069            | -31.378                        | 0.5479                                    | 0.4435                           | 0.4957        |
| 11        | 750                 | 30                    | 1-2         | 37.35                     | 51.56            | 31.446            | -34.246                        | 0.9249                                    | 0.3794                           | 0.6522        |
| 12        | 750                 | 30                    | 3-4         | 13.35                     | 14.86            | 22.510            | -23.440                        | 0.3333                                    | 0.8325                           | 0.5829        |
| 13        | 750                 | 45                    | 11-12       | 24.35                     | 35.06            | 27.730            | -30.896                        | 0.5704                                    | 0.4564                           | 0.5134        |
| 14        | 750                 | 45                    | 1-2         | 37.31                     | 50.46            | 31.437            | -34.059                        | 0.9233                                    | 0.3830                           | 0.6532        |
| 15        | 750                 | 45                    | 3-4         | 13.35                     | 18.48            | 22.510            | -25.334                        | 0.3650                                    | 0.6884                           | 0.5267        |
| 16        | 750                 | 60                    | 11-12       | 28.52                     | 32.18            | 29.103            | -30.152                        | 0.6716                                    | 0.4780                           | 0.5748        |
| 17        | 750                 | 60                    | 1-2         | 35.51                     | 70.72            | 31.007            | -36.991                        | 0.8556                                    | 0.3333                           | 0.5945        |
| 18        | 750                 | 60                    | 3-4         | 14.14                     | 12.48            | 23.009            | -21.924                        | 0.3457                                    | 1.0000                           | 0.6729        |
| 19        | 500                 | 30                    | 11-12       | 29.35                     | 35.42            | 29.352            | -30.985                        | 0.6414                                    | 0.4540                           | 0.5477        |
| <b>20</b> | <b>500</b>          | <b>30</b>             | <b>1-2</b>  | <b>38.36</b>              | <b>46.98</b>     | <b>31.678</b>     | <b>-33.438</b>                 | <b>0.9663</b>                             | <b>0.3955</b>                    | <b>0.6809</b> |
| 21        | 500                 | 30                    | 3-4         | 15.12                     | 15.90            | 23.591            | -24.028                        | 0.3940                                    | 0.7817                           | 0.5879        |
| 22        | 500                 | 45                    | 11-12       | 28.20                     | 28.08            | 29.005            | -28.968                        | 0.6631                                    | 0.5168                           | 0.5900        |
| 23        | 500                 | 45                    | 1-2         | 38.35                     | 47.42            | 31.675            | -33.519                        | 0.9659                                    | 0.3938                           | 0.6798        |
| 24        | 500                 | 45                    | 3-4         | 16.98                     | 21.90            | 24.599            | -26.809                        | 0.4258                                    | 0.6066                           | 0.5162        |
| 25        | 500                 | 60                    | 11-12       | 26.12                     | 38.48            | 28.339            | -31.705                        | 0.6112                                    | 0.4351                           | 0.5232        |
| 26        | 500                 | 60                    | 1-2         | 39.14                     | 47.00            | 31.852            | -33.442                        | 1.0000                                    | 0.3954                           | 0.6977        |
| 27        | 500                 | 60                    | 3-4         | 13.35                     | 23.20            | 22.510            | -27.310                        | 0.3650                                    | 0.5831                           | 0.4741        |

Footnote: The values showed in bold are optimized values.

Fig. 3.1 shows the residual plots for grey grade. Table 3.4 shows the ANOVA table, which indicates the significance of process parameters on the Weight Reduction Rate and Moisture Content. In the Table 3.4, the F-test is a matter of including the correct variances in the ratio. The F-statistic is this ratio of variation between sample means to the variation within the samples.

**Table 3.3.** Response table for the grey relational grade.

| Process parameters             | Level 1                  | Level 2       | Level 3       |
|--------------------------------|--------------------------|---------------|---------------|
| Initial mass $M_i$ (g)         | 0.5330                   | 0.5851        | <b>0.5886</b> |
| Inclination Angle( $0^\circ$ ) | <b>0.5698</b>            | 0.5674        | 0.5696        |
| Time period                    | 0.5256                   | <b>0.6458</b> | 0.5353        |
| *Optimum Levels                | Mean grey grade = 0.5466 |               |               |

**Table 3.4.** ANOVA for grey relation grade.

| Factors           | Design of Factor | Sum of squares | Mean square=sum of squares / 2 | F value | F <sub>0.05</sub> | % of contribution=sum of squares/total sum of squares |
|-------------------|------------------|----------------|--------------------------------|---------|-------------------|-------------------------------------------------------|
| Initial Mass      | 2                | 0.002141       | 0.00107                        | 13.55   | 0                 | 1.56 significant                                      |
| Inclination Angle | 2                | 0.015905       | 0.007952                       | 100.69  | 0                 | 11.62 significant                                     |
| Time Period       | 2                | 0.117244       | 0.058622                       | 742.26  | 0                 | 85.66 significant                                     |
| Error             | 20               | 0.00158        | 0.000079                       |         |                   | 1.15                                                  |
| Total             | 26               | 0.13687        |                                |         |                   | 100%                                                  |

$S = 0.00888699$ ;  $R\text{-Sq} = 98.85\%$ ;  $R\text{-Sq (adj)} = 98.50\%$ .

Footnote: The values showed in bold are optimized values.

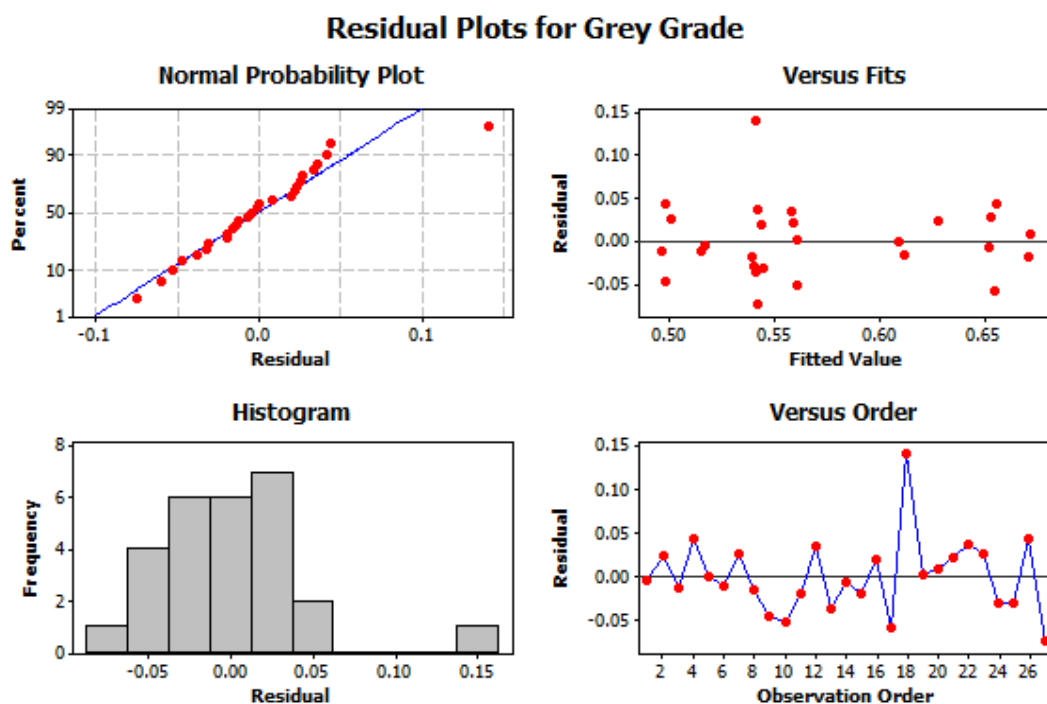


**Figure 3.1.** Residual plots for grey grade.

### 3.2. Analysis for weight reduction rate and Moisture Content of copra

The optimal values for the maximum weight reduction rate has an initial mass of 500 g, inclination angle is 30° and time period is between 1-2 AN (after noon). The interaction has been plotted to pictorially depict the interaction process parameters on weight reduction rate and moisture content. In the full interaction plot, two panels per pair of process parameters have been shown in Figs. 3.2 and 3.3.

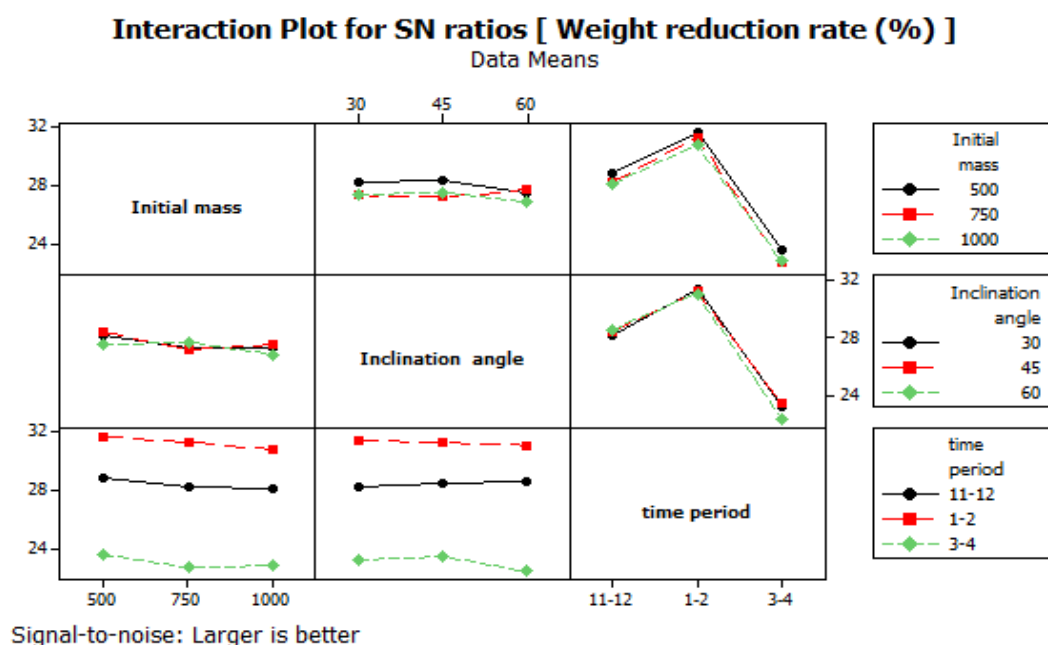


Figure 3.2. Interaction plot for WRR.

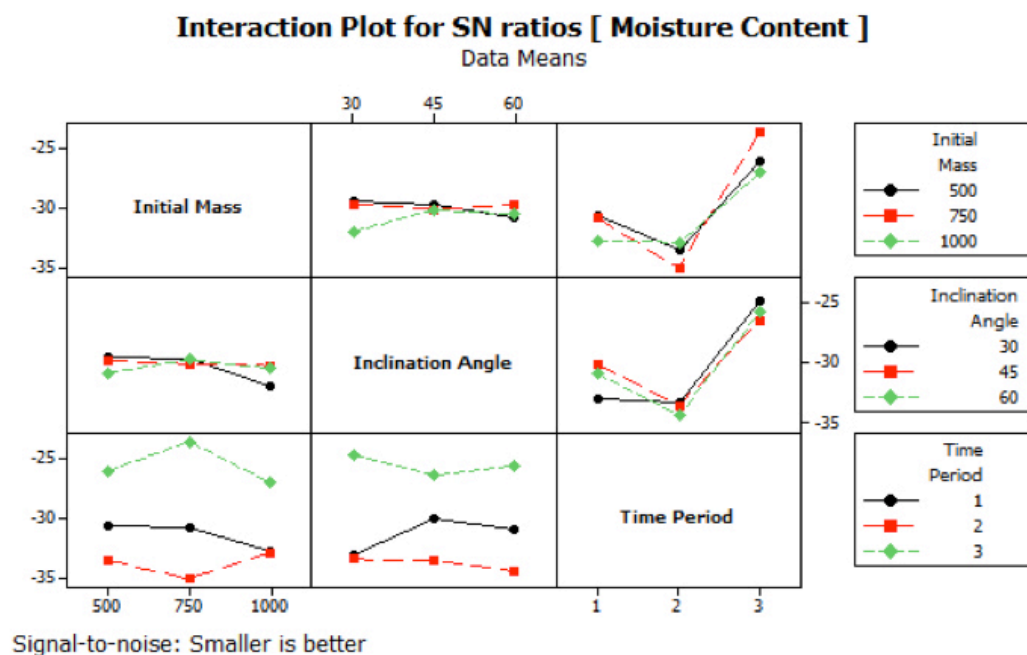


Figure 3.3. Interaction plot for MC.

### 3.3 Confirmation test

After identifying the most influential parameters, the final phase is to verify the predicted results (WRR and Moisture content) by conducting the confirmation test. The  $A_3B_1C_2$  through the GRA is an optimal parameter combination of the drying process. Therefore, the combination  $A_3B_1C_2$  was seen as a confirmatory test. The predicted Grey relational grade can be calculated using the optimum parameters as

$$\alpha_{predicted} = \alpha_m + \sum_{i=1}^3 (\alpha_o - \alpha_m)$$

Where,  $\alpha_o$  = average grey relational grade of the optimal level,  $\alpha_m$  = overall mean grey relational grade.

$$\alpha_{predicted} = 0.57045 + (0.58534 - 0.57045) + (0.645329 - 0.57045) + (0.58534 - 0.57045) = 0.6722$$

Table 3.4 shows the confirmatory test for weight reduction and moisture content of copra. Based on the confirmatory test, the weight reduction and moisture content were improved by 37.36 % and 32.28 % respectively, with respect to the initial parametric setting. The optimized parameter combination suggested for the higher WRR and Lower Moisture content has an initial mass of 500 g, the inclination angle of 30° and time period of 1-2 hours. The percentage contribution of factors on grey relational grade within a period of time is 86% and inclination angle is 12%. The error and initial mass percentage of contribution of factors are 1% respectively.

**Table 3.4.** Confirmatory test table.

|                  | Initial levels of drying parameters | Optimal combination levels of drying parameters |             |             |
|------------------|-------------------------------------|-------------------------------------------------|-------------|-------------|
|                  |                                     | Prediction                                      | Experiment  | Improvement |
| Level            | $A_1B_1C_1$                         | $A_3B_1C_2$                                     | $A_3B_1C_2$ |             |
| WRR (gr)         | 23.25                               | 38.36                                           | 38.36       | 37.36%      |
| Moisture content | 69.38                               | 46.98                                           | 46.98       | 32.28%      |
| Grey grade       | 0.5123                              | 0.6722                                          | 0.6811      | 32.94%      |

The following data were observed from the present investigation which focuses on optimization and analysis of the solar drying process parameters of copra using Grey Relational Analysis and ANOVA.

- Based on the confirmatory test, improvement in Weight Reduction Rate and Moisture content is 37.36 % and 32.28 % respectively.
- The grey relational grade is improved by 32.94 %.
- The parameter combination suggested for the higher WRR and lesser Moisture Content have an Initial mass of 500 g, inclination angle of 30° and time period 1-2 AN.
- The results of ANOVA, the time period and Inclination angle are the significant drying parameters which affect the weight reduction rate and moisture content.

The modified design of the direct air dryer modelled during this research will maximize the efficiency of drying using solar radiation. This modified design can be used to replace

mechanical and thermal dryers which are dependent on high cost fuel. This modified design is brought into existence by keeping the copra as the food component. The researches on carrying out direct drying of other food components using this modified design can be carried out as future research work.

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# EFFECTIVENESS OF A TEMPERATURE CONTROL SYSTEM IN HOME INDUCTION HOBS TO REDUCE ACRYLAMIDE FORMATION DURING PAN FRYING

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## ABSTRACT

Three trials were conducted to determine the influence of the use of temperature control systems on physico-chemical characteristics and acrylamide formation in the domestic preparation of potatoes. French fries were pre-treated by soaking in water or acidified water, and then they were cooked using a range of home-cooking procedures. Soaking raw potatoes in acidified water (pH=3.17) before frying at a controlled temperature (180 °C) was the most efficient pretreatment for reducing acrylamide formation (76%). For the same temperature, roasted frozen par-fried potatoes contained less fat and acrylamide than similar pan-fried potatoes. Potatoes butter fried at 140 °C had an acrylamide concentration similar to that of potatoes fried in oil at 180 °C, but this value was reduced by 71% when the frying was carried out using a temperature control system. Controlling the frying temperature reduced acrylamide formation at all the temperatures studied.

*Keywords:* acrylamide, frying, induction hob, temperature control, potatoes, home-cooking, pretreatments, roasting

## 1. INTRODUCTION

In 2002, TAREKE *et al.* detected high concentrations of acrylamide in heat-processed foodstuffs. This compound is classified as being likely carcinogenic to humans (ROSEN and HELLENÄS, 2002). Since then, scientists have identified different factors affecting the formation of acrylamide in food. STADLER *et al.* (2002) have shown that acrylamide can be released by the thermal treatment of certain amino acids such as asparagine, particularly in combination with reducing sugars, and by the thermal treatment of early Maillard reaction products. Because potato products are especially high in asparagine, it is currently thought that this amino acid is responsible for the majority of the acrylamide formation in potato crisps and French fries. Considerable research has been carried out to develop strategies to reduce acrylamide formation (GÖKMEN, 2006; FRIEDMAN and LEVIN, 2008; KUMAR *et al.*, 2014; URBANCIC *et al.*, 2014; KAHKESHANI *et al.*, 2015; ZUO *et al.*, 2015). The main problem in developing such strategies is that the main pathway is the same as that for the formation of compounds with desirable aromas during frying (MATTHÄUS, 2009). Therefore, the use of reliable temperature control systems during frying to avoid overheating is necessary to prevent further acrylamide formation (GERTZ and KLOSTERMANN, 2002; MAJCHER and JELEN, 2007; ARIAS-MENDEZ, 2013). Some authors have studied the effect of different pretreatments on acrylamide formation during frying. For example, lowering the pH by adding acids before frying has been found to be an efficient way of considerably diminishing acrylamide formation for French fries (JUNG *et al.*, 2003; PEDRESCHI *et al.*, 2007). Roasting produces lower acrylamide contents than microwaving. Hence, the important factors influencing acrylamide formation are not only the heating temperature and time but also the modality of the heat transfer process (CLAEYS *et al.*, 2005; YUAN *et al.*, 2007).

The heating source is one of the most sensitive parts of the equipment for the formation of acrylamide in the product. It is important to ensure a consistent transfer of heat from the heating source to the product (MATTHÄUS, 2009). With most household appliances, the temperature settings are uncertain. Recently, induction hobs have been introduced on the market. The hob is equipped with infrared sensors that measure the heat emitted by the pan and accordingly adjust the temperature of the food being fried so that the desired temperatures are maintained within  $\pm 2$  °C.

The main objective of the present study is to evaluate the possible effect of the use of temperature control systems on the formation of acrylamide, taking into account the organoleptic characteristics of the fried product together with color, water loss and oil uptake. Three trials were conducted for this purpose. The aim of the first test was to determine the effect of using induction hobs with temperature control systems on the abovementioned factors. In addition, the effect of preparation by par-frying and freezing of fresh potatoes was studied. In the second trial, the possible additional effects of two pretreatments (water immersion or immersion in water acidified with vinegar) on the temperature-controlled frying were studied. Finally, in the third trial the aim was to establish a comparison between pan-frying and other cooking methods for potatoes (oven roasting and pan frying in butter at medium temperature), given the lack of data in the literature on the presence of acrylamide in home-cooked potato dishes.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Fresh Potatoes (variety Bintje) and frozen par-fried potatoes (variety Fontana) were provided by Flensted A/S (Ansager, Denmark). High oleic sunflower oil (Riquísimo Koipesol, Deoleo, Madrid, Spain), vinegar (X-tra vinegar, FDB, Copenhagen, Denmark) and butter (X-tra butter, FDB, Copenhagen, Denmark) were obtained from local supermarkets.

### 2.2. Cooking procedures

Fresh potatoes were stored at 4 °C and subsequently washed, peeled and cut into strips (5 cm x 1 cm x 1 cm) or slices of 3 mm thickness before frying. The samples were fried on an induction hob provided with an automatic control system to maintain a specific temperature once it was reached (Power induction, BOSCH PIB675L34E, BSH, Munich, Germany). Pan frying was done in a saucepan (18 cm diameter) using a ratio of 100 g of potatoes per 100 mL of oil. To evaluate the effect of the temperature control (trial 1), fresh and frozen par-fried potato strips were fried for 10 min at initial oil temperatures of 160 °C, 180 °C and 200 °C with and without temperature control. The aim of trial 2 was to test if soaking pretreatment of the potato strips could mitigate the amount of acrylamide even in the potatoes fried using the temperature control. Fresh potato strips (100 g) were soaked for 20 min in a tap water bath (1 L) at room temperature or in an acidified water bath with vinegar (pH= 3.17). Once the superficial water retained in the samples was eliminated with paper towels, the potatoes were then fried following the same frying conditions as used in trial 1. In trial 3 alternative domestic preparations to frying were carried out: a) fresh potato slices (100 g) were fried for 7 min on each side in butter (5 g) at lower than usual initial frying temperatures (140 °C), with and without temperature control, and b) frozen par-fried potatoes (100 g) were placed in a universal pan and roasted for 25 min at 170, 175 and 180 °C in a pre-heated electric oven with forced-air circulation (iQ500 Siemens, BSH, Munich, Germany). During frying the temperature profiles of the oil were monitored by a type K thermocouple probe and recorded by a Data-logger (Testo Loger 177.T4). Three batches were made for each of the experimental conditions. Superficial oil retained in the potatoes was eliminated with paper towels and the samples were analyzed.

### 2.3. Acrylamide determination

The liquid chromatography-tandem mass spectrometry method described by NIELSEN *et al.* (2006) was used for the determinations, with slight modifications. Thirty mL of MilliQ water was added to a 0.3 g aliquot homogenate. Acrylamide (2-propene amide) [CAS No. 79-06-1] (>99.5%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Labelled  $d_3$ -acrylamide (> 98%) was supplied by Polymer Source Inc. (Dorval, Quebec, Canada). The potato strips were homogenized using a mixer (model 4169/4297, Braun AG, Kronberg, Germany). The acrylamide analysis was performed on 3 g aliquots of homogenized fried potato samples with an added internal standard comprised of 150 µL of 10 µg mL<sup>-1</sup>  $d_3$ -acrylamide and 30 mL of deionized water. The sample was extracted by a homogenizer (model Ultra Turrax T25, Janke and Kunkel, Staufen, Germany) at 1,000-1,200 rpm for 2 min. The sample was then centrifuged at 500 g for 20 min (Heraeus Multifuge, Osterode, Germany) and an aliquot of 2 mL was transferred to an Eppendorf vial, frozen to -18 °C for at least 30 min and subsequently centrifuged in an Eppendorf centrifuge at 12,100 g for 10 min (Minispin Centrifuge, Eppendorf Ag, Hamburg, Germany). The sample was

thawed during centrifuging, and starch precipitated from the supernatant at this low temperature. The SPE (Solid Phase Extraction) cleanup was performed by an automated sampler (Gilson Aspec XL, Gilson Company Inc., Lewis Center, OH, USA) using LiCrhoLut Rp-C18 SPE-cartridges (500 mg) from Merck (Darmstadt, Germany). The SPE columns were conditioned with 2 mL of methanol, 2×2 mL of water, and 0.5 mL of sample lead to waste. Subsequently, 1.75 mL of sample was loaded onto the cartridge and the eluate transferred to Miniprep PTFE filter HPLC vials with a pore diameter of 0.45 µm (Whatman Inc., Little Chalfont, UK). The LC system consisted of a liquid chromatograph (model HP1100, Agilent Technologies, Santa Clara, CA, USA). Separation was performed with 0.1% formic acid in water, with a flow of 0.2 mL min<sup>-1</sup> on a Hypercarb column (dimensions 2.1 mm × 100 mm, particle size 5 µm). The MS-MS detection was performed using a Micromass Quattro Ultima triple quadrupole instrument (Waters Corporation, Milford, MA, USA). The source was maintained at 120 °C and the desolvation gas at 400 °C. Nitrogen was used as the cone and desolvation gas with flow rates of 150 and 500 L h<sup>-1</sup>, respectively. Argon was used as the collision gas and maintained at a pressure of 0.24 Pa. The detection was performed by multiple reactions monitoring (MRM). Acrylamide was detected in positive ion mode. Quantification of the fragmentations was done using the MassLynx software version 4.1 including QuanLynx. Each determination was performed in triplicate.

## 2.4. Color determination

The potato strip color was measured using digital image analysis. For image acquisition an HP Scanjet G4010 scanner (Hewlett-Packard, Palo Alto, CA, USA) delivering 4800 × 9600 dpi hardware resolution was used. The image resolution was set at 200 ppp. Digital image processing was performed using the Matrox Inspector 8.0 software (Matrox Electronic Systems Ltd., Quebec, Canada). Calibration of the digital system was done using the UNE 48-103-94 Spanish color norm (AENOR). The correlation with CIELab values was calculated using a quadratic model (LEÓN *et al.*, 2006). Once tempered, the surfaces of 5 potatoes for each batch (15 potatoes for each condition) were scanned. The corresponding RGB (Red, Green and Blue) coordinates were obtained and, applying the transformation model, the corresponding CIELab coordinates were calculated.

## 2.5. Fat content

Samples previously finely ground in a cooled mill homogenizer IKA A10 (Janke and Kunkel, Staufen, Germany) were extracted with 80 mL of petroleum ether to a 2055 Soxtec (Foss, Hillerød, Denmark). The extraction program was operated at 115 °C and included 30 min of immersion and 1 h 20 min of extraction and draining. The fat content was determined by weight difference, Method 30-25(AACC, 2000). Each determination was performed in triplicate.

## 2.6. Statistical analysis

Statistical analysis was performed using XLSTAT 2014 software (Addinsoft, New York, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple range test for comparisons of means and least significant differences ( $p < 0.05$ ) were performed with the data. All the data were expressed as the mean ± standard deviation. The standard deviation was calculated from 3 (for acrylamide and fat content) or 5 (for color) determinations for each of the three independent experiments.

### 3. RESULTS AND DISCUSSION

#### 3.1. Temperature monitoring

The oil temperature evolution recorded at different initial temperatures with and without activating the temperature control system is shown in Fig. 1 for fresh potatoes. During the frying, the oil temperature undergoes sharp changes, even in the case of frying with temperature control, due to the abrupt temperature decrease caused by the addition of the potatoes (up to 45 °C). The oil temperature evolution in the frying of frozen par-fried potatoes follows a similar trend, but the initial temperature drop is more marked (up to 49 °C). For the same initial temperature, the oil is hotter if frying is carried out without control. After a decreasing stage, when the control of temperature is switched off, the temperature begins to increase in an almost linear fashion overshooting the initial value by 36.8 °C when the initial temperature of the oil is 200 °C, by 23.5 °C when the oil is at 180 °C, by 17.2 °C when the oil is at 160 °C and by 11.8 °C when the oil temperature is at 140 °C. However, in the event that temperature control is switched on, the temperature trends asymptotically to its initial value  $\pm 2$  °C. The temperature differences between frying with or without temperature control decrease as the initial oil temperature decreases.

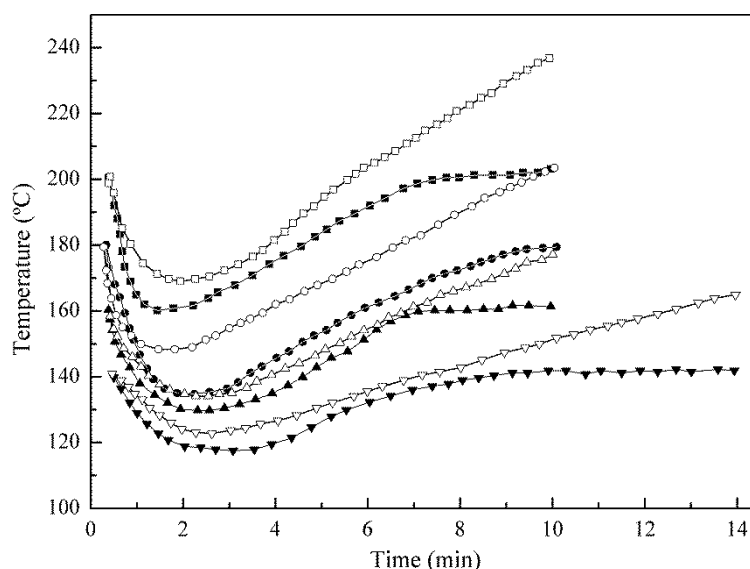


**Figure 1.** Oil temperature during the frying of raw potatoes. Filled symbols correspond to frying with temperature control and hollow symbols to frying without temperature control. Initial temperature: squares, 200 °C; circles, 180 °C; triangles, 160 °C and inverted triangles, 140 °C.

#### 3.2. Effect of par-frying-freezing and temperature controlled frying

Table 1 shows the weight loss, color, fat and acrylamide concentration of fresh and frozen par-fried potatoes of trial 1. The weight loss increased with the temperature, and there were no differences in this respect between samples that were fried at the same initial temperature. The oil uptake in fresh potatoes during temperature-controlled frying was unchanged with increasing temperature. However, it increased in the absence of temperature control. Furthermore, these samples had a higher oil content than the corresponding samples that were fried with temperature control. In the case of frozen par-fried potatoes, the oil concentration increased as the temperature increased, and again the

use of the temperature control had a positive effect by reducing the uptake of oil at a higher frying temperature (200 °C). Temperature is a factor that directly affects the three main mechanisms of absorption (DANA and SAGUY, 2006; HUANG and FU, 2014).

**Table 1.** Effect of par-frying-freezing and temperature controlled frying on the physico-chemical data, fat and acrylamide contents of potatoes.

| Potatoes         | T <sub>i</sub><br>(°C) | Temperature control | Weight loss (%)         | Color                   |                       | Fat content (g/100 g)  | Acrylamide content (mg/kg) |
|------------------|------------------------|---------------------|-------------------------|-------------------------|-----------------------|------------------------|----------------------------|
|                  |                        |                     |                         | L*                      | a*                    |                        |                            |
| Fresh            | 160                    | with                | 32.6±2.9 <sup>b</sup>   | 69.3±1.3 <sup>f</sup>   | -1.7±0.8 <sup>a</sup> | 6.4±1.7 <sup>a</sup>   | 403±40 <sup>a</sup>        |
|                  | 180                    | without             | 45.3±2.2 <sup>cde</sup> | 60.8±2.5 <sup>c</sup>   | 5.3±0.7 <sup>d</sup>  | 9.4±0.4 <sup>b</sup>   | 1547±120 <sup>d</sup>      |
|                  |                        | with                | 44.0±1.6 <sup>cde</sup> | 68.7±0.3 <sup>ef</sup>  | 2.6±0.7 <sup>c</sup>  | 6.5±1.3 <sup>a</sup>   | 1154±150 <sup>c</sup>      |
|                  | 200                    | without             | 52.1±0.7 <sup>e</sup>   | 59.5±0.8 <sup>c</sup>   | 6.6±0.1 <sup>e</sup>  | 12.3±0.6 <sup>c</sup>  | 5600±560 <sup>h</sup>      |
|                  |                        | with                | 50.8±0.6 <sup>e</sup>   | 64.1±0.6 <sup>cde</sup> | 4.9±0.9 <sup>d</sup>  | 6.9±0.8 <sup>a</sup>   | 3030±180 <sup>f</sup>      |
|                  | 200                    | with                | 50.8±0.6 <sup>e</sup>   | 64.1±0.6 <sup>cde</sup> | 4.9±0.9 <sup>d</sup>  | 6.9±0.8 <sup>a</sup>   | 3030±180 <sup>f</sup>      |
| Par-fried-frozen | 160                    | with                | 28.7±1.1 <sup>a</sup>   | 65.7±2.8 <sup>def</sup> | 0.9±0.2 <sup>b</sup>  | 16.4±1.1 <sup>cd</sup> | 450±30 <sup>a</sup>        |
|                  | 180                    | without             | 38.2±3.9 <sup>bc</sup>  | 61.7±2.5 <sup>cd</sup>  | 5.5±0.2 <sup>d</sup>  | 18.9±1.3 <sup>d</sup>  | 1905±97 <sup>e</sup>       |
|                  |                        | with                | 41.2±1.5 <sup>cd</sup>  | 64.3±0.6 <sup>cde</sup> | 3.2±0.6 <sup>c</sup>  | 18.0±1.3 <sup>d</sup>  | 1632±121 <sup>d</sup>      |
|                  | 200                    | without             | 47.6±0.2 <sup>de</sup>  | 47.0±1.7 <sup>a</sup>   | 10.7±0.3 <sup>f</sup> | 36.6±1.9 <sup>f</sup>  | 5247±260 <sup>h</sup>      |
|                  |                        | with                | 46.7±3.3 <sup>de</sup>  | 53.1±1.2 <sup>b</sup>   | 10.0±1.0 <sup>f</sup> | 24.9±1.3 <sup>e</sup>  | 4267±304 <sup>g</sup>      |
|                  | 200                    | with                | 46.7±3.3 <sup>de</sup>  | 53.1±1.2 <sup>b</sup>   | 10.0±1.0 <sup>f</sup> | 24.9±1.3 <sup>e</sup>  | 4267±304 <sup>g</sup>      |

Significant differences (p<0.05) between potato types and temperatures are indicated by different letters.

A higher frying temperature causes further degradation of the surface of the potato structure, favoring oil absorption. It also favors a more rapid removal of water, enhances the absorption of oil during cooling, and leads to a faster degradation of the frying medium producing the formation of surface-active substances promoting an increase in the oil uptake. The frozen par-fried potatoes had an initial fat content of 6.0 g/100 g, and showed a markedly higher final fat content than fresh potatoes under the same frying conditions. Color changes in French fries during frying caused by the Maillard reaction depend on factors such as the content of reducing sugars, the temperature and the frying time (MÁRQUEZ and AÑÓN, 1986). The L\* and a\* coordinates are color parameters that best reflect this darkening (PEDRESCHI *et al.*, 2006) and therefore show greater differences between samples. The L\* decreases when increasing the frying temperature of fresh and frozen par-fried potatoes. For the same initial temperature, fresh potatoes are lighter than frozen par-fried potatoes. As a result of frying, the a\* coordinate value increases from negative values in fresh potatoes at the lowest initial temperature, 160 °C, (-1.7±0.8), to values which become greater when increasing the frying temperature. These changes in the L\* and a\* coordinates occur as a result of the Maillard reaction, generating compounds that give brownish tones. The rate at which the Maillard reaction occurs is strongly dependent on temperature and the concentration of the compounds involved (PEDRESCHI *et al.*, 2006). The frozen par-fried potatoes previously acquire some color in the industrial pre-frying process which could explain the lower value of L\* and higher a\* for the same frying conditions. French fries fried with the temperature control activated had a lower a\* and a higher L\* than those fried without controlled temperature. Numerous studies suggest a link between acrylamide formation and the development of non-enzymatic browning during frying (PEDRESCHI and MOYANO, 2005; PEDRESCHI *et al.*, 2006; ROMANI *et al.*, 2009). This is a result of the good correlations between changes in the color parameters L\* and a\* and the concentration of acrylamide. The concentrations of

acrylamide in French fries under different conditions are shown in Table 1. The color changes show that the acrylamide content increases with temperature. This is consistent with data found in the literature (GERTZ and KLOSTERMANN, 2002; RYDBERG *et al.*, 2003; PEDRESCHI *et al.*, 2006; MIAO *et al.*, 2014). Furthermore, it can be observed that the temperature control reduces the formation of acrylamide. GERTZ and KLOSTERMANN (2002), FISELIER *et al.* (2006), and MATTHÄUS (2009) noted the importance of using efficient temperature control fryers to ensure the recovery of the initial oil temperature after adding the food to achieve the formation of a crispy crust on the fried product and to avoid overheating during frying that could produce an increase in the formation of acrylamide. In all cases, frozen par-fried potatoes had higher acrylamide contents than the corresponding fresh potatoes.

### 3.3. Pre-treatments

Pre-treatment by soaking 20 min in tap water or in acidified water (vinegar pH= 3.17) had no impact on the weight loss or oil uptake, but it had an effect on the color of the potatoes; they were paler and less brown (higher  $L^*$  and lower  $a^*$ ), especially when the initial frying temperature was 200 °C, as shown in Table 2. Acidification of the soaking water exerted a positive effect with respect to the coordinate  $L^*$ ; therefore, the French fries were slightly brighter. In the control and the pre-treated samples, the  $a^*$  increased with the temperature. However, soaking in acidified water reduced the development of brownish tones as the  $a^*$  color coordinate was lower than in the control sample. Other authors have found the same trend after soaking in an aqueous NaCl solution (PEDRESCHI *et al.*, 2007; SANTIS *et al.*, 2007). This reduction of the coordinate  $a^*$  could be due to a more limited development of brownish compounds caused by the lixiviation of the precursors of the Maillard reaction, reducing sugars and asparagine in the soaking water (MÁRQUEZ and AÑÓN, 1986). The pretreatments influenced the concentration of acrylamide in the French fries (Table 2). The pre-soaked samples had a lower concentration of acrylamide at all frying temperatures. The results also indicate that the addition of vinegar to the soaking water makes this pretreatment more effective when potatoes are fried at the initial temperatures of 160 and 180 °C. At 200 °C, acidification of the soaking water did not reduce the concentration of acrylamide compared to the sample soaked in water at a higher pH. Soaking produced a reduction in the acrylamide content of between 23% and 50%. The lower the temperature, the greater the relative reduction. With the pretreatment with acidified water, the acrylamide content was reduced up to 76%.

**Table 2.** Influence of soaking on the physico-chemical data, fat and acrylamide contents ( $\mu\text{g kg}^{-1}$ ) of potatoes fried with temperature control.

| $T_i$ (°C) | Pre-treatment        | Weight loss (%)         | Color                  |                       | Fat content (g/100 g) | Acrylamide content ( $\mu\text{g/kg}$ ) |
|------------|----------------------|-------------------------|------------------------|-----------------------|-----------------------|-----------------------------------------|
|            |                      |                         | $L^*$                  | $a^*$                 |                       |                                         |
| 160        | Without soaking      | 32.6±2.9 <sup>a</sup>   | 68.8±0.5 <sup>d</sup>  | -2.1±0.5 <sup>a</sup> | 6.3±1.5 <sup>ab</sup> | 412±34 <sup>c</sup>                     |
|            | Water bath           | 33.6±2.3 <sup>a</sup>   | 72.2±3.7 <sup>d</sup>  | -2.2±0.3 <sup>a</sup> | 5.1±0.6 <sup>a</sup>  | 206±23 <sup>b</sup>                     |
|            | Acidified water bath | 33.1±3.4 <sup>a</sup>   | 73.1±0.7 <sup>d</sup>  | -2.2±1.0 <sup>a</sup> | 5.2±1.2 <sup>a</sup>  | 160±12 <sup>a</sup>                     |
| 180        | Without soaking      | 42.0±0.3 <sup>b</sup>   | 60.0±3.1 <sup>b</sup>  | 2.3±0.3 <sup>b</sup>  | 6.4±1.4 <sup>ab</sup> | 1109±134 <sup>e</sup>                   |
|            | Water bath           | 42.6±3.0 <sup>b</sup>   | 64.0±3.0 <sup>bc</sup> | 1.9±0.4 <sup>b</sup>  | 7.1±0.1 <sup>bc</sup> | 734±58 <sup>d</sup>                     |
|            | Acidified water bath | 45.2±2.7 <sup>bcd</sup> | 66.0±2.0 <sup>c</sup>  | 1.8±0.5 <sup>b</sup>  | 7.2±0.9 <sup>bc</sup> | 267±43 <sup>b</sup>                     |
| 200        | Without soaking      | 52.4±4.1 <sup>de</sup>  | 54.6±0.9 <sup>a</sup>  | 5.5±0.4 <sup>d</sup>  | 6.9±0.7 <sup>bc</sup> | 3056±155 <sup>h</sup>                   |
|            | Water bath           | 50.0±2.3 <sup>cd</sup>  | 58.9±1.7 <sup>b</sup>  | 5.6±0.9 <sup>d</sup>  | 7.1±0.5 <sup>bc</sup> | 2348±134 <sup>f</sup>                   |
|            | Acidified water bath | 50.±2.0 <sup>cd</sup>   | 61.3±3.0 <sup>bc</sup> | 4.0±0.7 <sup>c</sup>  | 6.9±0.2 <sup>bc</sup> | 2457±113 <sup>fg</sup>                  |

There are significant differences ( $P < 0.05$ ) between values with different letters within the same column.

Some authors, such as PEDRESCHI *et al.* (2004) and MESTDAGH *et al.* (2008), have noted that reducing sugars by blanching or soaking prior to frying reduces acrylamide levels up to 60% in potatoes. Furthermore, PEDRESCHI *et al.* (2007) found that the reduction in the acrylamide concentration was related to the soaking time. Compared to the control, they found a reduction of 15% after 60 min and 30% after 120 min of soaking. Acidifying the soaking water with citric acid further reduced the acrylamide in the potato slices, especially at lower temperature frying (PEDRESCHI *et al.*, 2004). This is consistent with the results of the vinegar addition in the present study. JUNG *et al.* (2003) found the same trend in potatoes pretreated in a solution of citric acid, achieving a 25% reduction in the acrylamide concentration in French fries. They attributed this decline to the decrease in the pH as well as the leaching of free asparagine and reducing sugars from the surface of the potatoes into the solution. The formation of acrylamide is strongly dependent on the pH (RYDBERG *et al.*, 2003).

### 3.4. Alternative cooking methods

Table 3 shows the physico-chemical parameters such as weight loss and color as well as the fat and acrylamide contents of roasted or butter fried potatoes. No significant differences between oven temperatures were obtained ( $P>0.05$ ) for the studied parameters except the acrylamide content. Comparing roasted potatoes with those fried in oil (trial 1), it can be concluded that the roasted potatoes had lower levels of fat than the fried ones and they were less brown. The roasted potatoes had acrylamide values between 213 and 742 mg/kg. The temperature strongly influences the acrylamide concentration, as the concentration increased with the oven temperature. However, in this case there was no correlation with the color development, as there was no significant difference in the  $L^*$  or  $a^*$  coordinates with the temperature. Even at the highest roasting temperature (180 °C), the acrylamide formation was lower than that generated when frying with oil temperature control at the same initial temperature (1632 mg/kg). Butter fried potatoes showed significant differences in the color coordinate  $a^*$  and in the acrylamide content depending on whether or not the temperature control was used. Thus, good temperature control markedly diminished the acrylamide content (71% reduction) and reduced the appearance of the brown tones while maintaining the other parameters such as weight loss and fat content. The high concentration of acrylamide in butter fried potatoes, even at a lower temperature than usual for frying (140 °C), could be explained by the reaction between butter, sugars and amino acids such as lysine (URIBARRI *et al.*, 2010; NIQUET-LERIDON *et al.*, 2015).

**Table 3.** Physico-chemical data, fat and acrylamide contents of roasted and butter fried potatoes.

| Cooking condition            | Weight loss (%)        | Color                 |                       | Fat content (g/100 g)   | Acrylamide content (mg/kg) |
|------------------------------|------------------------|-----------------------|-----------------------|-------------------------|----------------------------|
|                              |                        | $L^*$                 | $a^*$                 |                         |                            |
| Roasted                      |                        |                       |                       |                         |                            |
| 170 °C                       | 47.0±2.4 <sup>b</sup>  | 63.0±1.3 <sup>a</sup> | 2.1±0.6 <sup>ab</sup> | 11.2±0.5 <sup>c</sup>   | 213±60 <sup>a</sup>        |
| 175 °C                       | 47.6±1.4 <sup>b</sup>  | 63.1±4.4 <sup>a</sup> | 2.6±1.4 <sup>bc</sup> | 10.2±1.4 <sup>bc</sup>  | 625±45 <sup>c</sup>        |
| 180 °C                       | 48.4±1.4 <sup>b</sup>  | 64.9±2.0 <sup>a</sup> | 1.9±0.7 <sup>ab</sup> | 11.4±1.1 <sup>abc</sup> | 742±47 <sup>d</sup>        |
| Butter fried at $T_f=140$ °C |                        |                       |                       |                         |                            |
| Without control              | 44.9±1.1 <sup>a</sup>  | 63.3±4.0 <sup>a</sup> | 3.3±1.2 <sup>c</sup>  | 10.4±1.3 <sup>bc</sup>  | 1123±80 <sup>e</sup>       |
| With control                 | 45.3±2.0 <sup>ab</sup> | 64.7±6.1 <sup>a</sup> | 2.3±0.6 <sup>ab</sup> | 9.4±1.1 <sup>ab</sup>   | 323±43 <sup>b</sup>        |

Significant differences ( $p<0.05$ ) between cooking conditions and temperatures are indicated by different letters.

## 4. CONCLUSIONS

The results of this study indicate that it is important to include a system of temperature control in domestic cooking equipment to optimize culinary quality while at the same time mitigating the acrylamide content. Such control prevents overheating during frying and enables the formation of acrylamide to be limited in domestic preparations. During frying, potatoes lose more water and capture more oil under higher temperature conditions. Potatoes prepared at higher temperatures become darker as they develop more brown colors. Frying at controlled and moderate temperatures (180 °C or less) reduces the acrylamide concentration of potatoes up to 46% with respect to potatoes fried without temperature control. The reduction improves to 76% if the potatoes are previously soaked in acidified water (pH = 3.17 for 20 min). The formation of acrylamide varies greatly depending on the method of cooking the potatoes. For the same temperature, roasted potatoes contained less acrylamide than fried potatoes. Not only is the temperature control an important factor in acrylamide formation during frying, but the type of frying fat is also critical. The dissociation of the lactose present in butter into reduced sugars is suggested as an explanation for this high concentration in butter fried potatoes. In addition, frying with butter must be performed at moderate temperatures because of its lower smoke point. The substitution of butter with a mixture of sunflower oil and butter could be a solution to obtain a higher smoke point. Frozen par-fried potatoes gained more fat, developed a darker color and contained more acrylamide than fresh potatoes when prepared under the same conditions; therefore, frozen par-fried potatoes should be prepared at lower heating conditions than those selected for fresh potatoes.

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# EFFECT OF STORAGE ON THE CONTENT OF SELECTED ANTIOXIDANTS AND QUALITY ATTRIBUTES IN CONVECTION AND FREEZE-DRIED PEARS (*PYRUS COMMUNIS* L.)

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## ABSTRACT

Fresh, convection dried and freeze-dried pears were examined for selected quality parameters - vitamin C and E, total polyphenols, antioxidant activity, rehydration, and colour. Both products were analyzed immediately after drying and after long-term (12 months) storage at  $2\pm1$  °C and  $20\pm2$  °C. Retention in freeze-dried pears was superior to that in convection-dried products for vitamins and was similar for polyphenols and antioxidant activity. There were no significant differences in lightness between convection and freeze-dried products, either immediately after drying or throughout the storage period. 12-month storage led to a significant increase in the proportion of yellow color in both types of dried product compared to the raw material, and compared with the product after drying. The differences were significant in most cases except for the convection dried pear kept in cold store.

*Keywords:* fruit, drying, storing, antioxidants, rehydration, colour

## 1. INTRODUCTION

Fruits are recognized as a good or very good source of antioxidants in the human diet. These substances form a large group, which comprises polyphenols, vitamins, carotenoids and many others. Medical studies have shown a correlation between the consumption of antioxidants and decreased risk of cardiovascular disease and some cancer types (LILA, 2004; JOHN *et al.*, 1996; OLLSON *et al.*, 2004). In view of the seasonal availability most of the fresh fruits, there is a need to find relatively inexpensive methods of preservation that will give products with a similar nutritive value to that of the raw material.

Although dried fruits have long been a part of the human diet, there is little in the literature on the levels of antioxidant compounds they contain, not excluding even the popular fruit. One such species known is pear *Pyrus communis* (SANSAVINI, 2002). Pears are a good source of many valuable nutrients (CHEN *et al.*, 2007; KOMES *et al.*, 2013). It is a typical fruit of temperate zones. Due to its nutritive values and organoleptic properties, the pear is popular fruit among consumers. It is consumed as fresh fruit but also is popular as processed products, and it is used in juices, nectars, marmalades and purees, dried product, milk products (PARK *et al.*, 2003). Drying fruits allows their preservation by removing most of the free water content, and thus inhibiting microbial and fruit own enzymes activity. Dehydration also reduces the weight and volume of the raw material. This method gives the benefit due to the cost of packaging, transport and storage (BRENNAN and LANCASTER, 1994; GUINÉ and CASTRO, 2003).

Convection drying (using air circulation) is more widely used in industrial processing than freeze-drying due the high costs of the latter, both in terms of equipment and the process itself. Although convection drying is a cheaper process, the resulting product is less abundant in nutritive compounds and more difficult to rehydrate owing to the higher drying temperature and intensive aeration of the material among other factors (MICHALCZYK *et al.*, 2008). Apart from the drying method applied, the quality of the dried product may also be affected by the conditions and length of storage, two factors which have received little attention in the literature.

The aim of this paper was, therefore, to compare convection dried and freeze-dried pears in terms of the selected quality parameters, antioxidants, rehydration and colour, in each product and the extent to which quality is affected by the conditions and length of storage.

## 2. MATERIAL AND METHODS

### 2.1. Material

The experimental material consisted of whole and sound pears of the Conference cultivar, of uniform size gathered at consumption maturity. Fruits were obtained from the orchard experimental station of the University of Agriculture in Cracow (Garlica Murowana, Cracow district, 50°08'23.3N, 19°55'45.6E). Healthy and shaped fruit with a weight of 150.0-180.0 g were washed, peeled, removed the seeds, and sliced into eighths. Peeled and sliced pears were blanched in water containing 0.1 % sodium metabisulfite and 0.5% citric acid. Blanching time required to inactivate the peroxidase was 60 seconds at a temperature of 96-98 °C. After blanching the material is cooled by spraying cold water and allowed sieves for 30 minutes to drain any residual water and dried in a stream of air. Representative samples were then taken to determine the level of the selected indicators in the raw material. The remaining fruits were divided into two batches, one each for convection (CD) and freeze-drying (FD).

For convection drying, electric dryers designed for drying fruits, vegetables and mushrooms (Zorpot Zalmet. Poland) were used. The process was carried out at 60 °C for 10 hours to a moisture content of about 10%. For freeze-drying, pears were first frozen at -40 °C in a Feutron 3626-51 (ILKA Feutron, Germany) fast freezing chamber (KORUS, 2012). Next, sublimation was performed using a Gamma 1-16 LSC freeze dryer (Christ, Germany). The process was conducted under the following parameters: initial temperature of the frozen raw material: -30 °C; condenser temperature: -52 °C, shelf temperature: +20 °C; duration of secondary drying: 6 hours; shelf temperature: +30 °C. The overall time required to achieve a water content of less than 3% using this method was 20 hours.

Immediately after drying, the pears in each separate type of dried product (convection and freeze-dried) were thoroughly mixed, placed in airtight plastic containers, left for 7 days to allow for any equilibration of humidity, and mixed once more. Next, the containers were opened in conditions of low humidity (< 40%) in order to collect samples for analysis of indicators of chemical composition and to determine rehydration ability at the stage described in this work as "immediately after drying - 0 months storage". The remaining dried product was then packed in a twist off jars, divided into two groups and stored without exposure to light. One group was placed in chilled storage (2±1 °C) and the other stored at room temperature (20±2 °C).

## 2.1. Chemical analysis and colour evaluation

The content of vitamin C, E, total polyphenols and antioxidant activity were determined in the raw material, and in products immediately after drying and after 4, 8 and 12 months of storage. Additionally, rehydration ability and colour were determined immediately after drying and again after 12-month storage. Water content was established by the oven method (AOAC, 1984), vitamin C and E content using high-performance liquid chromatography (HPLC) (PN-EN, 2003; PN-EN, 2002). Total polyphenols were determined by the Folin-Ciocalteu spectrophotometric method (SINGLETON *et al.*, 1999) while total antioxidant activity was measured by means of the DPPH (2,2-diphenyl-1-picrylhydrazyl) (PEKKARINEN *et al.* 1999). Immediately after production and after 12-month storage, dried products were also examined for water absorption ability (PN, 1990) as well as for colour by an instrumental method with a Minolta CM-3500d spectroscope setting L\*a\*b\* parameters. Analyses were made in four replications. The results were statistically evaluated using single-factor analysis of variance and LSD test (Statistica v. 12, StatSoft, Inc.). The standard deviation was calculated for the results obtained.

## 3. RESULTS AND DISCUSSIONS

Antioxidant levels in fresh fruits, including pears, have been discussed in the literature (PRIOR *et al.*, 1998; OMS-OLIU *et al.*, 2008; MARKOWSKI *et al.*, 2012). However, there are few works concerned exclusively with preserved products, including dried fruits (CHONG *et al.*, 2013; VEGA-GÁLVEZ *et al.*, 2012). Vitamin C, regarded as a fundamental antioxidant in fruits (SANTOS and SILVA, 2008), is susceptible to degradation by high pH, increased temperatures, exposure to light and the presence of oxygen, enzymes and such metals as iron and copper (MOSER and BENDICH, 1991). It has been observed that good L-ascorbic acid retention during technological treatment is accompanied by similar retention of other nutritive compounds (SANTOS and SILVA, 2008). The level of vitamin C may, therefore, be an indicator of the degradation of other biologically active substances.

Fresh pears contained 41.7 mg vitamin C/100 g dry matter (6.7 mg/100 g fresh matter) (Table 1). Similar values, less than 10 mg/100 g FM, gives SILVA *et al.* (2010) and TAVARINI *et al.* (2010), but OZTURKA *et al.* (2015) found in different cultivars of pears 9-30 mg/100 g FM.

**Table 1.** Effect of drying methods and storage temperature on the nutrient content in the dried pears.

| Object                                        |      | Vitamin C<br>[mg/100 g dry<br>matter] |          | Vitamin E<br>[mg/100 g dry matter] |           | Total polyphenol<br>[mg/100 g dry<br>matter] |        | Antioxidant activity<br>[µM Trolox /1g dry<br>matter] |      |
|-----------------------------------------------|------|---------------------------------------|----------|------------------------------------|-----------|----------------------------------------------|--------|-------------------------------------------------------|------|
| Raw material                                  |      | 41.7±1.9                              |          | 0.94±0.03                          |           | 597±27                                       |        | 100±3                                                 |      |
| Dried fruits, time and temperature of storage |      |                                       |          |                                    |           |                                              |        |                                                       |      |
| [months]                                      | [°C] | CD                                    | FD       | CD                                 | FD        | CD                                           | FD     | CD                                                    | FD   |
| 0                                             | -    | 12.9±0.7                              | 18.5±0.9 | 0.50±0.03                          | 0.79±0.05 | 528±20                                       | 555±21 | 71±3                                                  | 91±4 |
| 4                                             | 2±1  | 11.6±0.5                              | 17.6±0.8 | 0.31±0.02                          | 0.51±0.01 | 505±18                                       | 527±22 | 61±3                                                  | 61±4 |
|                                               | 20±2 | 10.7±0.5                              | 16.2±0.6 | 0.25±0.02                          | 0.48±0.01 | 486±19                                       | 501±21 | 54±3                                                  | 56±4 |
| 8                                             | 2±1  | 10.7±0.5                              | 16.6±0.6 | 0.44±0.01                          | 0.42±0.03 | 485±24                                       | 494±18 | 57±4                                                  | 61±3 |
|                                               | 20±2 | 9.9±0.5                               | 14.5±0.7 | 0.20±0.03                          | 0.34±0.01 | 472±21                                       | 453±22 | 50±3                                                  | 49±2 |
| 12                                            | 2±1  | 10.3±0.4                              | 15.6±0.4 | 0.22±0.01                          | 0.36±0.02 | 463±16                                       | 473±17 | 54±3                                                  | 56±3 |
|                                               | 20±2 | 9.6±0.6                               | 12.5±0.7 | 0.14±0.01                          | 0.27±0.01 | 439±16                                       | 427±18 | 45±2                                                  | 45±3 |
| LSD (α = 0.05)                                |      | 1.10                                  |          | 0.031                              |           | 28.8                                         |        | 4.5                                                   |      |

CD - convention drying, FD - freeze-drying.

Drying caused significant vitamin C loss in both convection and freeze dried pears: 69% and 56% respectively. This confirms the earlier findings for strawberry and American cultivars of blackberry, in which freeze-drying resulted in better L-ascorbic acid retention than other drying methods. This being attributed to lack of oxygen and lower temperature of the process (ASAMI *et al.*, 2003). Reduction of vitamin C losses can be achieved by using neutral gas instead of air in the convection drying (RAMESH *et al.*, 1999). Vitamin C content fell steadily throughout the 12 month period of storage at both storage temperatures. Although at every stage of evaluation. The freeze-dried product contained significantly more vitamin C than convection dried. In addition, vitamin C levels were higher in products stored at the lower temperature. After 12 months of storage vitamin C retention, compared with the raw material, was 23-25% in the convection dried product and 30-37% in the freeze-dried product; and 74-79% and 68-84% respectively compared with the product immediately after drying (the two values refer to the higher and lower storage temperature respectively).

Vitamin E, which comprises a number of tocopherol- and tocotrienol-derived compounds, is subject to degradation from exposure to oxygen and UV radiation and the presence of iron (LIN *et al.*, 2006). Vitamin E content in fresh pears was 0.94 mg/100g dry matter (0.157 mg/100 g FM) (Table 1). According to LIN *et al.* (2006), the edible part of the pears had about 0.2 mg vitamin E per 100 g FM.

The drying process caused significant though but moderate losses in vitamin E content compared with the raw material: 47% in the convection dried product and 26% in the freeze-dried product. Examination of vitamin content in apricots after microwave and radiation drying showed that the shorter exposure to high temperature in microwave drying resulted in better vitamin E retention (KARATAS and KAMIŞLI, 2007). DAOOD *et al.* (1996) comparing natural drying of paprika under ambient conditions with forced-air

dehydration, showed that the former method led to higher losses of  $\alpha$ -tocopherol. Vitamin E loss after 12 months' storage was significant; their levels in convection and freeze-dried products were 15-22% and 28-38% respectively of those found in the raw material and 28-44% and 34-46% of those in the product immediately after drying (the two values refer to higher and lower storage temperature respectively). Industrially dried peaches, pears, and plums contained respectively 75, 76, and 48% of the vitamin E levels in fresh fruits, although there is no information concerning the conditions and length of storage (CHUN *et al.*, 2007). In the convection dried and comminuted paprika observed falls in  $\alpha$ -tocopherol content were 70%, 90% and 100% in products stored for 30, 60 and 90 days respectively (DAOOD *et al.*, 1996).

Polyphenols form one of the principal groups of plant secondary metabolites. Pear fruits are characterized by moderate polyphenol content (NACZK and SHAHIDI, 2006). In fresh pears total polyphenols amounted to 597 mg/100 g dry matter (96 mg/100 g FM). The content of this substances can vary over a wide range, for example, catechin can range from 40-544 mg/kg FM although considerably lower levels of 525 mg/100 g and 429 mg/100 g FM (OZTURKA *et al.*, 2015). Total phenols can vary from 30 mg in Italian Coscia cultivar (TAVARINI *et al.*, 2010) up to 232 mg/100 g FM in unidentified Thai cultivar of *Pyrus pyrifolia* (CHONG *et al.*, 2013).

Convection and freeze-drying caused moderate though still significant reductions in polyphenol content of 47 and 26% respectively compared with the raw material. CHONG *et al.* (2013) reported losses in dried pears of 13-66%, depends on the used drying methods. Further slight losses in total polyphenols were observed throughout the 12-month storage period, becoming significant after 8 months. The effect of both the drying method and lower temperature was not always proved statistically. After 12 months' storage polyphenol retention in convection and freeze-dried products was 74-78% and 72-79% respectively compared with the raw material, and 82-88% and 77-85% compared with the product immediately after drying (the two values refer to storage at  $20 \pm 2$  °C and  $2 \pm 1$  °C respectively).

The level of antioxidant activity depends on the fruit species, cultivation conditions, the length of storage and method of measurement (CONNOR *et al.*, 2002; KALT *et al.*, 1999). Antioxidant activity in fresh pears was 100  $\mu$ M Trolox eq/1 g dry matter (16.1  $\mu$ M Trolox eq/1 g FM). CHONG *et al.* (2013) using an identical method, reported a value of 16.6  $\mu$ M Trolox eq/g, while KEVERS *et al.* (2011) who applied the oxygen radical absorbance capacity (ORAC) method, recorded 27.5  $\mu$ M Trolox eq/g in an extract of Conference pear, and 14.6-42.5  $\mu$ M Trolox eq/g FM for five other cultivars. Convection drying and freeze-drying caused 29 and 9% reductions in antioxidant activity. Storage of products, however, led to larger losses, becoming significant after 4 and 12 months in air-dried product and after first 4 and 8 months in freeze-dried ones. Antioxidant activity was not significantly higher in freeze-dried than in convection-dried products at all stages of storage experiment. The lower storage temperature was found to have a beneficial effect. After 12 months of storage, antioxidant activity in convection and freeze-dried products was lower by 46-55 %, and by 44-55% compared to the raw material, and by 63-76% and by 49-62% compared to the product immediately after drying (the two values refer to storage at  $20 \pm 2$  °C and  $2 \pm 1$  °C respectively).

The content of vitamin C and polyphenols in fresh berry fruits was positively correlated with the level of antioxidant activity (CONNOR *et al.*, 2002; KALT *et al.*, 1999; KEVERS *et al.*, 2007). In comparison with other fruit species, extracts of pears had moderate amounts of polyphenols and lower amounts of vitamin C (GARCIA-ALONSO *et al.*, 2004). Hence, WANG *et al.* (1996) reported that vitamin C did not account for more than 15% of total antioxidant activity. Our results showed that for dried pear products stored for 12 months the correlation coefficients calculated between antioxidant activity and polyphenols, and

vitamin C were 0.89 and 0.80 respectively, regardless of the drying method or storage temperature.

The water content in the raw material affects the yield of the dried product, its quality and tendency to go mouldy. Fresh pears contained 83.97 g water per 100 g. Convection drying removed water to the level of 9.13 g/100 g immediately after production. In freeze-drying, the respective value was 2.89 g/100 g. Therefore, both products conformed to the methodical assumptions of this research with water contents after 12-month storage of 9.58-9.66 g in the convection dried product and 2.95-2.97 g/100 g in the freeze-dried product. Good rehydration properties are an essential characteristic of quality in dried products (RATTI, 2001). When apples, bananas, carrots, and potatoes were dried using five different methods, freeze-drying resulted in the highest porosity and natural drying in the lowest [KROKIDA and MAROULIS, 1997]. This statement agrees with our observations because the higher water absorption ability of FD pears could be explained, above all, by higher porosity. Immediately after drying, 100 g of convection dried pears absorbed 360 cm<sup>3</sup> of water, while freeze-dried ones absorbed 19% more (Table 2).

This tendency remained unchanged after 12 months of storage. Although absorption power decreased by 12-14% and 9-11% in convection and freeze-dried products respectively (the two values refer to storage at 20±2 °C and 2±1 °C respectively).

**Table 2.** The ability of water absorption by dried pears immediately after drying and after 12 months of storage, ml/100 g dried fruits.

| Dried material after storage time [months] | Storage temperature [°C] | Convention drying | Freeze-drying |
|--------------------------------------------|--------------------------|-------------------|---------------|
| 0                                          | -                        | 360±12            | 428±10        |
| 12                                         | 2±1                      | 318±13            | 398±8         |
|                                            | 20±2                     | 308±10            | 380±8         |
| LSD ( $\alpha = 0.05$ )                    |                          | 12.3              |               |

Colour is a crucial factor determining the sensory attractiveness of fruits. Changes in colour may indicate deterioration in the quality of a product due to processing and storage. Colour is determined by the presence of natural pigments and the degree of their decomposition as well as the interactions and degradation of other components in fruit, which occur, for example, during the process of enzymatic and non-enzymatic browning (CHONG *et al.*, 2013; PASŁAWSKA, 2005). In the present work, the colour of the raw material and dried products was determined according to the CIE (L\*a\*b\*) system (Table 3). The drying process caused significant increase of lightness (the L\* value increased by 15-17% in convection and freeze-dried pears). Only freeze-drying resulted in a significant change in a\* value, i.e., the decrease in the red colour, as compared to the raw material. This occurrence can be explained by the use of blanching before drying. This operation in aqueous solution could cause rinsing of the ingredients responsible for a\* parameter in fresh fruits, e.g., water-soluble polyphenols. Then, low FD drying temperatures and lack of oxidation enzymes in the blanched material caused that the darkening no took place. In turn, the CD pears were dehydrated at a temperature that could induce Maillard reactions (VEGA-GALVEZ *et al.*, 2012) and thus increase a\*. The above explanation also seems to confirm minor changes in b\*. Positive values of b\* parameter (b\* > 0) correspond to the yellow colour formed mainly by water-insoluble carotenoids (GUINÉ and BARROCA, 2012). However, it should be noted that the values of parameters a\* and b\* were small, with a\* close to zero and parameter L\* was over 80, which translated into the colour of

fresh and dried pears similar to white or cream-white. Conversely, the  $b^*$  value determined in the convection dried product was 14% higher than in the raw material, while in the freeze-dried product this difference was insignificant.

**Table 3.** Effect of drying method and storage temperature on changes of colour parameters  $L^*a^*b^*$  in the dried pears.

| Object                                        |        | L*         |            | a*         |            | b*         |            |
|-----------------------------------------------|--------|------------|------------|------------|------------|------------|------------|
| Raw material                                  |        | 76.66±4.14 |            | 4.46±1.31  |            | 17.49±2.04 |            |
| Dried fruits, time and temperature of storage |        |            |            |            |            |            |            |
| [months]                                      | [°C]   | CD         | FD         | CD         | FD         | CD         | FD         |
| 0                                             |        | 88.01±4.50 | 89.92±4.72 | 3.46±0.19  | -0.52±0.19 | 20.05±2.23 | 18.03±1.88 |
| 12                                            | 2 ± 1  | 85.97±4.09 | 80.98±3.98 | -1.97±0.09 | 0.96±0.08  | 19.74±1.95 | 20.59±2.24 |
|                                               | 20 ± 2 | 85.23±4.17 | 80.59±4.02 | -1.38±0.07 | 0.96±0.09  | 21.55±2.37 | 20.31±2.21 |
| LSD (α = 0.05)                                |        | 4.511      |            | 0.561      |            | 1.193      |            |

CD - convention drying,. FD - freeze-drying.

## 4. CONCLUSIONS

Retention rates in dried products stored for 12 months were similar for vitamin C and vitamin E, 23-37% and 15-38% respectively, and over 70% total polyphenols. Retention rates for antioxidant activity against the DPPH radical were between these values, 45-56%. Retention in freeze-dried products was superior to that in convection-dried products for both vitamins and was similar for total polyphenols and total antioxidant activity. In addition, retention rates were almost ever significantly higher at the lower storage temperature. Average losses of vitamin C and total polyphenols were higher during drying than over the 12-month storage period, while for vitamin E and antioxidant activity the losses were lower, slightly in the case of the former, and distinctly so for the latter. There were no significant differences in  $L^*$  value between convection and freeze-dried products, either immediately after drying or throughout the storage period. 12-month storage led to a significant increase in the proportion of yellow colour in both types of dried product compared to the raw material; however, compared with the product immediately after drying, the differences found were significant in most cases except for the convection dried product kept in chilled storage.

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## QUALITY AND SENSORY PROFILE OF ULTRASOUND-TREATED BEEF

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### ABSTRACT

The effects of high-intensity ultrasound treatment on beef (*M. longissimus dorsi*) quality and sensory attributes were evaluated. Ultrasound treatment (40 kHz, 11 Wcm<sup>-2</sup>) was applied for 60 min. Control and ultrasound-treated samples were stored at 4°C and evaluated at 0, 7, and 14 days. After 14 days of storage, lipid oxidation of the ultrasound-treated samples increased ( $p < 0.0089$ ), shear force decreased ( $p < 0.0001$ ), and the treated meat was perceived as more tender and juicy. The application of ultrasound increased perception of tenderness without changing other sensory attributes.

*Keywords:* lipid oxidation, meat aging, meat tenderness, sensory attributes, ultrasonic treatment

## 1. INTRODUCTION

Various technological alternatives have been explored to enable minimally processed meat preservation, including novel thermal and nonthermal processing tools that have been successfully applied throughout the food supply chain (DEMIRDÖVEN and BAYSAL, 2009) without affecting the functional or sensory properties of fresh meat and meat products. Sensory attributes are important quality factors in the meat industry and are responsible for consumers' meat choices (MANDOUR *et al.*, 2014). For this reason, methods are needed to ensure the safety, nutritional, and sensory qualities of meat.

The use of ultrasound technology in meat processing is emerging (GALLEGO-JUÁREZ, 2010; CHEMAT *et al.*, 2011). Ultrasound is an acoustic energy, and is considered mechanical, nonionizing, and nonpolluting (ÜNVER, 2016) with great potential for use in high-quality food production processes. Ultrasound changes the physical, chemical, and functional properties (TEREFE *et al.*, 2016) of food products; can therefore, influence the quality of various food systems (KENTISH and FENG, 2014). Low intensity ultrasound has been used to evaluate the composition of meat, fish, and poultry products through food quality analysis (KNORR *et al.*, 2004) but also it has been reported as successful in the processes of mass transfer (CÁRCEL *et al.*, 2007), marination, softening, and inactivation of microorganisms (ÜNVER, 2016). Ultrasound is an alternative to traditional meat aging methods for the tenderization and improvement of meat quality. Exposure to high-intensity ultrasound can induce tenderness due to the cavitation effects that weaken the cell structure, release lysosomes and proteases, and cause protein denaturation (SIRÓ *et al.*, 2009). The muscle tissue can be weakened to increase meat tenderness (STADNIK and DOLATOWSKI, 2011; HAI-JUN *et al.*, 2012). Therefore, the aging period can also be reduced while preserving the quality parameters of meat (DOLATOWSKI *et al.*, 2007) without compromising the oxidative stability of meat (STADNIK *et al.*, 2008). However, this method must be developed further before it can be considered for industry-wide use. To date, no study has examined changes in the sensory properties of fresh or aged meat caused by high-intensity ultrasound. Thus, the aim of this study was to evaluate the effects of high-intensity ultrasound treatment on sensory quality, texture, and lipid oxidation (LO) of beef stored at 4°C.

## 2. MATERIALS AND METHODS

### 2.1. Meat and sample preparation

The samples for all experiments were beef from *M. Longissimus dorsi* (Hereford), obtained from a local supplier 2 days *post mortem* and then vacuum-packed. Muscles were stored at 4°C for 24 h prior to treatment. The pH of the meat was between 5.6-5.9. Visible fat was manually removed from each muscle prior to treatment. Samples were sliced similarly in terms of weight and size (130 × 90 × 25 mm, length × width × height). The location of the sample was randomly assigned to each treatment and a new muscle was used for each experimental replication. A total of 12 replicates were used.

### 2.2. Treatments

Samples were designated as; control (C) and ultrasound-treated (U). Based on the storage length (0, 7, or 14 days at 4°C), samples were further identified as; C<sub>0</sub>, C<sub>7</sub>, and C<sub>14</sub> and U<sub>0</sub>, U<sub>7</sub>, and U<sub>14</sub>, respectively. Ultrasound treatment (40 kHz, 11 Wcm<sup>-2</sup>) was applied to the U samples at the end of each storage period. The samples were treated for 60 min (30

min/side) in a modified-intensity ultrasonic bath (Branson® 1510 model 1510R-MTH; Branson Ultrasonics Corporation, Danbury, CT, USA) using distilled water as the diffusion medium. The effective power of the ultrasound system was determined using a calorimetric technique previously described (MARGULIS and MARGULIS, 2003). Temperature was kept constant at 4°C and the intensity was modified to obtain 11 Wcm<sup>-2</sup>. After sonication (or no treatment), meat was vacuum packed and prepared for analysis.

### 2.3. Shear force measurements

Shear force (SF) was measured using the method outlined by MAHER *et al.* (2004); the samples were placed in airtight plastic bags and cooked in a water bath (Isotemp 215; Fisher Scientific, Pittsburgh, PA, USA) until the temperature at the geometrical center of the sample reached 72°C. Cooked samples were tempered at room temperature and cooled at 4°C overnight, then drained and stored at 4°C for 24 h. After this period, 1 cm diameter cylinders were cut in the muscle parallel to the fibers using a punch. Cored samples were sheared using a TA-XT2i (Stable Micro Systems, Surrey, UK) with a V-shaped blade (Warner-Bratzler meat shear-compression) attached to a 100 N load cell and a crosshead speed of 200 mm min<sup>-1</sup>. Average values of 8 replicates for each sample were performed and the SF values were reported as Newtons.

### 2.4. Lipid oxidation measurement

The degree of lipid oxidation (LO) was determined by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) according to the technique described by PICCINI *et al.* (1986). Ten grams of muscle were homogenized (ESGE Bio Homogenizer model M133/1281-0; Bio Spec products Inc., Bartlesville, OK, USA) with a 10% solution of 6 N HCl for 40 s, the resulting suspension was subjected to distillation and 50 ml aliquots were collected. Afterwards a 2.5 ml of distillate was taken and mixed with 2.5 mL TBA at 0.02 M TBA. The mixture was incubated in a boiling water bath (100°C) for 40 min. A sample containing 2.5 mL of distilled water and 2.5 mL of TBA was used as a blank. Both were cooled for 10 min in running tap water and absorbance was measured at 535 nm on a spectrophotometer (Genesys 20, model 4001/4; Thermo Spectronic, Waltham, MA, USA). The results were plotted against a standard curve prepared with known concentrations of tetraethoxypropane. This determination was performed in triplicate and the results expressed as mg of malondialdehyde (MDA) per kg of meat (mg MDA/kg meat).

### 2.5. Sensory evaluation

#### 2.5.1 Selection and training of panellists

Twelve panellists were recruited and trained using the quantitative descriptive analysis technique described by STONE *et al.* (2004). The panellists were selected by the basic taste test. In the second stage of selection, the Farnsworth-Munsell 100 Hue test for color sensitivity and test was used for taste, using the triangular test (International Organization for Standardization, ISO, 8586-1, 1993). Total training duration was 80 h, training included familiarization with relevant descriptive terms and ways of perceiving the selection and quantification of the sensory characteristics of cooked meat as well as the use of intensity scales ISO 4121 (2003). Representative samples were offered to the panel to determine relevant attributes. For the evaluation of appearance (i.e., color), a modified version of the AMSA (2012) protocol was used. Meat color was evaluated using reference scales. The panellists then generated individual lists of descriptors for each sensory characteristic (i.e.,

odor, appearance, flavor and texture) to characterize the meat samples (ISO 8586-2, 1994). Next, consensus lists of five descriptors per sensory modality were created and these terms were used for preparation of a lexicon that was used for further panellist training. The selected attributes were: whitish, pink, grayish, light-brown, and pale appearance; raw meat, grilled meat, fresh-cooked meat, boiled meat, and metallic odors; fresh bovine cooked meat, greasy, dry meat, bovine meat, and metallic flavors; soft, juicy, fibrous, tough, and elastic textures. The descriptor intensities per attribute were evaluated on a 10 cm linear scale with two anchor points. The final lexicon terms (descriptors) and definitions used to train the panellists are shown in Table 1.

**Table 1.** Lexicon developed (descriptors that characterize a beef sample) by panellists and used in the evaluated quantitative descriptive analysis.

| Attribute  | Descriptor               | Definition                                                                                                                           |
|------------|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| Appearance | Whitish                  | Perception of greater amount of white light on the surface of the meat.                                                              |
|            | Pink                     | Pale shade of red.                                                                                                                   |
|            | Grayish                  | Meat with less intense hue and brown tone.                                                                                           |
|            | Light-brown              | Brown hue reflecting more light.                                                                                                     |
|            | Pale                     | Meat color is observed to be less saturated.                                                                                         |
| Odor       | Raw meat                 | Amount of beef odor in the sample; beef identity.                                                                                    |
|            | Grilled meat             | Full aromatic generally associated with beef suet that has been grilled.                                                             |
|            | Fresh-cooked meat        | Odor or note of aromatic fresh-cooked beef.                                                                                          |
|            | Boiled meat              | Aromatic notes associated with boiled meat or soup stock.                                                                            |
|            | Metallic                 | Aromatics associated with impression of slightly oxidized metal.                                                                     |
| Flavor     | Metallic                 | Taste associated with undercooked meat (bloody taste).                                                                               |
|            | Fresh bovine cooked meat | Taste characteristic of all meat, the aromatics associated commonly in partially cooked meat.                                        |
|            | Greasy                   | Flavor associated with fat heated to a high temperature.                                                                             |
|            | Dry meat                 | Flavor associated with meat that is overcooked and charred on the outside.                                                           |
|            | Bovine meat              | The aromatics commonly associated with matured cooked beef muscle products (boiled beef broth).                                      |
| Texture    | Soft                     | Describes beef meat that is easy to bite between the teeth (low hardness).                                                           |
|            | Juicy                    | Perception of the amount of water released by the product during the first bites.                                                    |
|            | Fibrous                  | Indicate that the orientation of particles in meat beef is similar to that perceived in celery.                                      |
|            | Tough                    | The number of chews required to masticate beef meat into a state ready for swallowing is similar to that necessary for old cow meat. |
|            | Elastic                  | Describes the rapidity of recovery from a deforming force.                                                                           |

Sources: AMSA, 1995; BYRNE *et al.*, 2001; NOLLET and TOLDRA, 2011.

Panellists' performance was evaluated by applying a test of homogeneity of variances using the PROC GLM procedure in the SAS statistical package (SAS Institute, Cary, NC, USA). Consistency among the panel on sensory modalities was statistically significant for appearance (color) ( $p < 0.0001$ ), odor ( $p < 0.05$ ), flavor ( $p < 0.0001$ ), and texture ( $p < 0.0001$ ). The coefficient of the descriptors was estimated using XLSTAT-Sensory software (version 2015.6.01.25740; Addinsoft, Paris, France).

### 2.5.2 Sensory test

Samples were cooked in an oblong electric skillet (The West Bend Company, West Bend, WI, USA) to an internal temperature of 72 °C, following AMSA (1995)-established methods. Samples were cut into six equal pieces and maintained at 35°C until sensory analysis ( $\leq 30$  min). The test was conducted under white light. Panelists were instructed to cleanse their palates with water between samples. The sensory tests were conducted for the sonicated (U) and untreated (C) samples in three sessions. In each session, panelists received randomly a (30 g) sample from each treatment, identified by a three-digit code. The panelists evaluated the samples using an unstructured 10-cm linear scale (0 = none, 10 = very). Data were recorded (values in cm) as intensity points for each descriptor.

### 2.6. Statistical analyses

The test variables (SF, LO, and sensory attribute intensity) were analyzed using the generalized linear model procedure (SAS software, SAS Institute) and the statistical model

$$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + E_{ijk},$$

where  $Y_{ijk}$  = response variables,  $\mu$  = general average,  $A_i$  = effect of ultrasound treatment,  $B_j$  = effect of storage time,  $(AB)_{ij}$  = effect of interaction between ultrasound treatment and storage time, and  $E_{ijk}$  = random error. When the effect of a factor or interaction on one or more variables was significant ( $p \leq 0.05$ ), Tukey's statistical test was performed to compare the averages. Analysis of variance was also performed to determine the discriminant power of the descriptors and their estimated coefficients, using the XLSTAT-Sensory software package (version 2015.6.01.25740; Addinsoft).

## 3. RESULTS AND DISCUSSIONS

### 3.1. Shear force

SF differed significantly between treatments and storage periods ( $p < 0.0001$ ; Fig. 1). SF values were higher on day 0 of storage and declined significantly by day 14 for both U and C samples. U samples had significantly reduced SF ( $p < 0.0001$ ) compared to the C samples, which showed higher SF at all storage times. These results corroborate those reported previously (JAYASOORIYA *et al.*, 2007; ZHOU *et al.*, 2010). STADNIK and DOLATOWSKI (2011) highlighted the potential of using low-frequency and low-intensity *postmortem*. They found reduced meat toughness at 48 and 72 h postmortem. The effect of high-intensity ultrasound on SF reduction has also been reported for the following parameters: 24 kHz and 12 Wcm<sup>-2</sup> for 4 min in bovine meat (JAYASOORIYA *et al.*, 2007), 24 kHz and 12 Wcm<sup>-2</sup> for 4 min in poultry after 7 days of storage (XIONG *et al.*, 2012), and 2.5-3 Wcm<sup>-2</sup> for 180 min in pork (SIRÓ *et al.*, 2009). SIKES *et al.* (2014) also observed a reduction in SF with aging at 4°C for 7 days ( $p < 0.001$ ), but no interaction between ultrasound treatment and storage. Other results have differed; as no effect was observed on SF with 62 Wcm<sup>-2</sup> (LYNG *et al.*, 1998), 22 Wcm<sup>-2</sup> (POHLMAN *et al.*, 1997a), or 4-19 Wcm<sup>-2</sup> (MCDONNELL *et al.*, 2014), although ultrasound treatment decreased gumminess and cohesiveness of salted pork in the latter study.

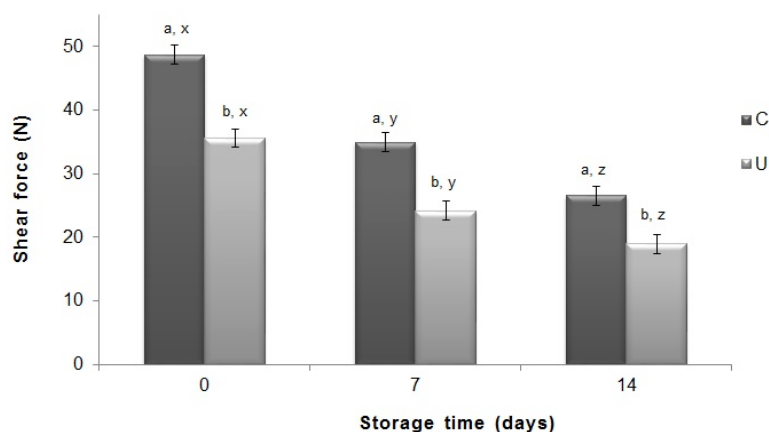


**Figure 1.** Effects of treatment and storage time on instrumental texture, measured as shear force (N) for bovine *M. Longissimus dorsi* treated and not treated with ultrasound (40 kHz, 11 Wcm<sup>-2</sup>) and stored at 4°C for 0, 7, or 14 days (mean ± standard error bars). C= control (no ultrasound); U= ultrasound-treated.

<sup>a, b</sup> Different letters indicate significant differences with ultrasound ( $p < 0.0001$ ).

<sup>x, y, z</sup> Different letters indicate significant differences between storage time ( $p < 0.0001$ ).

Ultrasound treatment affects meat tenderization via acoustic cavitation, as bubble formation, growth, and eventual collapse have thermal, chemical, and mechanical effects (YUSAF and AL-JUBOORI, 2014). Asymmetric collapse causes an eruption of fluid, producing a microburst affecting the integrity of muscle structure (BHASKARACHARYA *et al.*, 2009). This process is associated with *postmortem* hydrolysis of myofibrillar proteins in the aging stage, which leads to greater meat tenderization (GEESINK *et al.*, 2001) and explains the SF reduction observed in the present study. Likewise, depending on ultrasound frequency, alternating positive and negative pressures are produced, causing expansion or compression and resulting in cell rupture. This process also causes water hydrolysis (AWAD *et al.*, 2012), leading to the formation of chemically active free radicals (H and OH), which intervene in the structural stability and catalytic functions of proteins. Thus, ultrasound treatment may modify the availability of adenosine triphosphate in the *pre-rigor* muscle (SIKES *et al.*, 2014), which also accelerates the start of *rigor mortis* (DOLATOWSKI *et al.*, 2004; STADNIK and DOLATOWSKI, 2011) and therefore increases the aging rate of meat (CHANDRAPALA, 2015).

### 3.2. Lipid oxidation

The degree of LO in the samples differed significantly according to the interaction of treatment and storage factors ( $p < 0.01$ ; Fig. 2). Both ultrasound and control meat presented lower lipid oxidation at day 0 of storage and these values increased significantly after 14 days of storage in treated samples ( $p < 0.01$ ). The degree of LO in all samples fell below the rancidity threshold of 1-2 mg MDA/kg (VIEIRA *et al.*, 2009) and was also lower than the oxidation odor detection threshold (0.5-1 mg MDA/kg) (TARLADGIS *et al.*, 1960). These results agree with those reported by STADNIK (2009), who obtained TBARS values that indicated no compromise to the oxidative stability of ultrasound-treated (45 kHz, 2 Wcm<sup>-2</sup> for 120 s) meat samples stored under refrigeration. Ultrasound breaks down cell membranes, fragments collagen, denatures proteins by bubble pulsation and cavitation, and promotes the formation of free radicals (KUIJPERS *et al.*, 2002). Consequently, it intensifies meat oxidation by increasing the speed of chemical reactions (AWAD *et al.*,

2012). Furthermore, aging represents a change in both structures and chemical composition of beef. For example, free radicals are produced during aging, mainly from metal release. Furthermore, fat and fat-like membrane molecules are degraded to fatty acids during aging (DASHDORJ *et al.*, 2016). These two factors together may explain why ultrasonicated meat is slightly more oxidized after 14 days of storage. Possibly, polyunsaturated fatty acids (PUFAs) released from phospholipids (membranes) during aging become more exposed to released free radicals during sonication (i.e., iron), interacting more rapidly during the same processes. Since lipid peroxidation is more strongly influenced by oxidation of membrane components such as PUFAs (FAUSTMAN *et al.*, 2010), the exposition of these fatty acids could be responsible for the slight increase in sonicated meat. However, the values obtained from treated samples in our study indicated minimal changes in LO during storage.

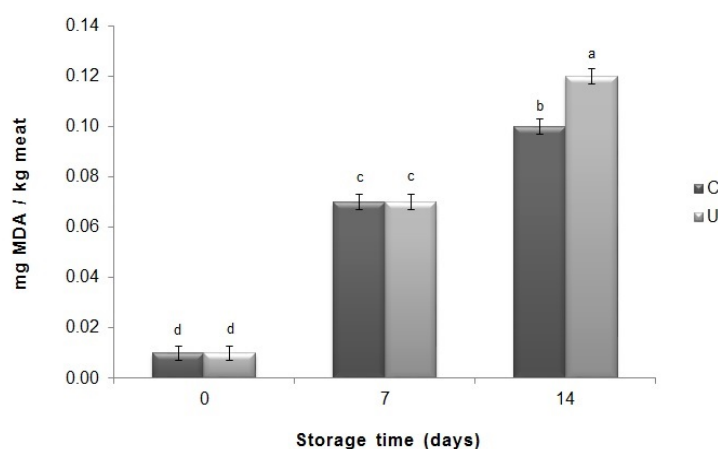


**Figure 2.** Effects of treatment and storage times on the lipid oxidation index (mg MDA/kg of meat) for bovine *M. Longissimus dorsi* treated and not treated with ultrasound (40 kHz, 11 Wcm<sup>-2</sup>) and stored at 4°C for 0, 7 or 14 days (mean ± standard error bars). C= control (no ultrasound); U= ultrasound-treated.

<sup>a, b, c</sup> Different letters indicate significant differences by interaction treatment of ultrasound and storage time ( $p < 0.0089$ ).

### 3.3. Sensory properties

The effects of ultrasound treatment and storage on odor and flavor characteristics differed significantly with an interaction between these factors ( $p < 0.01$  and  $p < 0.0001$ , respectively; Fig. 3A and 3C). After 7 and 14 days of storage, untreated samples had a more intense odor and flavor (raw meat odor,  $p < 0.0001$ ; fresh-cooked meat odor  $p < 0.0006$ ; and fresh bovine cooked meat flavors,  $p < 0.0001$ ) than meat without storage, but also a more intense pleasant boiled meat odor ( $p < 0.0001$ ) compared to samples treated with ultrasound. Ultrasound treatment also increased the perception of unpleasant greasy flavor ( $p < 0.0034$ ), which was more noticeable after storage for 14 days ( $p < 0.0001$ ).

The untreated samples were perceived as less greasy on day 0 ( $p < 0.0001$ ) maybe because of the structural damage or the liberation of cooked-meat flavor precursor lipids. Metallic flavor, dry meat flavor, and metallic odor showed no significant difference according to storage period and treatment. The intensity of fresh-cooked meat odor was lower after storage for 7 days in ultrasound-treated samples ( $p < 0.0001$ ), which may be due to the concentration of volatile compounds (aromatic molecules) that may be lower during this

period. STETZER *et al.* (2007, 2008) reported that positive flavor compounds decrease with aging (between 7 and 14 days of storage) and negative compounds increase. Pentanal and 3-hydroxy-2-butanone decrease with aging while nonanal, butanoic acid and 1-octene-3-ol increase. Both sonicated and untreated meat showed more whitish and pink colors at 14 days of storage ( $p < 0.0001$ ; Fig. 3B) compared to treated and untreated samples in other periods of storage.

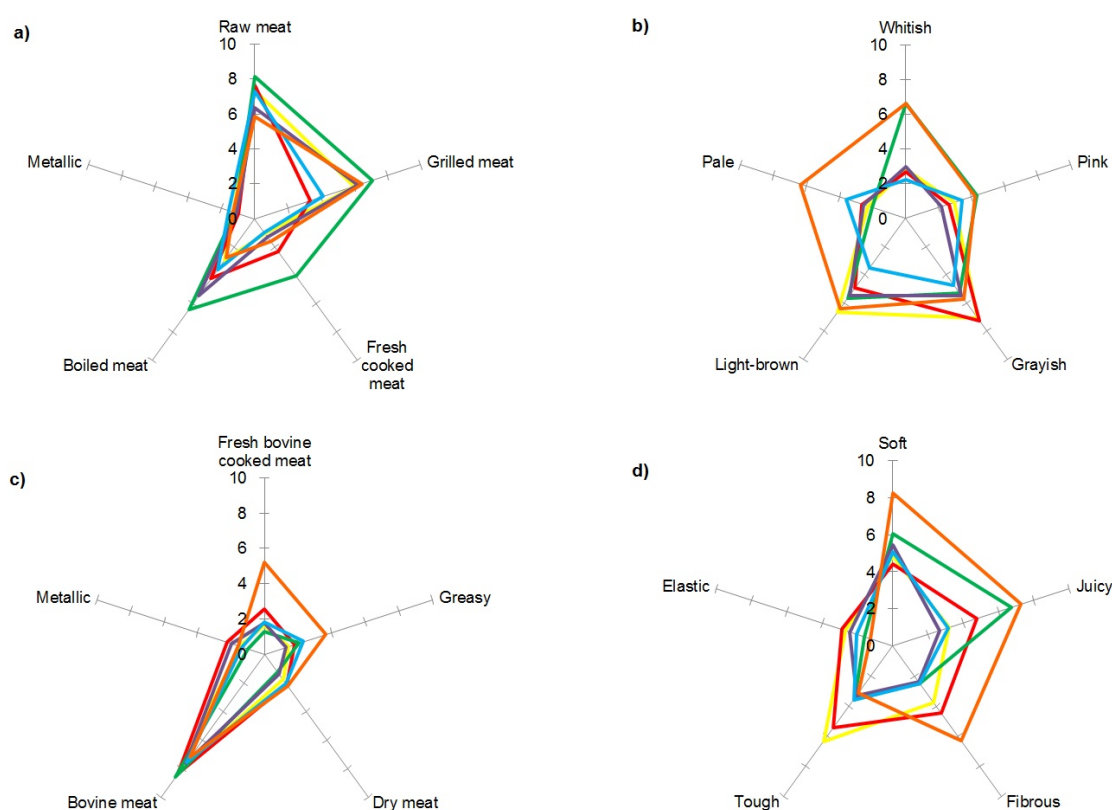


**Figure 3.** Quality descriptors for bovine *M. Longissimus dorsi* with and without ultrasound treatment (40 kHz, 11 Wcm<sup>-2</sup>) after storage at 4 °C for 0, 7 and 14 days. a) Odor descriptors, b) Color descriptors, c) Flavor descriptors, d) Texture descriptors. C<sub>0</sub>= control (not ultrasound, yellow); 0 days of storage; C<sub>7</sub>= control (not ultrasound, red); 7 days of storage; C<sub>14</sub>= control (not ultrasound, green); 14 days of storage; U<sub>0</sub>= ultrasound, 0 day of storage, (purple); U<sub>7</sub>= ultrasound, 7 days of storage, (blue); U<sub>14</sub>= ultrasound, 14 days of storage, (orange).

Untreated meat tended to have a grayish color, with significant interaction observed for 0 and 7 days of storage ( $p < 0.0001$ ). On day 0, untreated meat had a more intense light-brown color ( $p < 0.0002$ ) than sonicated meat; contrarily, the lowest intensity of this attribute was observed in ultrasound-treated samples after 7 days of storage. The palest color was registered for ultrasound-treated meat at 14 days of storage ( $p < 0.0001$ ). This may be related to the results obtained by JAYASOORIYA *et al.* (2007) and HAI-HUN *et al.* (2012), who indicated that ultrasound application generates an increase in muscle temperature. Therefore, the thermal denaturation and oxidation of the meat pigments could affect the color of the meat, making it paler and less red.

The ultrasound-treated meat stored for 14 days was the softest and juiciest of all samples ( $p < 0.0111$  and  $p < 0.004$ , respectively; Fig. 3C), but it had a more fibrous texture, associated with lower SF values. Meat not treated with ultrasound and stored for 7 days had the most elasticity ( $p < 0.0058$ ). Untreated meat on day 0 of storage had the toughest perceived texture, in agreement with the instrumental texture results (greater SF value). These results coincide with LYNNG *et al.* (1998) who indicated that lamb treated with ultrasound and storage for 7 days was perceived as softer probably associated with the process of proteolysis during storage and the cavitation effect of ultrasound. In contrast, POHLMAN *et al.* (1997b) reported no effect of treatment with ultrasound and storage time on bovine *M. pectoralis* because of the greater presence of connective tissue. It has been asserted (DOLATOWSKI *et al.*, 2007; STADNIK and DOLATOWSKI, 2011) that a softer meat texture after ultrasound can be explained by the physical weakening of the muscular structure, affecting the cellular membranes by accelerating proteolysis and releasing cathepsins from the lysosomes and/or calcium ions of the intracellular storage. The descriptors with the strongest discriminating factors for sample characterization were texture attributes, with the exception of untreated meat after 14 days of storage and ultrasound-treated meat after 7 days of storage (Fig 4).

Human perception is conditioned by the sensory interaction of physical processes, such as chewing; thus, sensory properties are linked to physical characteristics (CAINE *et al.*, 2003) and ultrasound wave propagation in meat depends on meat properties (DAMEZ and CLERJON, 2008). The results of the present study show that exposure to high-intensity ultrasound increases meat tenderness, as perceived by trained panellists who characterized the ultrasound-treated sample that had been stored for 14 days as the most tender. Sensory attributes resulting from proteolysis, such as odor, flavor, tenderness, and juiciness became evident due to the aging process, as the storage period increased. Ultrasound treatment resulted in additional softness and juiciness effects over the storage period. These panel results and SF values are similar to those obtained in other studies. POHLMAN *et al.* (1997a) conducted a sensory analysis of beef samples (*M. pectoralis* and *M. Longissimus thoracis*) subjected to ultrasound aging (20 kHz, 1000 Wcm<sup>-2</sup>) or cooked by convection, and found increased myofibrillar tenderness ( $p < 0.05$ ) and reduced flavor intensity in ultrasound-treated samples. Muscle treated with ultrasound had greater postcooking moisture, but no difference in juiciness was observed; the quantity of connective tissue and tenderness in general were unaffected by the aging method. LYNNG *et al.* (1998) reported that sensory evaluation of bovine *M. Longissimus thoracis*, *M. lumbarum*, and *M. semimembranosus* treated with ultrasound (20 kHz and 62 Wcm<sup>-2</sup> for 15 s) showed no difference in tenderness, general texture, or global acceptance after 0, 3 or 14 days of storage. However, they found that storage time significantly improved chewability. In spite of the difficulties with comparing different experiments due to differences in frequency/intensity/time combinations of the ultrasound applied to meat, the majority of studies describe the favorable effects of ultrasound on meat texture (ALARCON-ROJO *et al.*, 2015) and that effect has been corroborated herein.

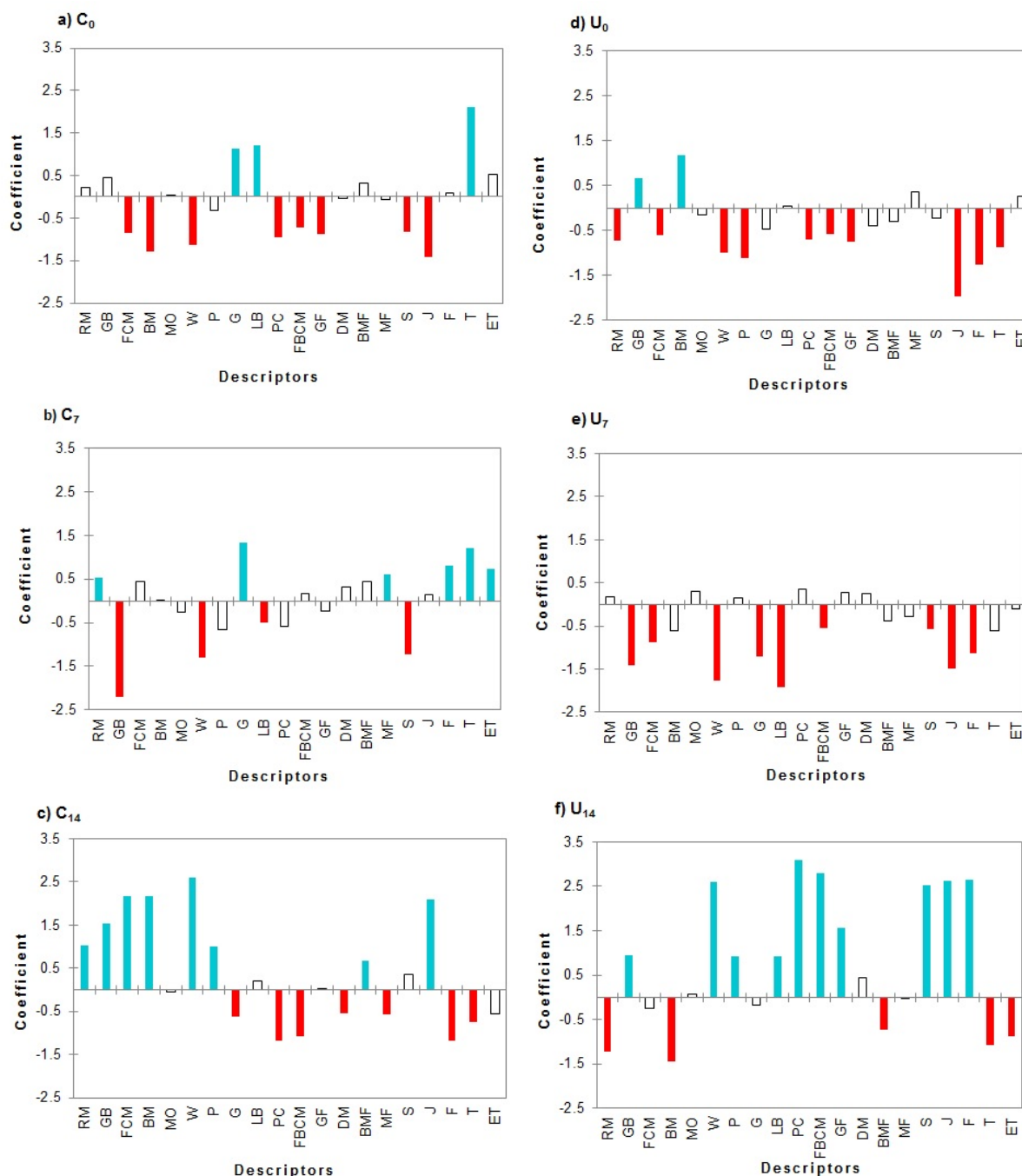


**Figure 4.** Estimated coefficient of descriptors for bovine *M. longissimus dorsi* with ultrasound treatment after storage at 4 °C for 0, 7, or 14 days. (confidence interval 95 % model  $Y = P + J$ ). C<sub>0</sub> = control, 0 days of storage; C<sub>7</sub> = control, 7 days of storage; C<sub>14</sub> = control, 14 days of storage; U<sub>0</sub> = ultrasound, 0 days of storage; U<sub>7</sub> = ultrasound, 7 days of storage; U<sub>14</sub> = ultrasound, 14 days of storage. RM= raw meat; GB= grilled beef; FCM= fresh-cooked meat; BM= boiled meat; MO= metallic odors; W= whitish; P= pink; G= grayish; LB= light-brown; PC= pale color; FBCM= fresh bovine cooked meat; GF= greasy flavor; DM= dry meat; BMF= bovine meat flavor; MF= metallic flavor; S= soft; J= juicy; F= fibrous; T= tough; ET= elastic texture.

## 4. CONCLUSIONS

High-intensity ultrasound reduces the Warner-Bratzler SF of beef, and although meat LO increases, it does not negatively affect the quality. Thus, ultrasound application may be a feasible way to preserve the sensory properties of meat while significantly reducing aging time. Ultrasound technology can be applied to improve meat texture, as confirmed by our finding that high-intensity ultrasound increased meat tenderness. In this context, factors related to muscle (species, gender, age, diet or muscle type) and those related to ultrasound (frequency, intensity, time or ultrasound system) should be considered. Results of sensory analyses indicate that ultrasound does not change panellists' perception of beef quality. These findings should be complemented by consumer evaluation to rule out any detriment to meat quality.

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## CHLORINE DIOXIDE GAS TREATMENT ON POSTHARVEST QUALITY OF RASPBERRY

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### ABSTRACT

The study investigated the effects of chlorine dioxide ( $\text{ClO}_2$ ) gas on the postharvest quality of raspberries (cv Grandeur) during storage. Weight loss, color, total soluble solids content (TSSC), titratable acidity (TA), pH, vitamin C, total phenols, anthocyanins and antioxidant capacity were evaluated. The  $\text{ClO}_2$  positively influenced weight loss, color, TA, TSSC and antioxidant capacity. Moreover,  $\text{ClO}_2$  treatment decreased the total yeast and mold count. In contrast, the vitamin C, anthocyanins and total phenolics were not influenced by the  $\text{ClO}_2$  treatment.

*Keywords:* chlorine dioxide, fruit quality, pad, postharvest, red raspberries, storage

## 1. INTRODUCTION

Red raspberries (*Rubus idaeus* L.) are rich in ascorbic acid, total phenols and anthocyanins. Therefore, consumption of these berries is an important source of antioxidant compounds with a significant role in the preservation and promotion of health, such as preventing diabetes, cardiovascular risk factors and oxidative stress (ATIENZA *et al.*, 2015; SEN and CHAKRABORTY, 2016). Red raspberry is a highly perishable product in which modification of quality compounds are related to the growth of microorganisms, and natural decay (GIACALONE and CHIABRANDO, 2012). Raspberry harvest and storage are complex operations and can affect the quality and aroma compounds of the fruit (GIUGGIOLI *et al.*, 2014). Thus, the raspberry postharvest control strategies are critical to preserving the fruit quality, particularly on a long distribution chain (BRIANO *et al.*, 2015a; HAFFNER *et al.*, 2002). Many technologies, like active packaging, modified atmosphere packaging, edible coating, Difolatan, hexanal, vapor essential oils or sanitizer treatments, such as chlorine dioxide ( $\text{ClO}_2$ ) and ozone have been studied to reduce the growth of microorganism, control postharvest decay and preserve the quality and freshness of berries (APPENDINI and HOTCHKISS, 2002; BRIANO *et al.*, 2015b; CHIABRANDO and GIACALONE, 2008; CHIABRANDO and GIACALONE, 2015a; HAJIZADEH and KAZEMI, 2012; SUN *et al.*, 2014).

Chlorine dioxide is an alternative sanitizer, approved by the U.S. Food and Drug Administration (FDA) and by the U.S. Environmental Protection Agency (EPA).  $\text{ClO}_2$  is legally used in China and USA for sanitizing fruit and vegetables (Ministry of Health of the People's Republic of China, 2008 and USFDA, 2010). Particularly,  $\text{ClO}_2$  is authorized in the US for use in washing, whole fresh fruit, vegetables, shelled beans and peas with intact cuticles at a concentration not exceeding 5ppm. In EU, however, there are no clear regulations regarding the use of  $\text{ClO}_2$  for fresh produce. Therefore, as a provisional solution, it was agreed that the individual member states will be given the ability to establish enforcement levels at the national level until risk management can take place based on European Food Safety Authority (EFSA) scientific opinion and monitoring data (BANACH *et al.*, 2015).  $\text{ClO}_2$  has been postulated as an alternative to sodium hypochlorite ( $\text{NaClO}$ ) for fresh and fresh-cut produce sanitization. CORDIS (Community Research and Development Information Service), designing new decontamination approaches for fresh-cut food and sanitation strategies, include  $\text{ClO}_2$  among the most promising sanitation methods.

In the European Union,  $\text{ClO}_2$  can be used in other forms, like active packaging, as a gas-generating pad (DUKAN *et al.*, 1999). The active substances of the pad are molecules of  $\text{HClO}$  (hypochlorous acid), found in silica gel and toxic to microorganisms. Chlorine dioxide gas is also a great sanitizer for food, at low concentration (CHEN *et al.*, 2010; CHANG *et al.*, 2000). The principal advantage of  $\text{ClO}_2$  gas is its high penetrability (HAN *et al.*, 2001). Different studies (CHEN *et al.*, 2010; CHANG *et al.*, 2000) showed the positive effects of gaseous  $\text{ClO}_2$  on the storage quality of lettuce, mulberries, plums and strawberries.

The objective of this study was to evaluate the impact of  $\text{ClO}_2$  treatment as a pad applied on the top lids of the clamshells, on the postharvest quality, nutraceutical aspects and microbiological decay of raspberries during four different storage conditions.

## 2. MATERIALS AND METHODS

### 2.1. Samples and treatment

Red raspberries (*R. idaeus* L.) cv. Grandeur were handpicked from a commercial orchard of the Agrifrutta Soc. Coop. SRL (Piedmont, Italy) at full ripeness and directly placed in commercial plastic boxes, clamshell type (13.5 x 9.0 x 2.5 cm, perforated, polyethylene terephthalate, 120 g of fruits). The samples were transported immediately to the laboratory of the DISAFA, University of Turin, and only sound fruit was selected for the experiment. The boxes were randomly divided into two groups. On one group, a chlorine dioxide gas-generating pad (CDP) (Oplon Pure Science, Ltd., Ness Tsiyona, Israel) was placed on the top lids of the clamshells (U.S. Food and Drug Administration (FDA)-approved technology). The second group was used as the control. The effect of the CDP on the berries quality and antimicrobial activities during storage were measured. Each treatment included three replicates.

### 2.2. Storage treatments

Samples were stored in the dark in a controlled temperature room, and four different storage conditions were evaluated:

S1: 4 days at 1 °C (short storage)

S2: 8 days at 1 °C (long storage)

S3: 4 days at 1 °C and 3 days at 4 °C (short storage + short-range international transport)

S4: 8 days at 1 °C and 3 days at 4 °C (long storage + short-range international transport)

The hypothesized transport time was 3 days, simulating a refrigerated transport from Italy to Northern Europe.

Three repetitions were performed for all chemical analyses each time.

### 2.3. Weight losses

Weight loss was determined by weighing the numbered samples boxes at the beginning of the experiment (time 0) and at the end of the four different storage conditions. The values were reported as the percentage of weight loss per initial boxes weight, as shown in equation (1).

$$\% \text{ weight losses} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} * 100 \quad (1)$$

### 2.4. Quality measurements

The physicochemical quality attributes of the berries were measured at the beginning of the testing (time 0) and at the end of the four different storage conditions.

#### 2.4.1 Color

The color of the berries was measured using a tristimulus CR-400 Chroma Meter (Konica Minolta Sensing, Inc. Osaka, Japan) with the D65 lamp and 2° observation angle. The instrument was calibrated against a standard white plate ( $Y = 93.7$ ,  $x = 0.3158$ ,  $y = 0.3321$ ) before the analysis. Thirty measurements (30 berries) per treatment and sampling time

were made. The results were expressed as CIELAB ( $L^*a^*b^*$ ) color space. The  $L^*$  values describe the lightness, and  $a^*$  and  $b^*$  values express the red-greenness and blue-yellowness, respectively. The color of berries was also expressed as  $C^*$  (chroma or saturation). This parameter indicates the color variation  $C^* = [(a^*)^2 + (b^*)^2]^{1/2}$  (FRANCIS, 1980). The reported values were the mean  $\pm$  SD of 30 determinations.

#### 2.4.2 Total soluble solid content (TSSC), titratable acidity (TA) and pH

For each treatment, a digital refractometer (Atago refractometer model PR-32; Atago Italia, Milan, Italy) was used to determine the TSSC ( $^{\circ}$ Brix) in three undiluted filtered juice samples, each extracted from 30 berries. The instrument was calibrated against distilled water. The TA and pH were determined by adding 50 mL of distilled water into 10 mL of filtered juice and titrated with 0.1 N NaOH to pH 8.2 with an automatic titrator (Titration Workstation TitraLab AT1000 Series, Hach, Milan, Italy). Titration data were expressed as meq  $L^{-1}$ .

#### 2.5. Extraction and evaluation of total anthocyanins content, total phenolic contents, and total antioxidant capacity

The anthocyanins, phenolics and antioxidant capacity were determined on the fruit extracts, obtained using 12.5 mL of extraction solvent (500 mL of methanol, 28.3 mL nanopure water and 1.4 mL 37% HCl) and 5 g of fresh fruit. After 60 min at 25  $^{\circ}$ C under reduced light conditions, the extracts were homogenized at 24000 rpm for 1 min, with an Ultra-Turrax T-25 tissue homogenizer (Janke and Kunkel, IKA<sup>®</sup>-Labortechnik, Saufen, Germany) and centrifuged at 3000 rpm for 15 min (Centrifuge AVANTITM J-25, Beckman Instruments Inc.). The supernatant was recovered and stored at -26  $^{\circ}$ C. Three replicates for each treatment were performed at day 0 and at the end of the four different storage conditions.

Anthocyanins were determined using the pH differential method of CHENG and BREEN (1991) by measuring the absorbance of the aqueous phase at 515 and 700 nm using a UV-visible spectrophotometer (U-5100, Hitachi, Tokyo, Japan). Anthocyanins were estimated by the difference in absorbance at 515 and at 700 nm in buffer at pH 1.0 and 4.5, where  $A = (A_{515} - A_{700})_{pH1} - (A_{515} - A_{700})_{pH4.5}$ .

Results were expressed as mg cyanidin-3-glucoside per 100 g fresh berries.

Total phenolics contents were quantified using the SLINKARD and SINGLETON protocol (1977) with Folin-Ciocalteu reagent. Absorbance was measured at 765 nm. The results were calculated as gallic acid equivalents (GAE) (mg GAE 100  $g^{-1}$  of fresh berries).

The antioxidant activities of the berries were measured by the ferric reducing antioxidant power assay, as described by BENZIE and STRAIN (1996), with some modifications (PELLEGRINI *et al.*, 2003). Results were expressed as mmol  $Fe^{2+}$   $kg^{-1}$  fresh berries.

#### 2.6. Extraction and evaluation of vitamin C

The vitamin C content was performed in agreement with SANCHEZ-MORENO *et al.* (2003) and GONZALEZ MOLINA *et al.* (2008) at day 0 and after 4 and 8 days of storage. Fruit flesh (10 g) was homogenized in 10 mL of methanol/water (5:95 v/v) using an Ultra-Turrax T-25 for 3 min. Then, the pH was adjusted to 2.2–2.4, and the extract was filtered through a C18 Sep-Pak cartridge (Waters Associates, Milford, MA, USA). The resultant solution was combined with 1,2-phenylenediamine dihydrochloride (Fluka Chemika, Neu-Ulm, Switzerland) for 37 min before HPLC analysis. Three replicate analyses of 10 fruits were performed for each treatment. The chromatographic system (Agilent) was

equipped with a diode array detector and Kinetex-C18 column (4.6 x 150 mm, 5 µm, Phenomenex., Torrance, CA, USA) and controlled through HPLC online software (Agilent). The mobile phase (isocratic) consisted of 50 mM monobasic potassium phosphate and 5 mM cetrimide (Sigma-Aldrich Corporation, Saint Louis, USA) in methanol:water (v/v) 5:95. The flow rate of 0.9 ml/min. The temperature was 40 °C, and the detector was set at 261 nm for ascorbic acid (AA) and 348 nm for dehydroascorbic acid (DHAA). The vitamin C content (AA and DHAA contents) was expressed as mg 100 g<sup>-1</sup> of fresh weight. All standards and reagents were of analytical purity and were purchased from Sigma Italiana SRL (Ozzano Emilia, Italy).

## 2.7. Yeast and mold evaluation

The yeasts and molds content was evaluated at day 0 and after 4 and 8 days of storage as described by the *Compendium of Methods for the Microbiological Examination of Foods* (VANDERZANT and SPLETTSTOESSER, 1992). A 30 g sample of fresh berries was blended with 270 mL of peptone buffered water (Sigma Italiana SRL, Italy) for 1 min in a Stomacher® bag using a blender (Stomacher®400 Circulator, Seward, Worthing, UK). Rose bengal agar (Sigma Italiana SRL, Italy) was used for the yeast and molds evaluations. All the plates were incubated at 30 °C for 5 days. Microbial counts were expressed as log colony forming units (CFU) g<sup>-1</sup>.

## 2.8. Statistical analysis

Analysis of variance (ANOVA) was performed on the data, and the means were compared by Tukey's honestly significant differences test. The source of variation was the treatments (control and CDP) and the storage time. Differences between mean values were considered significant when  $p \leq 0.05$ . SPSS software was used for all data analyses (SPSS Statistics version 22 IBM).

# 3. RESULTS AND DISCUSSIONS

## 3.1. Weight loss

In general, raspberries are affected by considerable weight loss because the tissues are exposed to a high level of respiration and transpiration (KRÜGER *et al.*, 2011). In the present study, low weight losses were observed in all the samples compared to previous studies (KRÜGER *et al.*, 2011; HAFFNER *et al.*, 2009). After 4 days of storage (S1), the weight loss of the CDP samples was 0.71%, which was significantly lower than the control (0.98%). Therefore, in this postharvest storage condition, the CDP positively influenced the weight loss of raspberries, maintaining lower values compared to the control, according to ADAY and CANER (2011). The same result was also obtained in the S3 and S4 storage conditions, with 1.16 and 2.13% weight loss, respectively of the CDP treated fruit, while the corresponding control values were 1.52 and 2.61%. The effect of ClO<sub>2</sub> on the weight loss depends on the inactivity of the microbial population, the inhibition of enzyme activity, such as polyphenol oxidase, and the inhibition of respiration rate and ethylene biosynthesis (ADAY and CANER 2011; GUO *et al.*, 2013; WANG *et al.*, 2011; SUN *et al.*, 2014).

## 3.2. Quality measurements

### 3.2.1 Color

Color change is an important factor that affects the visual appearance and the postharvest quality of fresh raspberry fruit. The  $L^*$  and  $C^*$  values of raspberry after 4 and 8 days of storage are reported in Table 1. The  $L^*$  values after 4 and 8 days of storage showed a significant decrease in both treatments (control and CDP treatment), compared to day 0. Significant differences were observed between treatments after 4 days of storage at 1 °C, with higher values of  $L^*$  in the CDP treated berries (fruit with a higher luminosity). As described by KRÜGER *et al.* (2011) and SHIN *et al.* (2008), a decrease in lightness values during storage, indicates that the fruit became darker, less red and bluer with advance ripening. In the present work, samples treated with the CDP maintained the original color of the berries, in concurrence with the study of ADAY and CANER (2011) on strawberries. The  $ClO_2$  gas did not influence the lightness during the S3 and S4 storage conditions. In these instances, there were no significant differences ( $p \geq 0.05$ ) in  $L^*$  value between the CDP treatment ( $L^*$  27.9 and 29.4 in S3 and S4, respectively) and the control ( $L^*$  28.4 and 29.2 in S3 and S4, respectively).

The  $C^*$  values initially increased and then decreased during storage as shown in Table 1. Considering the short period of storage (S1), results showed significantly higher values for the CDP samples compared to the control. In this instance, the CDP treatment significantly improved the redness intensity of berries during storage. In previous studies, the CDP treatment caused no pigment degradation and consequently, no changes in external color of berries (ZHENG *et al.*, 2008; GOMEZ LOPEZ *et al.*, 2009). The same result was also obtained under the S4 storage condition, where the CDP samples showed  $C^*$  values of 23.3, which was significantly higher compared to the control ( $C^*$  20.8).

**Table 1.** Effect of  $ClO_2$  on color parameters (Lightness and Chroma) of raspberries after 4(S1) and 8(S2) days of storage at +1°C. The data are average of 30 replicates  $\pm$  SD.

| Color Parameters       | Treatments       | Storage time (days)          |                              |                               |
|------------------------|------------------|------------------------------|------------------------------|-------------------------------|
|                        |                  | 0                            | 4                            | 8                             |
| Lightness<br>( $L^*$ ) | Control          | 35.1 $\pm$ 1.9 <sup>aA</sup> | 29.1 $\pm$ 3.0 <sup>bB</sup> | 29.8 $\pm$ 1.74 <sup>aB</sup> |
|                        | Chlorine dioxide | 35.1 $\pm$ 1.9 <sup>aA</sup> | 30.4 $\pm$ 2.2 <sup>aB</sup> | 30.0 $\pm$ 2.24 <sup>aB</sup> |
| Chroma<br>( $C^*$ )    | Control          | 22.7 $\pm$ 4.0 <sup>aB</sup> | 25.9 $\pm$ 4.5 <sup>bA</sup> | 25.4 $\pm$ 4.1 <sup>aA</sup>  |
|                        | Chlorine dioxide | 22.7 $\pm$ 4.0 <sup>aB</sup> | 28.9 $\pm$ 5.3 <sup>aA</sup> | 25.9 $\pm$ 5.7 <sup>aA</sup>  |

### 3.2.2 TSSC, TA and pH

TSSC, TA and pH values after 4 (S1) and 8 (S2) days of storage are shown in Table 2. The TSSC values were significantly different between treatments after 4 and 8 days of storage at +1°C, while the differences were not significant under S3 and S4 conditions (data not shown). The higher TSSC levels in the control than CDP treated berries may be due to the higher respiration rate and weight losses and, consequently, more concentrated juice of the control berries. WU *et al.* (2011) showed that  $ClO_2$  treatment maintained the TSSC similar to the value recorded at harvest, which was better than untreated fruit, probably due to the reduction of postharvest infections.

The TA decreased significantly during storage for both treatments, but without significant differences. The TA values decreased significantly for all samples during storage. In the

control samples, the lowest recorded TA value was under S4 conditions, and in the CDP sample, it was under S3 conditions. The decrease in TA and increase in TSSC during storage were associated with the enhancement of temperature from 1 to 4 °C in S3 and S4 that caused an increase in respiration rate and the natural consumption of organic acids by the metabolism (HAFFNER *et al.*, 2009). The changes in TA probably depended more on temperature than the effect of ClO<sub>2</sub>, according to the study of ADAY and CANER (2011) on strawberries (Table 2).

The pH level increased in the control samples in agreement with the TA result, while in the CDP treated samples no significant differences were observed during storage. ADAY and CANER (2011) reported that ClO<sub>2</sub> treatment maintained stable pH levels in samples during storage probably due to the antimicrobial activity of ClO<sub>2</sub> on yeast and mold, and consequently, to the inhibition of natural decay.

**Table 2.** Effect of ClO<sub>2</sub> on quality parameters of raspberries after 4(S1) and 8(S2) days of storage at + 1°C. Total Soluble Solid Content (TSSC), Titratable Acidity (TA) and pH. The data are average of 3 replicates ± SD.

| Treatments           |                  | Storage time (days)       |                           |                           |
|----------------------|------------------|---------------------------|---------------------------|---------------------------|
|                      |                  | 0                         | 4                         | 8                         |
| <b>TSSC</b><br>°Brix | Control          | 8.4±0.1 <sup>aB</sup>     | 9.0±0.3 <sup>aA</sup>     | 8.6±0.0 <sup>aB</sup>     |
|                      | Chlorine dioxide | 8.4±0.1 <sup>aA</sup>     | 8.3±0.2 <sup>bA</sup>     | 8.1±0.1 <sup>bA</sup>     |
| <b>TA</b><br>meq/L   | Control          | 447.65±17.1 <sup>aA</sup> | 450.98±5.6 <sup>aA</sup>  | 390.83±17.6 <sup>aB</sup> |
|                      | Chlorine dioxide | 447.65±17.1 <sup>aA</sup> | 447.06±12.0 <sup>aA</sup> | 402.03±8.1 <sup>aB</sup>  |
| <b>pH</b>            | Control          | 3.04±0.01 <sup>aB</sup>   | 3.07±0.01 <sup>aAB</sup>  | 3.10±0.04 <sup>aA</sup>   |
|                      | Chlorine dioxide | 3.04±0.01 <sup>aA</sup>   | 3.02±0.01 <sup>bA</sup>   | 3.16±0.12 <sup>aA</sup>   |

Means sharing the same letters in rows (A, B) and in column (a, b) are not significantly different from each other (Tukey's HSD test,  $p \leq 0.05$ ).

### 3.3. Total anthocyanin content

Total anthocyanin content is one of the functional constituents in raspberries that are associated with their bright red color. The content of anthocyanins increased during the storage at 1 °C (S1 and S2) (Table 3) and then decreased during storage at 4°C (S3 and S4). This trend may be due to the decomposition of procyanidins at the beginning of storage (Chun *et al.*, 2013). The results of the present study agree with the research of HAFFNER *et al.* (2002) that reported a similar increase in anthocyanin content after 7 days of cold storage (1.7°C), but contrasts with the study of MULLEN *et al.* (2002) that did not show a significant increase during cold storage. Moreover, the results indicated that the content of anthocyanins increased in the same manner as the pH after 8 days of cold storage at 1 °C. According to ORAK (2007), a correlation exists between anthocyanins and pH in grapes. Hence, the current results suggest a similar trend in raspberries. Considering the treatment, ClO<sub>2</sub> does not seem to directly influence the anthocyanins level because no significant differences between treatments were found.

### 3.4. Total phenolics content

As shown in Table 3, according to the total anthocyanin content results, no significant differences in total phenolic content were observed during storage time or between

treatments. In general, an increase in total phenols was observed throughout the storage. A similar result was also noted previously in raspberries (KRÜGER *et al.*, 2011) in strawberries (CHENG and BREEN, 1991) and in blueberries (CHIABRANDO and GIACALONE, 2015b) and it seems to depend on the weight loss. In this work, the lack of significant difference between the CDP treated and control samples was probably due to the low level of  $\text{ClO}_2$ . GOMEZ LOPEZ *et al.* (2009) and NAPOLITANO *et al.* (2005) found that a high level of  $\text{ClO}_2$  can react with phenolic compounds, decreasing their content in the food. Considering the storage at 4 °C (S3 and S4), the  $\text{ClO}_2$  gas did not influence the total phenolic content. Indeed, there were no significant differences ( $p \geq 0.05$ ) between the CDP treatment (202.24 and 232.26 mg GAE 100 g<sup>-1</sup> in S3 and S4, respectively) and the control (216.31 and 240.95 mg GAE 100 g<sup>-1</sup> in S3 and S4, respectively).

**Table 3.** Effect of  $\text{ClO}_2$  on nutraceutical parameters of raspberries after 4(S1) and 8(S2) days of storage at +1 °C. The data are average of 3 replicates  $\pm$  SD.

| Treatments                                                                              |                  | Storage times (days)            |                                  |                                  |
|-----------------------------------------------------------------------------------------|------------------|---------------------------------|----------------------------------|----------------------------------|
|                                                                                         |                  | 0                               | 4                                | 8                                |
| <b>Total anthocyanin content</b><br>mg cyanidin 3-gluc100 g <sup>-1</sup> FW            | Control          | 43.62 $\pm$ 6.65 <sup>aB</sup>  | 54.5 $\pm$ 5.24 <sup>aAB</sup>   | 78.11 $\pm$ 7.66 <sup>aA</sup>   |
|                                                                                         | Chlorine dioxide | 43.62 $\pm$ 6.65 <sup>aB</sup>  | 52.24 $\pm$ 5.71 <sup>aAB</sup>  | 68.22 $\pm$ 7.47 <sup>aA</sup>   |
| <b>Total phenolics content</b><br>mg gallic acid equivalent (GAE)100 g <sup>-1</sup> FW | Control          | 198.3 $\pm$ 16.55 <sup>aA</sup> | 235.66 $\pm$ 19.44 <sup>aA</sup> | 219.45 $\pm$ 23.41 <sup>aA</sup> |
|                                                                                         | Chlorine dioxide | 198.3 $\pm$ 16.55 <sup>aA</sup> | 217.65 $\pm$ 17.33 <sup>aA</sup> | 204.21 $\pm$ 9.87 <sup>aA</sup>  |
| <b>Total antioxidant capacity</b><br>mmol Fe <sup>2+</sup> kg <sup>-1</sup> FW          | Control          | 27.81 $\pm$ 2.35 <sup>aA</sup>  | 28.93 $\pm$ 0.60 <sup>aA</sup>   | 30.96 $\pm$ 1.25 <sup>aA</sup>   |
|                                                                                         | Chlorine dioxide | 27.8 $\pm$ 2.35 <sup>aA</sup>   | 28.11 $\pm$ 1.42 <sup>aA</sup>   | 28.11 $\pm$ 0.80 <sup>bA</sup>   |
| <b>Vitamin C content</b><br>mg100 g <sup>-1</sup> FW                                    | Control          | 12.45 $\pm$ 0.40 <sup>aA</sup>  | 12.97 $\pm$ 1.09 <sup>aA</sup>   | 9.39 $\pm$ 0.85 <sup>aB</sup>    |
|                                                                                         | Chlorine dioxide | 12.45 $\pm$ 0.40 <sup>aA</sup>  | 12.87 $\pm$ 0.57 <sup>aA</sup>   | 8.76 $\pm$ 1.30 <sup>aB</sup>    |

Means sharing the same letters in rows (A, B) and in column (a, b) are not significantly different from each other (Tukey's HSD test,  $p \leq 0.05$ ).

### 3.5. Total antioxidant capacity

As shown in Table 3, the total antioxidant capacity increased slowly during storage. Statistical analysis showed significant differences between samples after 8 days of storage, with a higher value in the control than CDP treated sample. This result is in accordance with MULLEN *et al.* (2002) and KALT *et al.* (1999). Considering the storage at 4 °C (S3 and S4), the  $\text{ClO}_2$  did not influence the antioxidant capacity and there was no significant difference ( $p \geq 0.05$ ) between the CDP treatment (28.54 and 28.27 mmol Fe<sup>2+</sup> kg<sup>-1</sup> in S3 and S4, respectively) and the control (29.68 and 31.03 mmol Fe<sup>2+</sup> kg<sup>-1</sup> in S3 and S4, respectively).

### 3.6. Vitamin C content

Table 3 presents the vitamin C values of the untreated and treated samples after 4 and 8 days of storage. The vitamin C content decreased over time in both treatments, but without statistical differences between treatments. A similar trend was observed in the study of HAFFNER *et al.* (2002) and KALT *et al.* (1999) during storage at 0 °C. According to KRÜGER *et al.* (2011), the AA content depends particularly on the storage conditions and the genotype of the plant.

### 3.7. Yeast and mold evaluation

Raspberries are highly perishable and susceptible to microbial decay during postharvest storage. Therefore, decay is a primary factor of postharvest quality loss of raspberries, and a strategy is necessary to improve their shelf-life quality. No mold growth was visually observed during the present study. At day 0, the yeast and mold were present in relatively low amounts, at 2.9 and 4.2 log CFU/g<sup>-1</sup>, respectively (Table 4). After 4 and 8 days of storage at 1 °C, a reduced yeast and mold count was observed in the CDP treated samples compared to the control. The decay incidence of the raspberries gradually increased with time of storage only in the control samples. Hence, in this study, the CDP treatment was effective against the growth of yeast and mold throughout the storage period. This result was in agreement with the study of SUN *et al.* (2014) on blueberries. The same trend has also been found in lettuce, carrot, apples, peaches, tomatoes and fresh-cut produce (SY *et al.*, 2005).

**Table 4.** Effect of ClO<sub>2</sub> on nutraceutical parameters of raspberries after 4(S1) and 8(S2) days of storage at +1°C. The data are average of 3 replicates ± SD.

| Treatments                              |                  | Storage time (days) |                    |                    |
|-----------------------------------------|------------------|---------------------|--------------------|--------------------|
|                                         |                  | 0                   | 4                  | 8                  |
| <b>Yeast</b><br>Log CFU g <sup>-1</sup> | Control          | 2.85 <sup>aB</sup>  | 4.88 <sup>aA</sup> | 3.61 <sup>aA</sup> |
|                                         | Chlorine dioxide | 2.85 <sup>aA</sup>  | 2.56 <sup>bA</sup> | 2.60 <sup>bA</sup> |
| <b>Mold</b><br>Log CFU g <sup>-1</sup>  | Control          | 4.20 <sup>aA</sup>  | 4.79 <sup>aA</sup> | 4.41 <sup>aA</sup> |
|                                         | Chlorine dioxide | 4.20 <sup>aA</sup>  | 2.38 <sup>bB</sup> | 2.34 <sup>bB</sup> |

Means sharing the same letters in rows (A, B) and in column (a, b) are not significantly different from each other (Tukey's HSD test,  $p \leq 0.05$ ).

## 4. CONCLUSIONS

The present study showed the action of ClO<sub>2</sub> against the natural decay of raspberries and its efficacy in preserving berry quality under various postharvest storage conditions. Results suggest that ClO<sub>2</sub> treatment in active packaging is useful to reduce decay and maintaining raspberry quality during storage. In particular, ClO<sub>2</sub> slowed down the tissue metabolism and consequently, lower weight losses were found compared to the untreated fruit. Moreover, ClO<sub>2</sub> treatment significantly improved the redness intensity of berries during storage, but no significant effect on maintaining the stability of nutraceutical components was recorded. In summary, the CDP can be a valuable alternative sanitizer with a beneficial action against yeast and mold without reducing the quality of the final produce. This treatment also improved the shelf-life quality by inhibiting the weight loss and the color changes of raspberries during short period storage time.

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# IMPACTS OF PLASTICIZER AND PRE-HEATING CONDITIONS ON PROPERTIES OF BOVINE AND FISH GELATIN FILMS FABRICATED BY THERMO-COMPRESSSION MOLDING TECHNIQUE

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## ABSTRACT

Bovine and fish gelatin films were prepared by thermo-compression molding technique. This study investigated the effects of glycerol levels (15-35%) and resin pre-heating conditions including pre-heating temperatures (120, 140 and 160°C) and times (5 and 10 min) on film properties. Tensile strength (TS), elastic modulus (EM) and yellowness of films decreased, but elongation at break (EAB), water-vapor permeability (WVP) and transparency increased with increasing glycerol level. The gelatin films generally had decreased TS, EM, WVP and transparency, but increased yellowness as the pre-heating temperature and time increased. Therefore, the glycerol level and condition used for resin pre-heating directly influenced the film properties.

*Keywords: compression molding, film, gelatin, glycerol level, pre-heating conditions, WVP*

## 1. INTRODUCTION

Proteins are natural polymers frequently used for preparing bio-degradable/edible films. These films are alternatives to synthetic plastic films, which serve as one of the ways of reducing environmental pollution (GOMEZ-GUILLEN *et al.*, 2009). In general, protein films exhibit excellent barrier properties to oxygen, lipids and aromas, and have been reported to possess moderate mechanical properties, as well as a high nutritional value, but with low water vapor barrier property due to the hydrophilic character of these macromolecules (GENNADIOS, 2002; OU *et al.*, 2004). Protein films can be produced from several protein sources including vegetable proteins (corn zein, wheat gluten, soy protein, peanuts and cottonseed protein) and animal proteins (milk proteins, collagen, gelatin, keratin, egg albumin and myofibrillar protein) (CUQ *et al.*, 1998).

Gelatin is an animal protein derived from the partial hydrolysis of native collagens, which are the most abundant structural proteins found in skins, bones and connective tissues (KARIM and BHAT, 2009). It possesses a good film-forming property and is one of the first materials applied as edible coatings and films (KLOSE *et al.*, 1952; GENNADIOS *et al.*, 1994). Skin and bone from bovine and porcine sources are utilized commercially in gelatin production (JOHNSTON-BANKS, 1990; VENIEN and LEVIEUX, 2005). As a result of religious objections to the consumption of bovine materials and health concerns about the spread of diseases (such as bovine spongiform encephalopathy) to humans, fish gelatin is increasingly gaining attention as an alternative to replace mammalian gelatin (GOMEZ-GUILLEN *et al.*, 2009; KARIM and BHAT, 2009). Among all proteins, gelatin has received considerable attention in the development of edible films owing to its abundance and biodegradability (BIGI *et al.*, 2002; JONGJAREONRAK *et al.*, 2006). In addition, it is unique among hydrocolloids in forming thermo-reversible products with a melting point close to body temperature, which is particularly significant in edible and pharmaceutical applications (NORLAND, 1990; GOMEZ-GUILLEN *et al.*, 2009). With regards to film with many outstanding properties, such as transparency, biodegradability and barrier properties (gases and aroma), gelatin is suitable for application in biodegradable packaging (MARTUCCI and RUSECKAITE, 2009; NAGARAJAN *et al.*, 2015). However, the properties of gelatin films depend on the characteristics of the raw materials and manufacturing processes.

In general, there are two main methods used for preparing protein films: 1) the dry and 2) the wet processes. The wet process or solution casting is the most widely used film-forming method (ZHANG *et al.*, 2007; WANG *et al.*, 2009; LIMPISOPHON *et al.*, 2010). The dry process (or thermal processing method), such as compression molding and extrusion, is based on the thermoplastic properties of proteins when plasticized and heated above their glass transition temperature ( $T_g$ ) under low water content. Heating above  $T_g$  produces soft and rubbery material and may permit their incorporation into specific products. Cooling to room temperature can reconvert rubbery material to glassy materials, giving more or less rigid forms with the desired structure (CUQ *et al.*, 1997). This thermal process may affect film properties differently as compared with the casting method, but it enhances the commercial potential for large-scale production of biodegradable/edible films. Moreover, this method allows a much shorter period of time for film preparation and the use of conventional techniques which are more convenient for industrial applications than casting. Among various thermal processing techniques, thermo-compression molding can be applied for film preparation from different polymers. Although this technique is feasible at the laboratory level, it is not a potential technique for realistic packaging applications. This technique is still widely used especially in preliminary studies to ascertain the feasibility of using thermal processing technique and

to standardize the processing conditions prior to developing such biodegradable film via the continuous thermal processing techniques.

However, there is limited information regarding the preparation and properties of gelatin films fabricated by the thermal method. Among thermal processing methods, compression molding is the most common and simple method of molding, and is typically used to investigate the feasibility of converting any polymer to the desired product via thermoplastic processing. One of the requirements of thermal processing in film making from biopolymers including gelatin, is the possession of a sufficient melt flow under the processing conditions. Gelatin normally involves high molecular interaction, which results in high  $T_g$  and melt viscosity, but low melt flow-ability. As a consequence, the incorporation of a proper plasticizer to gelatin at suitable levels will allow a sufficient flow of the melt, thereby producing the gelatin film by thermal processing. Moreover, the processing conditions of the thermal technique used are also crucial for obtaining a gelatin film with good properties, which may vary based on gelatin type. The compression molding technique generally involves several fundamental steps including pre-heating (without applied pressure), degassing, heating and pressurizing (compressing), as well as cooling. The conditions used in each step have to be properly manipulated.

Therefore, this study was carried out to investigate the feasibility of gelatin film production by thermo-compression molding technique. In particular, the effects of plasticizer (glycerol) level, pre-heating temperature and time of compression molding on film-forming ability, as well as the properties of films from bovine-hide and fish-skin gelatins were studied.

## **2. MATERIALS AND METHODS**

### **2.1. Materials and chemicals**

This experiment utilized commercial bovine-hide gelatin (~240 bloom) and fish-skin gelatin (~240 bloom), purchased from Halamic Company (Bangkok, Thailand) and LAPI GELATINE S.p.A. (Empoli, Italy), respectively. The glycerol (food grade), used as plasticizer being approved as safe by the Food and Drug Administration (FDA), was purchased from Wako Pure Chemical Industry, Ltd., Tokyo, Japan.

### **2.2. Preparation of molding compound resin**

Prior to molding, compound resin based on gelatin and plasticizer, which is used as raw material for compression molding, was prepared. From the preliminary investigations of this study, the compound resin in the pellet form could not be successfully prepared by dry blending or melt compounding using twin-screw extrusion technique. For dry blending, the glycerol could not be well dispersed with gelatin powder and as such, the resin could not flow properly upon heat compression and became easily degraded. For melt blending, the gelatin melt was too viscous and tended to stick to the screw surface and became degraded upon mixing, mainly due to the evaporation of water acting as a plasticizer at high temperature. Thus, the plasticized-gelatin molding compound resin was prepared by solution blending. Molding compound resin (a mixture of gelatin and plasticizer) was prepared according to the method of PARK *et al.* (2008) with some modifications. Bovine or fish gelatin powders were first dissolved in hot (65 °C) deionized water to obtain a protein concentration (AOAC, 2000) of 20% (w/v). Glycerol was added to gelatin solution at different concentrations (15, 25 and 35% of protein, w/w). The film-forming solutions (FFS) were then heated at 90 °C for 2 h in a water bath. Upon heat-

pretreatment, the FFSs were gently stirred. Thereafter, the FFSs were poured onto a stainless tray and partially dried for 12 h at ambient temperature. These semi-dried resins were cut into small pellets ( $\sim 0.5 \times 0.3 \times 0.3$  cm<sup>3</sup>) and further dried in a vacuum oven at 35 °C for 48 h. The obtained resins (Fig. 1) were conditioned at 25 °C and 60% RH in an environmental chamber (TK120, NUVE, Belgium) for 48 h, before being used for the preparation of films via thermo-compression molding. The conditioned resins obtained had a moisture content of  $15 \pm 3\%$ .

## 2.3. Effects of glycerol level on film formation and film properties

### 2.3.1. Film fabrication by thermo-compression molding

The conditioned bovine and fish gelatin resins containing different glycerol levels (about 3 g) were placed between two stainless steel plates (10x10 inch<sup>2</sup>) covered with Mylar sheets. A spacer with a thickness of 0.1 mm was inserted between the plates. The set was inserted between heating platens of the compression molder previously heated to 120 °C. To melt the resin, it was pre-heated without applying pressure at the aforementioned temperature for 10 min. The molten resin was subsequently pressed to form a film in the compression molder at that temperature. A pressure of 20 MPa was applied for 2 min followed by the removal of the set from the compression molder. The samples were cooled down to room temperature. The gelatin film was then removed from the plates and subjected to analyses.



**Figure 1.** Photographs of molding compound resins from bovine and fish gelatins.

### 2.3.2. Analyses of thermo-compression molded gelatin films

Prior to testing, film samples were conditioned in an environmental chamber for 48 h at  $25 \pm 0.5$  °C and  $50 \pm 5\%$  RH.

#### 2.3.2.1 Film thickness

The film thickness was measured using a digital micrometer (Gotech, Model GT-313-A, Gotech Testing Machines Inc., Tawain) (CHUAYNUKUL *et al.*, 2015). Five random thickness measurements were recorded and the average was taken as the result.

### 2.3.2.2 Mechanical properties

The tensile strength (TS), elastic modulus (EM) and elongation at break (EAB) of the films were measured according to the ASTM-D882-01 (2002a) method, as described by IWATA *et al.* (2000), using a universal testing machine (Lloyd Instruments, Hampshire, UK). The specimen strip (50 x 20 mm<sup>2</sup>) was clamped between the grips with initial separation of 30 mm and then pulled apart at a cross-head speed of 30 mm/min until it was broken. The TS was calculated by dividing the maximum force at break by the cross-sectional area of the film. The EAB was calculated by dividing the length extended ( $\Delta L$ ) by the original length ( $L_0$ ) of the film. EM was derived from the initial slope of the linear portion of the stress-strain curve. Ten specimens were tested for each treatment.

### 2.3.2.3 Water vapor permeability (WVP)

WVP was determined using a modified ASTM E-96-01 (2002b) method as described by SHIKU *et al.* (2004). The pre-conditioned film was sealed onto the opening of an aluminum permeation cup (30 mm internal diameter) containing dried silica gel (0% RH) with silicone vacuum grease and rubber gasket. The cup was kept in a controlled chamber at 30±0.5 °C and 65±5% RH. The cup was weighed every 1 h until 8 h, under this controlled environment. The WVP of the gelatin film was calculated using the following equation:

$$WVP = \frac{q \cdot l}{t \cdot A \cdot \Delta P}$$

Where  $l$  is the average thickness of the film sample (mm);  $A$  is the exposed area of the film (m<sup>2</sup>);  $\Delta P$  is the difference of the partial vapor pressure (Pa) across the film and the term  $q/t$  was calculated by linear regression from the plot of weight gain and time, in the constant rate period. The WVP value was expressed in g.m/m<sup>2</sup>.s.Pa.

### 2.3.2.4 Color

The color of the film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA). Also,  $D_{65}$  (day light) and a measure cell with an opening of 30 mm were used. Dried film samples were directly placed on the sample compartment and covered with a white standard plate. The color of the film was expressed as  $L^*$ -(lightness/brightness),  $a^*$ -(redness/greenness) and  $b^*$ -(yellowness/blueness) values. The total difference in color ( $\Delta E^*$ ) was calculated according to the equation of GENNADIOU *et al.* (1996a) as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Where,  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differences between the color parameter of corresponding film samples and that of the white standard ( $L^* = 92.82$ ,  $a^* = -1.29$  and  $b^* = 0.51$ ).

### 2.3.2.5 Light transmittance and transparency value

The transmission of visible light of gelatin films was measured at a wavelength of 600 nm, using UV-Vis spectrophotometer (Model No. 1601, Shimadzu, Kyoto, Japan) as described

by HAN and FLOROS (1997). The transparency value of the films was then calculated using the following equation:

$$\text{Transparency value} = \frac{-\log T_{600}}{x}$$

Where  $T_{600}$  is the fractional value of transmittance at 600 nm and  $x$  is the film thickness (mm). According to this equation, a higher transparency value indicates a lower degree of film transparency.

## **2.4. Effects of pre-heating conditions on film formation and film properties**

The conditioned bovine and fish gelatin resins containing glycerol at 25% of protein were used to prepare films via compression molding using the same procedure as described above. In this study, different conditions in the pre-heating step prior to pressing were applied, including pre-heating temperatures (120, 140 and 160 °C) and times (5 and 10 min). Thereafter, the pre-heated resins were compressed at the aforementioned temperatures for 2 min with pressure of 20 MPa, followed by cooling as described above. The obtained films from bovine and fish gelatins were then subjected to analyses as mentioned earlier.

## **2.5. Statistical analysis**

All experiments were performed in triplicates ( $n=3$ ) and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and the mean comparisons were done by Duncan's multiple range tests (STEEL and TORRIE, 1980). Data are presented as mean±standard deviation and the probability value of  $p<0.05$  was considered as significant. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

# **3. RESULTS AND DISCUSSION**

## **3.1. Effect of glycerol levels on the properties of thermo-compression molded gelatin films**

### **3.1.1. Film forming ability and visualized appearance of films**

Glycerol, as a plasticizer, was incorporated into gelatin at different levels (15, 25 and 35% of protein, w/w). The addition of glycerol was expected to influence not only the properties of obtained films, but also the flow-ability and fusion behavior of molding compound resin during thermo-compression. The later effects were important for the feasibility of film-formation via thermal technique. It was found that all levels of glycerol used were able to promote sufficient flow-ability and fusion of the molten resins under hot-pressing (at 120 °C and 20 MPa). Thus, continuous, flexible films with average thickness in the range of 80.2-127.3 µm were formed, regardless of gelatin type. As the glycerol level increased, the average thickness of the films decreased (Table 1). This can be attributed to the fact that an increase in glycerol level resulted in increased flow-ability of the molten gelatin (at 120 °C), probably due to a decrease in its melt viscosity. Theoretically, the addition of a plasticizer to the polymer can also act as a processing aid

which can decrease the melt viscosity of the polymer, thereby enhancing the flow-ability of the polymer melt (PARK *et al.*, 2008; MARTUCCI and RUSECKAITE, 2009). PARK *et al.* (2008) prepared gelatin resins plasticized with 20% glycerol, sorbitol or a mixture of glycerol and sorbitol, and the resins were extruded to produce stretchable films. The results suggested that the film prepared only with sorbitol, as the plasticizer was not suitable for the extrusion film process due to its low fluidity. However, the films can be successfully extruded from resins plasticized with 20% glycerol and the glycerol-sorbitol mixture. Therefore, the glycerol level had an impact on the flow-ability and thus, the film-forming ability via compression molding of gelatin.

**Table 1.** Thickness and mechanical properties of thermo-compression molded gelatin films from resins containing different glycerol levels.

| Gelatin | Glycerol (%) | Thickness ( $\mu\text{m}$ )  | EM <sup>#</sup> ( $\times 10^2 \text{MPa}$ ) | TS <sup>#</sup> (MPa)         | EAB <sup>#</sup> (%)            |
|---------|--------------|------------------------------|----------------------------------------------|-------------------------------|---------------------------------|
| Bovine  | 15           | 127.3 $\pm$ 7.1 <sup>c</sup> | 5.22 $\pm$ 0.20 <sup>e</sup>                 | 26.68 $\pm$ 0.79 <sup>e</sup> | 6.86 $\pm$ 1.57 <sup>a*</sup>   |
|         | 25           | 98.5 $\pm$ 8.2 <sup>b</sup>  | 3.84 $\pm$ 0.24 <sup>c</sup>                 | 21.65 $\pm$ 0.55 <sup>d</sup> | 36.29 $\pm$ 14.67 <sup>c</sup>  |
|         | 35           | 83.2 $\pm$ 7.4 <sup>a</sup>  | 2.05 $\pm$ 0.16 <sup>b</sup>                 | 10.24 $\pm$ 0.85 <sup>b</sup> | 125.18 $\pm$ 11.83 <sup>d</sup> |
| Fish    | 15           | 122.7 $\pm$ 8.3 <sup>c</sup> | 4.38 $\pm$ 0.24 <sup>d</sup>                 | 25.45 $\pm$ 1.69 <sup>e</sup> | 4.98 $\pm$ 0.87 <sup>a</sup>    |
|         | 25           | 94.2 $\pm$ 9.4 <sup>b</sup>  | 3.46 $\pm$ 0.30 <sup>c</sup>                 | 19.74 $\pm$ 1.00 <sup>c</sup> | 24.10 $\pm$ 8.73 <sup>b</sup>   |
|         | 35           | 80.2 $\pm$ 8.4 <sup>a</sup>  | 1.30 $\pm$ 0.22 <sup>a</sup>                 | 7.83 $\pm$ 0.68 <sup>a</sup>  | 124.21 $\pm$ 6.79 <sup>d</sup>  |

\*Mean  $\pm$  SD (n=3).

<sup>#</sup>Different superscripts in the same column indicate significant differences (p<0.05).

### 3.1.2. Mechanical properties

The mechanical properties of thermo-compression molded (120 °C, 20 MPa) bovine and fish gelatin films from resins incorporated with glycerol at various levels are shown in Table 1. As shown in the table, gelatin films had decreased TS and EM, but increased EAB with increasing glycerol levels, regardless of gelatin type (p<0.05). Film containing 35% glycerol had the highest EAB (125.18 and 124.21% for bovine and fish gelatin films, respectively). However, it exhibited a rather sticky surface, which could be attributed to the excessive addition of glycerol. These results indicated the plasticizing effect caused by glycerol, as well as some adsorbed water in the film matrix. The addition of a miscible plasticizer can reduce inter-chain interactions to overcome the brittleness of films and improve film flexibility (LIMPISOPHON *et al.*, 2010). When glycerol is used, its hydroxyl groups could interact with the amino acid groups in the protein, thereby decreasing inter- and intra-molecular interactions between protein chains, such as hydrogen bonds, and improving the motion ability of protein macromolecules, thereby resulting in increased flexibility (i.e. reducing the EM) of the films. As a result, the behavior of gelatin films changed from brittle to flexible. Moreover, glycerol, a small molecule with highly hygroscopic characteristic, is easily inserted between protein chains and attracts more water to the structure of the film, thereby becoming more flexible. Similar results have been reported for protein films prepared using thermal techniques (CUNNINGHAM *et al.*, 2000; ZHANG *et al.*, 2001; SOTHORNVIT *et al.*, 2007).

However, the required amount of plasticizer, which is appropriate for the film-forming ability and better properties of film prepared from thermal processing, is dependent on protein type, technique and condition used (SOTHORNVIT *et al.*, 2007; PARK *et al.*, 2008). PARK *et al.* (2008) produced a gelatin film from resin that was plasticized with 20%

glycerol by extrusion. Thus, it was found that the extruded film could not be produced from resin plasticized with sorbitol, since it had low fluidity during processing. SOTHORNVIT *et al.* (2007) prepared a protein sheet from mixtures of whey protein isolates (WPI) and glycerol (30, 40 and 50%) using the compression molding method. They revealed that the increasing glycerol content not only decreased TS and EM, but also increased the EAB of the WPI-sheet. They suggested that by using the compression molding method, a minimum of 30% glycerol should be added in the production of WPI-sheets, since lower glycerol levels produce sheets which are too brittle to handle and test. ZHANG *et al.* (2001) prepared soy protein sheets with various glycerol levels (10, 20, 30, 40 and 50%) by extrusion. They reported that films with 10% glycerol were very difficult to produce and very brittle after losing moisture. When glycerol of 20 to 30% was added, the strength of the sheet decreased a little, but it was good in terms of greater EAB. An increase in glycerol level (above 30%) resulted in a large decrease in yield stress and continuous increase in EAB. From the results, it was found that the effect of the added glycerol on the compression-molded gelatin film showed a similar trend to the gelatin film developed using the casting technique (VANIN *et al.*, 2005; JONGJAREONRAK *et al.*, 2006; HOQUE *et al.*, 2011).

For the same level of glycerol used, bovine gelatin films showed greater mechanical resistance than fish gelatin films as indicated by the higher TS, EM and EAB of the films. This can be attributed to the higher imino acid content in bovine gelatin (ARVANITOYANNIS *et al.*, 2002; AVENA-BUSTILLOS *et al.*, 2006). The proline and hydroxyproline contents are approximately 30% for mammalian gelatins, 22-25% for warm-water fish gelatins (tilapia and Nile perch), and 17% for cold-water fish gelatins (cod) (AVENA-BUSTILLOS *et al.*, 2006; CHIOU *et al.*, 2008). Mammalian gelatins commonly have better physical properties and thermal stability than most fish gelatins (ARVANITOYANNIS *et al.*, 2002; MUYONGA *et al.*, 2004; AVENA-BUSTILLOS *et al.*, 2006). Nevertheless, the incorporation of glycerol at 25% protein, produced continuous gelatin films with sufficient flexibility to be handled, irrespective of gelatin type. Thus, the glycerol levels used had a significant influence on the film-forming ability and properties of the resulting compression-molded gelatin films.

### 3.1.3. Water vapor permeability (WVP)

The WVP of compression-molded bovine and fish gelatin films from resins incorporated with different glycerol levels is shown in Fig. 2. Generally, the WVP of all gelatin films increased with increasing glycerol levels ( $p < 0.05$ ), regardless of gelatin type. Glycerol, a highly hygroscopic substance, which consists of 3 hydroxyl groups, was able to attract water to the plasticized protein system (SOTHORNVIT *et al.*, 2001). Plasticizers not only improve the mechanical properties of protein films, but also increase the film permeability (CUQ *et al.*, 1997; SOTHORNVIT *et al.*, 2001).

Besides the contribution from solubility, polymer permeability is also dependent on its diffusion coefficient. The diffusion coefficient and thus, the permeability increased with an increase in free volume, as well as the interstitial space between the polymer molecules. From the result, the increase in WVP of both gelatin films with increasing glycerol level could also be explained by an increase in free volume of the film matrix. The insertion of plasticizers between protein molecules increased the free volume of the system and favored the mobility of polymeric chains. Consequently, the network structure of the film became less dense and more permeable (JONGJAREONRAK *et al.*, 2006). ORLIAC *et al.* (2003) reported that an increase in hydrophilic plasticizer levels enhanced the absorption of more water to the film network and increased water vapor transfer.

The results of this study are in agreement with the reports of various protein films prepared using solution casting such as gelatin films from cuttlefish (*Sepia pharaonis*) skin (HOQUE *et al.*, 2011), brown stripe red snapper and big-eye snapper skin (JONGJAREONRAK *et al.*, 2006), pig skin (VANIN *et al.*, 2005), muscle protein of Nile tilapia (PASCHOALICK *et al.*, 2003), wheat gluten (GONTARD *et al.*, 1993) and protein films prepared using thermal method such as sun flower protein film (ORLIAC *et al.*, 2003), soy protein sheet (ZHANG *et al.*, 2001) and soy protein isolate film (CUNNINGHAM *et al.*, 2000).

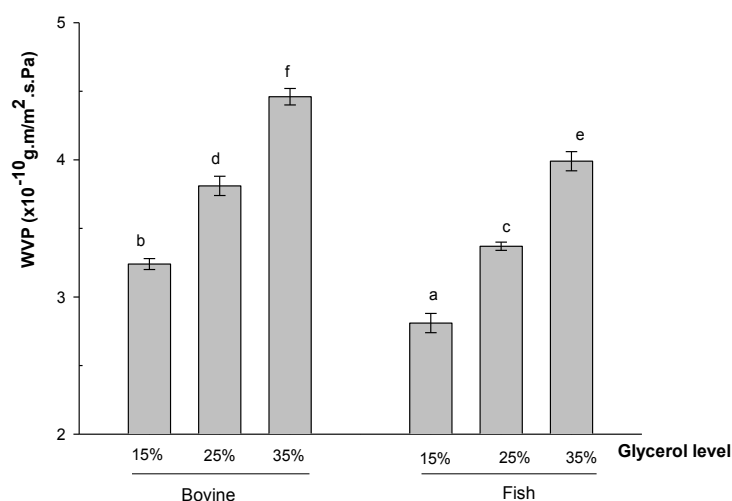


**Figure 2.** The WVP of thermo-compression molded gelatin films from resins containing different glycerol levels. Each bar represents the standard deviation (n=3). Different letters indicate significant differences (p<0.05).

At the same glycerol level, fish gelatin films showed lower WVP than bovine gelatin films (p<0.05). This may be attributed to the lower concentrations of proline and hydroxyproline in fish gelatins as compared with mammalian gelatins (NORLAND, 1990; GOMEZ-GUILLEN *et al.*, 2009). Moreover, it has been reported that the lower WVP of fish gelatin films could be useful, particularly for applications related to reducing water loss from encapsulated drugs and refrigerated or frozen food systems (NORLAND, 1990). Therefore, the amount of glycerol added influenced not only the flow-ability, film-forming ability and mechanical properties but also the barrier properties of the compression-molded gelatin films.

### 3.1.4. Color and transparency value

The color of the thermo-compression molded gelatin films from resins containing different glycerol levels is shown in Table 2. In general, as the glycerol concentration increased from 15 to 25%, both types of gelatin films exhibited almost similar  $L^*$ - and  $a^*$ -values (p>0.05). When the glycerol content was further increased to 35%, the fish gelatin film showed higher  $L^*$ - and  $a^*$ -values (p<0.05). However, the  $b^*$ - and  $\Delta E^*$ -values of films from both gelatins tended to decrease with increasing glycerol level. The behavior of total color difference ( $\Delta E^*$ ) was mainly as a result of the variation in  $b^*$ -value. Overall, the results indicated that when the concentration of glycerol increased, the film showed higher

lightness and less color, especially yellowness. This can be attributed to the effect of the dilution of proteins by the addition of glycerol, which is a colorless plasticizer (PASCHOALICK *et al.*, 2003; SOBRAL *et al.*, 2005). However, VANIN *et al.* (2005) did not observe the effect of plasticizer type (glycerol, polypropylene glycol, ethylene glycol and di-ethylene glycol) and plasticizer concentration (10, 15, 20, 25 and 30%) on the color difference and opacity of gelatin-based films. The different observations might be due to differences in amino acid composition and molecular weight of gelatin, as well as the alignment of gelatin molecules in the film network. At the same glycerol levels, bovine gelatin films were darker in color and had much higher yellowness and slightly lower lightness as evidenced by a greater  $b^*$ -value and lesser  $L^*$ -value, respectively, when compared with fish gelatin films ( $p<0.05$ ). This was mainly due to the differences in color of both gelatin powders used as raw materials.

**Table 2.** Color of thermo-compression molded gelatin films from resins containing different glycerol levels.

| Gelatin | Glycerol (%) | $L^*$                    | $a^*$                   | $b^*$                  | $\Delta E^*$           |
|---------|--------------|--------------------------|-------------------------|------------------------|------------------------|
| Bovine  | 15           | 88.98±0.02 <sup>a</sup>  | -1.58±0.02 <sup>a</sup> | 6.70±0.16 <sup>e</sup> | 7.95±0.14 <sup>g</sup> |
|         | 25           | 89.56±0.04 <sup>ab</sup> | -1.41±0.02 <sup>b</sup> | 4.88±0.22 <sup>d</sup> | 6.17±0.19 <sup>d</sup> |
|         | 35           | 89.85±0.08 <sup>bc</sup> | -1.33±0.04 <sup>b</sup> | 3.99±0.18 <sup>c</sup> | 5.31±0.18 <sup>c</sup> |
| Fish    | 15           | 90.53±0.08 <sup>c</sup>  | -1.21±0.04 <sup>c</sup> | 2.46±0.05 <sup>b</sup> | 3.81±0.05 <sup>b</sup> |
|         | 25           | 90.46±0.08 <sup>c</sup>  | -1.17±0.07 <sup>c</sup> | 2.27±0.09 <sup>b</sup> | 3.76±0.08 <sup>b</sup> |
|         | 35           | 92.01±0.88 <sup>d</sup>  | -0.44±0.10 <sup>d</sup> | 0.17±0.08 <sup>a</sup> | 1.60±0.20 <sup>a</sup> |

<sup>a</sup>Mean ± SD (n=3).

<sup>g</sup>Different superscripts in the same column indicate significant differences ( $p<0.05$ ).

The transparency value of compression-molded gelatin films, plasticized with glycerol at different levels, is shown in Fig. 3.

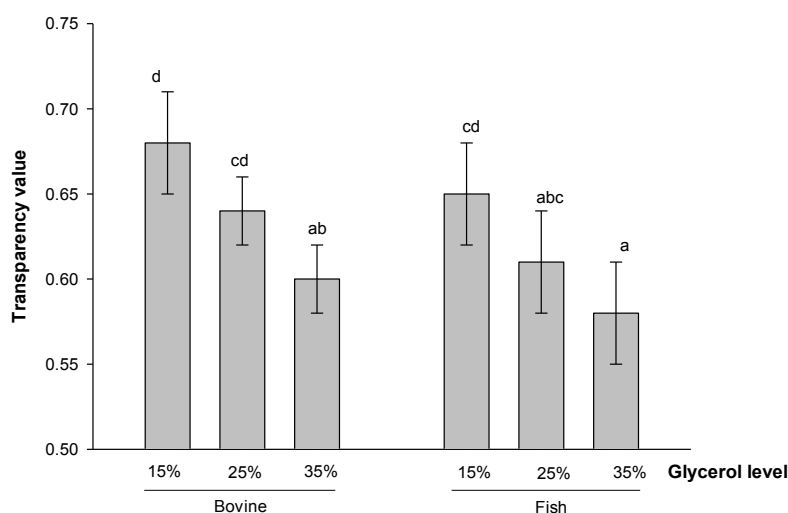


**Figure 3.** Transparency value of thermo-compression molded gelatin films from resins containing different glycerol levels. Each bar represents the standard deviation (n=3). Different letters indicate significant differences ( $p<0.05$ ).

Generally, the glycerol content used had a slight impact on the transparency of the compression-molded films of both gelatins. The transparency value of the film tended to decrease with increasing glycerol levels. For both gelatins, films plasticized with 35% glycerol showed a lower transparency value than those with 15% glycerol ( $p < 0.05$ ). Based on the equation used for the calculation of the transparency value, the film sample with lower transparency value was more transparent. From the results, films were more transparent when a high level of glycerol was incorporated. This could be attributed to the fact that the added glycerol decreased protein-protein interactions with concomitant increase in free volume in the film matrix; hence, light could pass through such a network more easily. This result is in accordance with other reports in which films became more transparent with increasing plasticizer level (PASCHOALICK *et al.*, 2003; SOBRAL *et al.*, 2005).

### 3.2. Effect of pre-heating conditions on properties of thermo-compression molded gelatin films

The result of this study showed that films from bovine and fish gelatins to which 25% glycerol was added had satisfactory mechanical and water vapor barrier properties. Furthermore, the film obtained was not sticky and easy to handle. Therefore, to study the effect of pre-heating conditions on gelatin resins, 25% glycerol level was used.

#### 3.2.1. Film forming ability and visualized appearance of films

To investigate the film forming ability of plasticized gelatins in the melt state via thermo-compression molding, pre-heating of resin prior to pressing was performed at various temperatures (120, 140 and 160 °C) and duration (5 and 10 min). Table 3 shows the overall visualized appearance of films obtained under different pre-heating conditions.

**Table 3.** Appearance of thermo-compression molded gelatin films prepared under different pre-heating conditions.

| Gelatin | Preheating conditions |            | Visual appearance                                       |
|---------|-----------------------|------------|---------------------------------------------------------|
|         | Temp.(°C)             | Time (min) |                                                         |
| Bovine  | 120                   | 5          | Film with many voids or pinholes                        |
|         |                       | 10         |                                                         |
|         | 140                   | 5          | Continuous, flexible, transparent and yellow film       |
|         |                       | 10         |                                                         |
|         | 160                   | 5          | Opaque and very dark yellow brittle film                |
|         |                       | 10         | Low fluidity of the melt; too brittle film              |
| Fish    | 120                   | 5          | Continuous, flexible, transparent and less yellow film. |
|         |                       | 10         |                                                         |
|         | 140                   | 5          |                                                         |
|         |                       | 10         |                                                         |
|         | 160                   | 5          | Rather opaque and slightly yellow brittle film          |
|         |                       | 10         | Low fluidity of the melt; too brittle film              |

The thickest films with a lot of visualized voids were obtained for both gelatin types when the processing temperatures used were lower than 120 °C. This might be due to the fact that the molten resins could not flow properly because of its high melt viscosity. Moreover, processing temperatures higher than 160 °C resulted in dramatic degradation. It was found that pre-heating of bovine gelatin resin at 120°C for 5 min rendered the film with numerous visualized voids or pin-holes. Under this pre-heating condition, the plasticized-bovine gelatin melt might still possess high viscosity. As a result, the air bubbles could not be evacuated from the melt before compressing and thus, were entrapped in the film after cooling. However, it was not noticed for fish gelatin resin pre-heated under the same condition, possibly due to the lower melt viscosity of fish gelatin as compared with bovine gelatin. Continuous and flexible films without visualized pin-holes were formed when the pre-heating temperature and time were increased up to 160 °C and 5 min, regardless of gelatin type.

The melt viscosity of both gelatin resins might decrease sufficiently and allowed the gelatin melt to flow easily. As a result, the gelatin molecules could form a continuous network structure of film. Generally, the melt viscosity of the polymer decreased with increasing processing temperature, which is a significant factor in determining the flowability and processibility of the polymer via molding processes (CUNNINGHAM *et al.*, 2000; ZHANG *et al.*, 2001; PARK *et al.*, 2008). However, very brittle films with obvious degradation were obtained for both gelatins when the pre-heating of resins at 160 °C for 10 min was implemented. The application of a higher temperature for a longer period of time resulted in a dramatic degradation of gelatin, as well as the evaporation of bounded water. CHUAYNUKUL (2011) reported that partial degradation was noticed in the gelatin film compression molded at 120 °C, as evidenced by the increased free-amino group content. Therefore, the condition used in the pre-heating step was crucial for determining the gelatin film processibility via compression molding. The studied pre-heating conditions also affected the properties of bovine and fish gelatin films differently.

### 3.2.2. Mechanical properties

The mechanical properties of gelatin films prepared by different resin pre-heating conditions are shown in Table 4. The TS and EM of the films decreased, whilst the EAB increased with increasing pre-heating temperature and time ( $p < 0.05$ ), regardless of gelatin type. Gelatin films exhibited the highest values of TS and EM at pre-heating temperature of 120 °C (20.82 MPa,  $6.17 \times 10^5$  MPa and 18.90 MPa,  $4.76 \times 10^5$  MPa for bovine and fish gelatin films, respectively). The TS and EM decreased when the pre-heating temperature was increased. This was probably due to the partial degradation of gelatin, which took place at higher temperature and extended time. The shorter gelatin molecules formed the lower intermolecular interactions, resulting in films with lower strength and stiffness (that is lower TS and EM, respectively). CHUAYNUKUL (2011) observed that the partial degradation of gelatins took place in resins and the resulting films when the resins were subjected to higher temperature prior to molding, as reconfirmed by the increased free-amino group content of the gelatin. The impact of heating on the degradation of gelatin has been investigated and reported by HOQUE *et al.* (2010). It was pointed out that the partial degradation of gelatin occurred when the gelatin solution was heated at a temperature higher than 70°C. This caused a decrease in the TS of the resulting films, because the shorter chains of gelatin molecules could not form the strong film network. The presence of degradation of protein molecules upon being processed at higher temperature was similarly observed in various protein-based films fabricated using

thermal processing techniques (SOTHORNVIT *et al.*, 2007; KATOH *et al.*, 2004; JANSSENS *et al.*, 2013).

**Table 4.** Mechanical properties and water vapor permeability (WVP) of thermo-compression molded gelatin films prepared under different pre-heating conditions.

| Gelatin | Pre-heating conditions |               | EM <sup>#</sup><br>(x10 <sup>2</sup> MPa) | TS <sup>#</sup><br>(MPa) | EAB <sup>#</sup><br>(%)   | WVP <sup>#</sup><br>(x10 <sup>-10</sup> g.m/m <sup>2</sup> .s.Pa) |
|---------|------------------------|---------------|-------------------------------------------|--------------------------|---------------------------|-------------------------------------------------------------------|
|         | Temp.<br>(°C)          | Time<br>(min) |                                           |                          |                           |                                                                   |
| Bovine  | 120                    | 5             | ND                                        | ND                       | ND                        | ND                                                                |
|         |                        | 10            | 6.17±1.37 <sup>f</sup>                    | 20.82±2.32 <sup>f</sup>  | 33.61±6.75 <sup>a</sup>   | 3.29±0.26 <sup>f</sup> ‡                                          |
|         | 140                    | 5             | 4.33±0.87 <sup>de</sup>                   | 17.15±0.43 <sup>de</sup> | 57.87±12.96 <sup>b</sup>  | 3.07±0.30 <sup>ef</sup>                                           |
|         |                        | 10            | 3.48±0.71 <sup>c</sup>                    | 13.85±1.33 <sup>c</sup>  | 126.29±11.49 <sup>e</sup> | 2.80±0.04 <sup>cd</sup>                                           |
|         | 160                    | 5             | 2.07±0.73 <sup>c</sup>                    | 11.56±1.40 <sup>a</sup>  | 90.12±9.63 <sup>d</sup>   | 2.47±0.11 <sup>ab</sup>                                           |
|         |                        | 10            | ND                                        | ND                       | ND                        | ND                                                                |
| Fish    | 120                    | 5             | 4.76±0.52 <sup>e</sup>                    | 18.90±1.41 <sup>e</sup>  | 24.68±5.94 <sup>a</sup>   | 3.12±0.05 <sup>ef</sup>                                           |
|         |                        | 10            | 3.63±0.07 <sup>cd</sup>                   | 16.67±1.63 <sup>d</sup>  | 31.50±5.79 <sup>a</sup>   | 2.97±0.26 <sup>de</sup>                                           |
|         | 140                    | 5             | 2.79±0.25 <sup>bc</sup>                   | 13.52±0.92 <sup>bc</sup> | 79.90±7.18 <sup>cd</sup>  | 2.61±0.08 <sup>bc</sup>                                           |
|         |                        | 10            | 1.91±0.57 <sup>ab</sup>                   | 11.81±1.72 <sup>ab</sup> | 105.52±15.1 <sup>e</sup>  | 2.50±0.07 <sup>ab</sup>                                           |
|         | 160                    | 5             | 1.60±0.36 <sup>a</sup>                    | 11.42±0.56 <sup>a</sup>  | 82.24±12.27 <sup>c</sup>  | 2.25±0.06 <sup>a</sup>                                            |
|         |                        | 10            | ND                                        | ND                       | ND                        | ND                                                                |

<sup>#</sup>Mean ± SD (n=3).

<sup>‡</sup>Different superscripts in the same column indicate significant differences (p<0.05).

ND = Not determined.

For the EAB value, pre-heating at higher temperature resulted in increased EAB of the films in both gelatins (p<0.05). At the same pre-heating temperature, films pre-heated for 10 min had higher EAB than those pre-heated for 5 min (p<0.05), regardless of gelatin type. This suggested an increase in film extensibility or stretch-ability. From the result, films prepared from the same type of gelatin had the highest EAB when pre-heating at 140 °C for 10 min was implemented. An increase in EAB was observed with a concomitant decrease in TS (GENNADIOU *et al.*, 1996b; JONGJAREONRAK *et al.*, 2006). Additionally, with increasing temperature from 120 to 140°C, gelatin might undergo partial degradation, leading to the formation of shorter chains. This may result in lower and/or weaker interaction between gelatin molecules (resulted in decreased TS), which could allow the molecules to slip around each other with more ease and a larger degree upon tensile deformation before breaking of the film specimen (leading to increased EAB) (HOQUE *et al.*, 2010). MUYONGA *et al.* (2004) observed that the gelatin extracted from Nile perch bones, which consisted of a higher proportion of low-molecular weight fractions, had considerably lower tensile strength, but higher percentage of elongation. However, when the pre-heating temperature was further increased to 160 °C, both gelatin films exhibited decreased EAB. At this temperature, a higher degradation of gelatin might take place resulting in shorter chains. These shorter gelatin chains may be unable to form a strong network, leading to a decrease in both TS and EAB.

When the films were compared based on different gelatin sources, fish gelatin films had lower TS, EM and EAB as compared with bovine gelatin films fabricated under the same pre-heating condition. This might be due to the presence of a higher number of imino acid residues in the α-chain of bovine gelatin, which contributed to the high number of

hydrogen bonds (NORLAND, 1990). CHIOU *et al.* (2008) prepared fish gelatin films from Alaska pollock (*Theragra chalcogramma*) and Alaska pink salmon (*Oncorhynchus gorbuscha*) and compared their mechanical properties to bovine and porcine gelatin films. They reported that pollock and salmon gelatin films had lower TS and EAB than mammalian gelatin films. Therefore, the pre-heating temperature and time used in thermo-compression molding are essential to controlling the mechanical properties of the resulting films.

### 3.2.3. Water vapor permeability (WVP)

The WVP of the thermo-compression molded gelatin films prepared from different pre-heating conditions is shown in Table 4. The WVP of the films seemed to decrease with an increase in pre-heating temperature and time used, irrespective of gelatin type ( $p < 0.05$ ). At the same pre-heating temperature, films prepared with pre-heating time of 10 min showed lower WVP than those prepared with pre-heating time of 5 min ( $p < 0.05$ ). Films obtained from higher pre-heating temperature also exhibited lower WVP ( $p < 0.05$ ). These results indicated that pre-heating at higher temperature led to increase in the water vapor barrier of the compression-molded gelatin films. This may be attributed to the exposure of the hydrophobic domains of gelatin chains upon heating, resulting in increased hydrophobic interaction and hydrophobicity of the films; thus, lowering the tendency of water absorbance by the film matrix (KIM *et al.*, 2002). Furthermore, the regular arrangement of shorter chain gelatin obtained after heating at higher temperatures, plausibly led to a compact matrix of gelatin during film formation (KIM *et al.*, 2002). As a result, the diffusion of water molecules could be retarded, which resulted in decreased WVP. KATOH *et al.* (2004) reported that molding at higher temperature caused the formation of a more compact structure of keratin film, which prevented the film from absorbing water. Moreover, the high temperature treatment of proteins might provoke additional interaction and crosslinking of protein molecules. JANSSENS *et al.* (2013) recorded an increase in water absorption of the compression-molded gluten protein film, as higher processing temperatures and longer times were used. This is mostly due to the presence of a higher degree of protein cross-linking during compression molding. A similar result was also reported by KIM *et al.* (2002) for heated soy protein films, in which the WVP of the film decreased during heating, and this was probably caused by the formation of crosslinks.

Under the same pre-heating condition, fish gelatin film had lower WVP as compared with bovine gelatin film ( $p < 0.05$ ). This can be attributed to the higher hydrophobicity of fish gelatin, which had lower concentrations of proline and hydroxyproline as compared with mammalian gelatins (NORLAND, 1990; KARIM and BHAT, 2009). AVENA-BUSTILLOS *et al.* (2006) reported the WVP of fish-gelatin films to be significantly lower than that of films made from mammalian gelatin. They explained the tendency of fish-gelatin films to exhibit lower WVP values than land animal-gelatin films in terms of amino acid composition. Fish gelatins, especially cold-water fish gelatins, are known to contain higher amounts of hydrophobic amino acids and lower amounts of hydroxyproline (AVENA-BUSTILLOS *et al.*, 2006).

### 3.2.4. Color and transparency value of films

The color and transparency value of compression-molded gelatin films prepared under different pre-heating conditions are shown in Table 5. As the pre-heating temperature and time increased, the films had increased yellowness ( $b^*$ -value) and total color difference ( $\Delta E^*$ -value), as well as slightly decreased film transparency (as indicated by an increase in

the transparency value) ( $p < 0.05$ ). The increased yellowness of the films pre-heated at higher temperature and longer time was plausibly due to Maillard reaction, which is associated with the heat-induced process (HOQUE *et al.*, 2010).

As the pre-heating temperature and time increased, the lightness ( $L^*$ ) of the bovine gelatin films showed a slight decreasing trend, whereas fish gelatin films showed no difference in  $L^*$ -value (90.12-90.62). PASCHOALICK *et al.* (2003) suggested that the increase in temperature caused a slight increase in the color of films, possibly due to the occurrence of a reaction among the glycerol molecules and the reactive group of lysine. For films fabricated under the same pre-heating condition, the fish gelatin films exhibited a lighter color but they were slightly more transparent than the bovine gelatin films. The differences in color and lightness of both films observed from the results were most likely influenced by the different gelatin types and manufacturing processes. The slight difference in transparency value of bovine and fish gelatin films might be due to differences in the intrinsic characteristics of each gelatin type, such as composition, density, aggregation or alignment of gelatin molecules in the films, as well as type of extraction treatments used to prepare the gelatin (BAILEY *et al.*, 1989). The purification procedure might increase the lightness of gelatin powder (NORLAND, 1990).

**Table 5.** Color and transparency values of thermo-compression molded gelatin films prepared under different pre-heating conditions.

| Gelatin | Pre-heating conditions |            | $L^{\#}$                 | $a^{\#}$                 | $b^{\#}$                | $\Delta E^{\#}$         | Transparency value <sup>#</sup>      |
|---------|------------------------|------------|--------------------------|--------------------------|-------------------------|-------------------------|--------------------------------------|
|         | Temp. (°C)             | Time (min) |                          |                          |                         |                         |                                      |
| Bovine  | 120                    | 5          | ND                       | ND                       | ND                      | ND                      | ND                                   |
|         |                        | 10         | 89.78±0.07 <sup>cd</sup> | -1.55±0.06 <sup>de</sup> | 5.09±0.14 <sup>d</sup>  | 6.05±0.12 <sup>c</sup>  | 0.64±0.03 <sup>ab</sup> <sup>†</sup> |
|         | 140                    | 5          | 89.10±0.05 <sup>bc</sup> | -1.82±0.03 <sup>c</sup>  | 7.49±0.11 <sup>e</sup>  | 8.42±0.09 <sup>d</sup>  | 0.70±0.03 <sup>c</sup>               |
|         |                        | 10         | 88.65±0.13 <sup>b</sup>  | -1.97±0.16 <sup>b</sup>  | 8.36±0.71 <sup>f</sup>  | 9.4±0.65 <sup>e</sup>   | 0.71±0.03 <sup>c</sup>               |
|         | 160                    | 5          | 87.16±1.41 <sup>a</sup>  | -3.06±0.12 <sup>a</sup>  | 12.04±0.62 <sup>g</sup> | 13.43±1.22 <sup>f</sup> | 0.81±0.02 <sup>d</sup>               |
|         |                        | 10         | ND                       | ND                       | ND                      | ND                      | ND                                   |
| Fish    | 120                    | 5          | 90.62±0.03 <sup>d</sup>  | -1.27±0.02 <sup>f</sup>  | 1.73±0.08 <sup>a</sup>  | 3.53±0.06 <sup>a</sup>  | 0.58±0.04 <sup>a</sup>               |
|         |                        | 10         | 90.59±0.03 <sup>d</sup>  | -1.30±0.01 <sup>f</sup>  | 1.90±0.06 <sup>a</sup>  | 3.60±0.05 <sup>a</sup>  | 0.60±0.03 <sup>a</sup>               |
|         | 140                    | 5          | 90.50±0.06 <sup>d</sup>  | -1.43±0.01 <sup>e</sup>  | 3.32±0.18 <sup>b</sup>  | 4.62±0.20 <sup>b</sup>  | 0.63±0.02 <sup>ab</sup>              |
|         |                        | 10         | 90.47±0.05 <sup>d</sup>  | -1.46±0.03 <sup>e</sup>  | 3.49±0.14 <sup>b</sup>  | 4.69±0.05 <sup>b</sup>  | 0.63±0.03 <sup>ab</sup>              |
|         | 160                    | 5          | 90.12±0.07 <sup>d</sup>  | -1.64±0.05 <sup>d</sup>  | 4.17±0.07 <sup>c</sup>  | 5.46±0.07 <sup>bc</sup> | 0.68±0.02 <sup>bc</sup>              |
|         |                        | 10         | ND                       | ND                       | ND                      | ND                      | ND                                   |

<sup>†</sup>Mean ± SD (n=3).

<sup>#</sup>Different superscripts in the same column indicate significant differences ( $p < 0.05$ ).

ND = Not determined.

#### 4. CONCLUSIONS

Films from bovine and fish gelatins were successfully prepared using the thermo-compression molding technique. Increasing the glycerol level resulted in decreased TS, EM and yellowness, but increased EAB, WVP and transparency of the resulting films. Gelatin films had generally decreased TS, EM, WVP and transparency, whilst increased yellowness occurred as pre-heating temperature and time increased. The compression-molded gelatin films incorporated with 25% glycerol (based on protein) had sufficient

flexibility (that is, EAB). Film processing conditions had significant impact on the processibility and properties of obtained compression-molded gelatin films. Among the major steps involved in the compression-molding of gelatin films, the pre-heating of resin prior to molding seemed to be the most crucial step in determining the processibility and properties of the obtained films. Therefore, the plasticizer level, pre-heating temperature and time have to be controlled for optimal flow-ability and fusion of the molten gelatin before compression/molding into a film.

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## IS LOCAL A MATTER OF FOOD MILES OR FOOD TRADITIONS?

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### ABSTRACT

In the last decade, the local food movement has achieved a growing popularity in the Italian food system. Nevertheless, the Italian food market still lacks a shared definition and labels indicating the local origin of the food products. In this study, we explore the meaning of “local food” in the Italian market using a qualitative approach. Results from twenty-three individual semi-structured interviews show that the meaning of “local” should be explained more in terms of connection between a community traditions and a geographical area than in terms of food miles.

*Keywords:* food traditions, Italian food market, local food, qualitative research

## 1. INTRODUCTION

In the Italian market, local food is defined with the expression "Chilometro Zero" (Zero Kilometers), since the first form of direct marketing was represented by the points of sales organized by producers within their farm, where the supply of food products to consumers occurred in the same location as the production (BUGNI, 2010). The popularity of local food products in Italy has been considerably growing: 1141 Farmers' Markets (FMs) organized by "Campagna Amica" (the most popular format of FMs in Italy) are recorded in 2016 (CAMPAGNA AMICA, 2016). In addition, the presence of Community Supported Agriculture (CSA) and direct marketing outlets as open markets, solidarity purchasing groups, small shops and farm-shops has been significantly growing in the last few years (Aldinucci, 2014; GIUCA, 2012; FRANCO *et al.*, 2015; PASCUCCI *et al.*, 2013; VASQUEZ *et al.*, 2017; WELLNER and THEUVSEN, 2015). With the so-called "De Castro" Decree, currently in force since the 1<sup>st</sup> of January 2008, guidelines have been set for the realization of marketplaces exclusively dedicated to direct retailing by farmers. Moreover, the Veneto Region, first in Italy, on the 25<sup>th</sup> of July 2008 issued regional law number 7 aimed at promoting the consumption of regional products in public food services in order to support the local economy. In addition, other Italian regions such as Emilia-Romagna and Abruzzo are tending to follow the same approach (COLDIRETTI, 2013). Given the increased popularity of the local food movement, large retail chains started, as well, to highlight the origin of the products that have been locally produced.

However, in the Italian market, labels certifying the local origin of the products are not present yet and what is local or not is not yet regulated. Admittedly, according to Italian and international literature reviews, it is difficult to identify a shared definition of "local food" (BAZZANI and CANAVARI, 2013:30).

Thus, the aim of the present study is to determine a definition of "local food" that can be shared throughout Italy, where the variety of resources in different territories and an ancient culinary art tradition lead to a high diversification in food consumption. In particular, the main goal of the research is to establish whether "local" can be better interpreted in terms of physical distance (i.e., food miles) or in terms of belonging to local community and food traditions.

We performed an explorative qualitative research, based on the use of semi-structured interviews. To the best of our knowledge, previous research related to the definition of local food was mostly based on an anthropological analysis of geographical and cultural conditions which lead to the starting up of local food networks (D'AMICO *et al.*, 2013; CHOLETTE, 2011; GIOVANNUCCI, *et al.*, 2010; MARTINEZ *et al.*, 2010; AMILIAN *et al.*, 2007; BRUNORI, 2007; SONNINO and MARSDEN, 2006; DUPUIS and GOODMAN, 2005; KIRWAN, 2004; HINRICHS, 2003; BARHAM, 2003; LA TROBE, 2001) or they were mainly focused on the description of consumers' perceptions towards local food (PENCARELLI *et al.*, 2015; APRILE *et al.*, 2012; DARBY *et al.*, 2008; ZEPEDA and DEAL, 2009). Therefore, this study represents one of the few attempts, the first one in Italy, to explore the meaning of "local food" using a qualitative approach. We interviewed twenty-three participants purposely chosen among consumers, farmers and experts of the food system asking about their opinions on local food consumption.

Through the exploration of concepts such as food values, quality perception, attitudes towards origin certification, we were able to highlight the main issues related to the definition of "local food" and we attempted to draw a possible scenario of the development of "local food" labels in the Italian market.

In the following sections we describe the methodology, then we summarize and discuss the results, and finally we draw our conclusions.

## 2. BACKGROUND ON THE CONCEPT OF "LOCAL FOOD"

BRUNORI (2007) suggested the distinction between "local food", "locality food" and "localist food". The term "local food" implies the instauration within a community of short-distance relationships, based on food habits and food traditions. On the other hand, the definition "locality food" is mainly focused on the origin of a product from a particular place, giving less importance to the "community factor". Finally, the concept of "localist food" implies consumers' willingness to reconstruct local identities by the regular consumption of food products, although they do not belong to the rural traditions of that local area. HAND and MARTINEZ (2010) stated that the re-valuation of local food was first supported by the Slow Food movement and defines whether a product is local or not on the basis of a maximum distance range of 100 Km (approximately 60 miles) within which the consumption and production locations are situated (SLOW FOOD, 2013). It is necessary to point out that the "Slow Food" association itself does not strictly respect this distance constraint. For example, at the Earth Market (the Farmers' Market organized by Slow Food) of Bologna seafood products originate from the coastal area of the Emilia-Romagna region, which is more than 100 Km away from the city of Bologna (BAZZANI *et al.*, 2016). Moreover, the concept of "local" has been often associated with regional, national boundaries (COSTANIGRO *et al.*, 2014; FEAGAN, 2007; HU *et al.*, 2012; LOMBARDI *et al.*, 2013; SCARPA *et al.*, 2005) or in terms of "traditional" food from a certain area (AKAICHI *et al.*, 2012). Some authors (AMILIEN *et al.*, 2007; APRILE *et al.*, 2012; BARHAM, 2003; GRACIA, 2013) associate to "local food" the well-known French term "*terroir*". This term highlights the influence of social and cultural factors in determining consumers' food habits: "the territorial reputation of a product is more often derived from a mixture of messages rather than the actual geography" (AMILIEN *et al.*, 2007:55). This term also refers to the so-called post-modern consumer, who is interested in the symbolic or cultural value rather than in the functional and utility value of products and services (VIGANÒ *et al.*, 2015).

## 3. MATERIALS AND METHODS

In order to account for the complexity and diversity of meanings embodied in the concept of "local food" we developed the study using an explorative qualitative analysis approach. This approach was chosen because it is more suitable for achieving a level of depth and understanding that is usually not easy to obtain with a quantitative survey based on statistical methods (MOLTENI and TROILO, 2012). Interviews were chosen as the most appropriate tool for analysing the social, cultural contexts through which informants can build cultural meanings (DENZIN, 2001; MOISANDER *et al.*, 2009). We performed in-depth interviews, supported by a semi-structured interview schedule, which served as a non-binding guideline for the interviewer.

A convenience, non-probabilistic sample of twenty-three individuals was selected. Three interviews were conducted by phone, the rest in person. The face-to-face interviews were performed in the cities of Bologna and Genoa. The selected sample consisted of six consumers, eight farmers and nine food market experts. We decided to interview different actors in the supply chain in order to have a broader interpretation of the issues related to the local food system. The consumers were recruited on the basis of their interest in the local food networks, indeed, four of them were regular Farmers' Markets shoppers and two of them were members of a CSA initiative. All the interviewed farmers regularly participated in Farmers' Markets and the selected experts were mainly involved in direct marketing activities or certification bodies (Table 1).

**Table 1.** Description of survey participants.

| No. | Consumer/Farmer/Expert | Location of the interview | Residence of the respondent | Activity                                                                                    |
|-----|------------------------|---------------------------|-----------------------------|---------------------------------------------------------------------------------------------|
| 1   | Consumer               | Bologna                   | Bologna                     | Regular Farmers' Markets shopper                                                            |
| 2   | Farmer                 | Bologna                   | Siracusa                    | Farmer participating in direct marketing activities                                         |
| 3   | Farmer                 | Bologna                   | Borgo Panigale (Bo)         | Farmer participating in direct marketing activities                                         |
| 4   | Farmer                 | Bologna                   | Crevalcore (Bo)             | Farmer participating in direct marketing activities                                         |
| 5   | Expert                 | Bologna                   | Bologna                     | Brand manager involved in the direct marketing of a wine company                            |
| 6   | Consumer               | Bologna                   | Treviso                     | Regular Farmers' Markets shopper                                                            |
| 7   | Farmer                 | Bologna                   | Crespellano (Bo)            | Farmer participating in direct marketing activities                                         |
| 8   | Farmer                 | Bologna                   | Rocca di Roffeno (Bo)       | Farmer participating in direct marketing activities                                         |
| 9   | Expert                 | Bologna                   | Imola (Bo)                  | Member of Coldiretti Association                                                            |
| 10  | Expert                 | Bologna                   | Bologna                     | Small Retailer of local food products                                                       |
| 11  | Farmer                 | Bologna                   | Casalfiumanese (Bo)         | Farmer participating in direct marketing activities                                         |
| 12  | Farmer                 | Bologna                   | Borgo Panigale (Bo)         | Farmer participating in direct marketing activities                                         |
| 13  | Consumer               | Bologna                   | Catania                     | Regular Farmers' Markets shopper                                                            |
| 14  | Consumer               | Bologna                   | Padua                       | Regular Farmers' Markets shopper                                                            |
| 15  | Expert                 | Bologna                   | Bologna                     | Researcher at the University of Bologna                                                     |
| 16  | Expert                 | Bologna                   | Bologna                     | Farm Assurance Technical Coordinator of a certification body                                |
| 17  | Consumer               | Bologna                   | Ravenna                     | Regular CSA shopper                                                                         |
| 18  | Expert                 | Bologna                   | Bologna                     | Member of Slow Food association, Bologna                                                    |
| 19  | Expert                 | Telephone interview       | Viterbo                     | Brand manager involved in the direct marketing of organic fresh fruit and vegetable company |
| 20  | Expert                 | Telephone interview       | Bologna                     | General manager of Italian vegetable seed company                                           |
| 21  | Expert                 | Genoa                     | Genoa                       | General manager of a retail company                                                         |
| 22  | Consumer               | Genoa                     | Genoa                       | Regular CSA shopper                                                                         |
| 23  | Farmer                 | Telephone Interview       | Lugo (Ra)                   | Farmer participating in direct marketing activities                                         |

Source: Data from the survey.

The recruitment of consumers was the most demanding among the three categories of respondents, since most of the consumers contacted affirmed that they had insufficient knowledge of the topic and did not accept the invitation to participate in the survey. In contrast, most of the contacted farmers and experts agreed to take part in the research (Table 2).

The interviews were administered during summer 2013. Once respondents were contacted, they were asked to take part in a research regarding the local food system. They were informed about the duration of the interview (30-45 minutes) and they were assured that their participation would be anonymous. Finally, the interviews were scheduled according to respondents' availability.

**Table 2.** Contacted and selected respondents.

|           | Contacted | Accepted | Response rate (%) |
|-----------|-----------|----------|-------------------|
| Consumers | 17        | 6        | 34                |
| Farmers   | 11        | 8        | 73                |
| Experts   | 12        | 9        | 75                |
| Total     | 40        | 23       | 57.5              |

Source: Data from the survey.

As previously mentioned, the interviews were structured according to a semi-structured interview schedule that was not strictly followed in order to minimize researcher influence and other sources of bias (ALVESSON, 2003). Therefore, general questions (open-ended questions) were posed to introduce the argument and, along the discussion, informants tended to be induced to raise issues that were considered important and relevant to the subject of interest (MYERS, 2009). All the interviews were recorded and transcribed verbatim. The transcribed interviews were analysed using, firstly, an open coding approach to examine the discrete parts. Then, axial coding was applied for the re-assembly of the data in categories and subcategories, which were finally brought together using selective coding (STRAUSS and CORBIN, 1998).

#### 4. RESULTS

The adoption of an explorative approach, based on the use of in-depth interviews, turned out to be appropriate for the aim of the research; we were able to collect a high variety of information that let us highlight the different aspects of the proposed topic. The semi-structured interview guideline was also effective in helping the interviewee to initially face the problem using a wide-angle lens, then turning the discussion into more specific issues related to the local food system (Fig. 1).

The definition of food values and respondents' perception of quality was essential in introducing the concept of origin, since nearly the totality of the interviewees marked the important issues as environmental and biodiversity safeguards, suitability of land and local traditions (Fig. 1).

Furthermore, the variety of issues mentioned in the interviews was also due to the choice of addressing different actors in the food supply chain. Indeed, results show that, generally, consumers were more focused on aspects such as organoleptic features of the products and support to the local economy, while farmers were more focused on environmental safeguards and, finally, experts highlighted the hygienic-sanitary safety aspect and cultural factors related to food consumption.

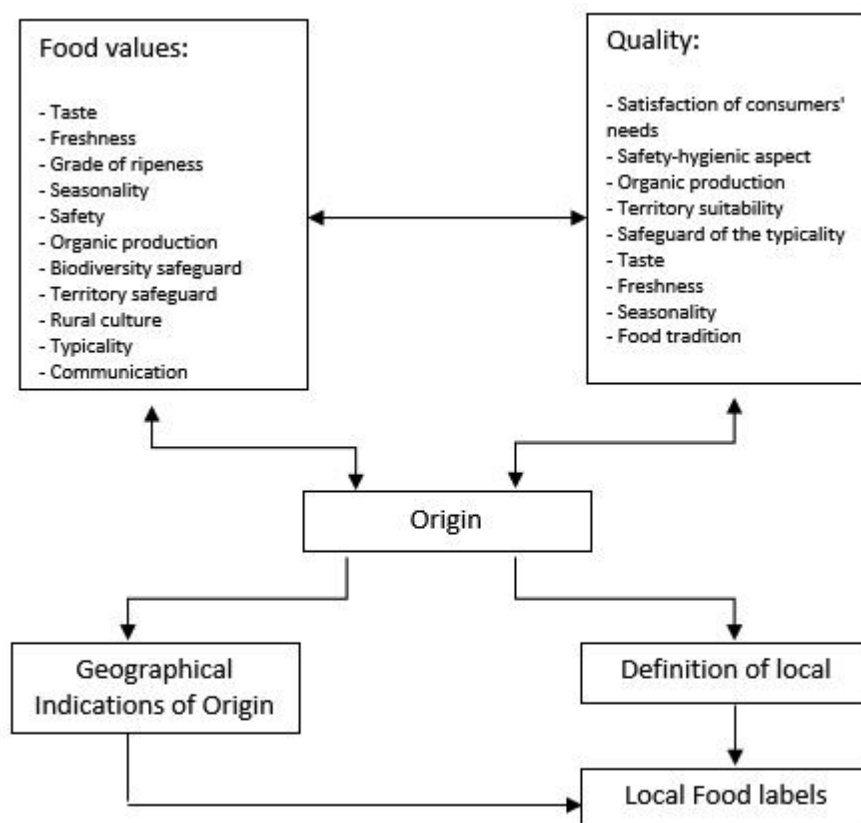


**Figure 1.** Issues extrapolated in the research.

#### 4.1. Food values

Respondents suggested different interpretations of the concept of food values: they referred to features such as organoleptic characteristics and nutritional value as well as to the environmental and ethical aspects related to the production and the supply of food products.

**Taste** was defined as the feature that mainly explained the value of a product. It is necessary to point out that, in the case of fresh food products, taste was mostly mentioned in combination with **freshness** and **correct grade of ripeness**; interviewees indicated “good products”, such as the ones that were harvested and sold within the day. **Seasonality**, as well, was mentioned as an important value in the food system, since the consumption of seasonal products implies both a better organoleptic quality and the respect of natural cycles. Furthermore, common opinion was that conventional agricultural techniques, early harvest and the post-harvest treatments, generally, were the main cause of quality loss, not just from the organoleptic, but also from the nutritional point of view. In fact, **safety** was pointed out as one of the primary factors in food consumption: a good food product is one that a “mom can give to his child without worrying whether it is healthy or not” (Interviewed farmer) and that “does not contain poisons” (Interviewed farmer). Accordingly, several respondents stated that an important value was whether the product had been **organically** produced. One expert argued that the industrialized food system lead to the research of agricultural techniques aimed at the production of “attractive” foods on large scale and he highlighted the necessity to turn to the use of techniques that were focused on the protection of soil fertility and the “respect of

nature". Indeed, **safeguarding biodiversity** became a crucial aspect in the definition of the food values, in order to preserve the variety of the products, which are typical of the different Italian regions. Particularly, **protecting the countryside** was defined as a very important aspect, both from the environmental and the social-cultural point of view: *"the respect of natural conditions of the countryside must be considered as an investment in improving our lifestyles, the economy of local farmers and the re-vitalization of rural areas"* (Interviewed expert). Indeed, re-valorisation of the role of farmers and of **rural culture** has been defined as the crucial point in the Italian food system, where the dominance of large retail chains tends more and more to large scale production and does not focus on peculiar characteristics (**typicalities**) of regional production, which represent the strength of products "made in Italy". Therefore, several interviewees argued that **communication** between farmers and consumers or information provided by labels and certifications are essential in a context where consumers are increasingly unaware of and less interested in food traditions. Finally, **price** was mentioned as a value that had a relative importance, but did not outweigh the items previously mentioned; only one consumer suggested price as one of the main attributes in purchasing food. In most of the cases, interviewees agreed on the fact that price had to be consistent with organoleptic characteristics of the product and quality of production techniques used. Therefore, an expert mentioned the slogan of Slow Food: "Buono, Pulito e Giusto" (Good, Clean, and Fair) in order to summarize the values that should be related to food consumption: food products must have a good taste, must comply with food safety regulations and environmental safeguard, and must be purchased at a price that is fair to consumers and profitable for farmers.

#### 4.2. The definition of quality

Most of the interviewees mentioned the word "quality", when they were asked to explain the values related to food products. The concept of quality was mainly interpreted in two different ways: some interviewees tended to be more focused on the definition of intrinsic characteristics such as taste, freshness and seasonality, while others referred mainly to cultural, geographical and environmental factors related to food consumption.

Some experts argued that quality is a subjective concept, it can be interpreted as the *"satisfaction of the needs of those receiving the product"* (Interviewed expert): consumers, for example, tend to look for good taste, flavour, while large retail chains are more interested in characteristics such as colour, standard shape, and long shelf life. In this respect, quality is therefore interpreted as excellence or differentiation according to consumer preferences, but it can also be interpreted as standardization and compliance with customers' contractual requirements. Indeed, quality was also defined as the respect of standards, laws and regulations that control the food system, thus potentially encouraging producers to aim no higher, quality-wise, than the minimum compliance requirements.

Food safety was mentioned as a basic feature or a prerequisite that food products must achieve at every stage of the food supply chain, therefore it should not lead to any differentiation among food products available on the market. In some cases, however, safety has been associated with organic production that, on the other hand, has been identified as a feature strictly related to quality. First of all, it implies the absence of synthetic chemicals, which allegedly alter the taste, flavour and healthiness of products. Secondly, but not less important, the continued use of artificial fertilizers (as it is linked to conventional agriculture) might encourage soil exploitation and damages to the food's nutritional value. Soil protection and use of sustainable agricultural techniques have been mentioned as crucial aspects in giving a definition of quality in the food system, but in this case interviewees, especially farmers and experts, highlighted the importance of the suitability of the land: *"the land must do what it can do"* (interviewed expert). Fruits and

vegetables should be grown in the most favourable soil and climate conditions, animals should be kept living in their natural habitat, their welfare should be respected, and food product processing (cheese and wine production, for example) should be applied where environmental conditions make a particular food product part of the community's traditions. On the basis of these issues, quality can be interpreted as the respect of natural cycles and the safeguard of food typicality.

Regarding organoleptic characteristics, interviewees stated that taste was the main attribute in defining food quality: "*it does not matter whether a product looks perfect, the important thing is that it tastes good!*" (Interviewed consumer). It is necessary to point out that respondents argued that a product is good and healthy when it is fresh, since avoidance of preservatives, and in the specific case of fresh fruits and vegetables, seasonality and sound harvesting time, allow products to develop their authentic aromas and flavours.

#### **4.3. The importance of the origin of food products and attitudes towards Geographical Indications**

The origin of food products was one of the most recurrent factors in relation to the concept of quality. In this paragraph, we will describe motivations that lead interviewees to explain the importance of this issue and their opinion regarding Geographical Indications (GIs).

First of all, they reaffirm the importance of land suitability and potential: soil and environmental conditions of a certain area are crucial for producing particular kind of food products. Respondents suggested the examples of Pachino cherry tomatoes and of Parma ham. A farmer argued that Pachino cherry tomatoes are typical from an area of Sicily where soils are characterized by a high salinity and a very dry climate, and it would be difficult to obtain their typical sweet flavour in different environmental conditions. One expert stated that Parma ham would not achieve its distinctive taste if raw materials were not kept exposed to the right grade of humidity that prevails in the Parma area. Generally, interviewees pointed out the variety of climate conditions in Italy, which determined the presence of different food traditions and their historical value in the different regions. Some experts stated that the process of selection by the population of a certain area, generation by generation, resulted in the best food products that they could obtain. They had learned, over the years, how to grow them and process them. The introduction and development of new varieties may cause confusion in local farmers and, therefore, result in lower quality products. For all these reasons, several informants agreed on the fact that, in a "*world of growing indifference towards food traditions*" (Interviewed expert), it is necessary to educate consumers to "*respect what the land can give*" (Interviewed expert) and to re-discover the value of agriculture's role in the Italian economy. Accordingly, when interviewees were asked their opinion about GI certification, some of them affirmed that this kind of certification may be a starting point for re-building a connection between consumers and land and to the re-evaluation of rural areas. However they highlighted the need to give more information about their function and meaning. Indeed, interviewed consumers affirmed that they could not give their opinion about GI certifications, since their knowledge of these certifications was not sufficient. On the other hand, several interviewees were sceptical regarding this kind of certification for different reasons: (1) they affirmed that it is not difficult to fake a food product, especially when they are unpackaged, (2) origin specification of a product does not provide crucial information such as agricultural techniques and treatments that have been used. One farmer suggested that collective self-certification within a community of farmers would be the appropriate tool to overcome these drawbacks.

It is necessary to point out that in several cases, when interviewees were asked their opinion regarding the importance of food product origin, they referred to proximity. The argument concerning the advantages of shortening the distance where the food is produced and where it is consumed will be the subject of the next section.

#### **4.4. Local food and its role in the food market**

In Italy, "local food" is widely defined as those products defined with the expression "Chilometro Zero" or "Km0" (zero kilometers). General opinion was that this label may be misleading, since it is barely possible to purchase food products that were produced in a range lower than one kilometre (around half a mile). Interviewees stated that "Km0" may have been developed just to persuade consumers to buy these products. Indeed, the interviewed consumers appreciated this expression, they affirmed that it explained clearly the concept of a food product sourced from a nearby location.

Most respondents suggested that the designation of a food product as local was closely related to the distance of the production area from the place of purchase, and that it should be defined in terms of miles; some of them suggested 50 km (30 miles) as a reasonable threshold. On the other hand, it is necessary to point out that, once interviewees had analysed the issue more deeply, they considered that food miles restrictions should depend on the kind of product. Several of them suggested the example of oranges, which are mostly cultivated in the South of Italy (in particular in Sicily and Calabria), but they are typically consumed all over the country, thus implying hundreds of miles of transportation. Interviewees agreed on the fact that in this case Italian oranges could be defined as a local product, whereas non-local products are those coming from other countries, such as Spain or Morocco. The same can be argued for olive oil: the interviewed farmers in Bologna were aware of the fact that very few olive groves were present within a range of 50 km, since the city is located at the northern limit of the natural distribution area of the olive tree. However, acknowledging that extra-virgin olive oil is consumed in significant quantities in the area, they affirmed that, in this case, the original olive oil from Emilia-Romagna, or from neighbouring regions (e.g., Tuscany), could be defined as "local". Indeed, the term has been frequently combined with food traditions and land suitability: Parmigiano-Reggiano (Parmesan) cheese, for example, is produced in an area that includes four different provinces of the region Emilia-Romagna, where similar environmental conditions and land configuration has induced the development of the same culinary traditions. Hence, local food has been valued as a factor linking farmers and food products to a certain area (VANDECANDELAERE *et al.*, 2009) and, especially, bringing farmers closer to consumers. Local food supply is generally limited to forms of short food supply chain such as farmers' markets, CSA, or direct marketing, where consumers come into direct contact with producers. This aspect has been considered crucial in educating consumers to build a connection with their traditions and rural areas. Thanks to the direct communication with farmers, consumers can obtain information about when and how to consume what they buy and especially about the agricultural techniques that have been used. They become an active participant of the local agriculture and are aware of helping the local economy to grow. Indeed, the interviewed farmers stated that these forms of short supply chains are the only ways for small farmers to maintain their business in a food system that is dominated by large retail chains. Local consumption has also been associated with environmental safeguards, because of the reduced need for transportation, and consequently of gas emissions along the supply chain, as well as favouring a reduced use of packaging. Another common opinion was that local products were fresher compared to non-local ones and that the face-to-face

relationships between farmers and consumers were an encouragement for producers to sell higher quality products.

On the other hand, interviewees agreed on the fact that these forms of short food supply chains have some limitations: first of all, food products supplied directly from farmers may be subject to less stringent food safety controls in comparison to conventional food streams. In fact, several small producers who take part in farmers' markets state that they cannot afford certifications. Moreover, Farmers' Markets and services organized by CSA may sometimes take place in periods and locations that are not convenient to consumers. Another inconvenience may be given by the difficulty in providing variety to consumers, supplying them all the kind of foods that a household may need. For these reasons, interviewees were asked about their opinion regarding the possibility, in large retail chains that some food products labelled as "locally produced" could be sold. In most cases, interviewees said they would appreciate this initiative, since it may also represent a way to teach less aware consumers on how to value-enhance their local, seasonal food products and to re-establish a connection with their food traditions. They suggested that a "local food label" should be mainly focused on farmers' identification and it should tell their "story": location and features of the farm where food has been produced, agricultural techniques used, how tradition suggests to consume the product, etc.; one expert suggested that the use of QR-codes may be appropriate to give this kind of information. On the other hand, the opinion of some interviewees was that information given to consumers in this way may not replace the information given directly by farmers. Most of them were sceptical about integrity of food certifications, especially where labels would define the origin of a product. Furthermore, interviewed farmers commented that large retail chains usually request amounts of products that small farmers are often unable to supply and that the reward that large retailers offer is not worth the higher cost of production. Finally, one expert argued that large retail chains would be interested in promoting the consumption of local food products only within the framework of a marketing strategy in which these products were characterized by a local brand owned by the large retail chain.

## 5. CONCLUSIONS

The results show that the meaning of "local" must be explained more in terms of connection to a geographical area than in terms of food miles. Some authors (AMILIEN *et al.*, 2007; BARHAM, 2003; GIOVANNUCCI *et al.*, 2010) suggest that the meaning of local can be associated to Geographical Indications. Our opinion is that the interpretation of "local" should be more related to the concept of belonging to a community within a certain area, where a culinary tradition has been preserved generation after generation. In accordance with BRUNORI's classification regarding local food systems (Brunori, 2007), we would rather associate the concept of Geographical Indications to the definition of "locality food" that is focused on the origin of a product from a particular place, while "local food" is more based on re-valuation of food traditions within a community. Furthermore, according to our results, distance restrictions and tolerance in defining "local" strictly depend on the kind of product. Therefore, the concept of local goes further than simply food miles, in cases when a food product is an expression of the identity of a region or of a country. Indeed, in several cases, respondents associated consumption of local food to re-valorisation of Italian food products and support to the national economy. Accordingly, "local food" labels would differ from "Food Miles" labels, since the latter are mainly associated (perhaps naively, according to CHOLETTE, 2011) to environmental impacts due to food transportation. "Local food" labels, instead, should highlight the

connection between a community and the land it occupies, and provide information not just regarding environmental benefits related to local food consumption, but also regarding support to local economy, and safeguard/conservation of land biodiversity, food traditions and, especially, characteristics and activities of food-producing farms.

The supply of local food products is mainly associated to forms of alternative food networks (KIRWAN, 2004; LA TROBE, 2001; MARTINEZ *et al.*, 2010; D'AMICO *et al.*, 2013) and respondents agreed upon the fact that the introduction of labels which determine the local origin of the products in mainstream food outlets may educate to local consumption even the more "distracted" consumer. Nevertheless, results show that different limitations would affect the supply of locally grown products at large retail chains' outlets. In the first place, general opinion was that consumers do not usually have a good knowledge of the meaning of certifications and the addition of a label may mostly generate confusion between consumers. In the second place, small farmers, who are generally the main actors in supplying local food (GOODMAN, 2004; RENTING *et al.*, 2003) may not be able to satisfy the volume requirements of large retail chains and they may not have the economic advantages that they usually obtain through alternative food networks. Finally, but not less important, quality and quantity of information given by a label could not replace information given by producers, and a lack of direct communication between farmers and consumers would imply a loss of the connection between urban and rural traditions that represents the main issue for local food networks. Local food seems to command a strong experiential content, authenticity, and low standardization of products and services (PENCARELLI *et al.*, 2015) therefore an innovative approach to marketing is required.

In future studies, it would be interesting to propose the same research question in other countries, with different climate conditions, culture and food habits. Our results suggest that, at least in Italy, local is strictly related to food traditions, but diverging cultural environments may induce to the value-enhancement of different food values and, therefore, to a different interpretation of the meaning of local food.

## NOTE

<sup>1</sup>DECRETO 20 Novembre 2007, MINISTERO DELLE POLITICHE AGRICOLE ALIMENTARI E FORESTALI, Attuazione dell'articolo 1, comma 1065, della legge 27 dicembre 2006, n. 296, sui mercati riservati all'esercizio della vendita diretta da parte degli imprenditori agricoli. Gazzetta Ufficiale N. 301 del 29 Dicembre 2007.

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# **SACCHAROMYCES CEREVISIAE BIODIVERSITY IN MONFERRATO, NORTH WEST ITALY, AND SELECTION OF INDIGENOUS STARTER CULTURES FOR BARBERA WINE PRODUCTION**

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## **ABSTRACT**

The aim of this study was to examine the biodiversity of *Saccharomyces cerevisiae* isolates from Barbera grapes and musts, from the Monferrato area, in the Piedmont region – North West Italy. An interdelta element PCR analysis was used to identify and discriminate 636 *S. cerevisiae* isolates at a strain level. Ninety-six *S. cerevisiae* that showed different molecular fingerprints were characterized through physiological tests and laboratory scale fermentations. A chemical analysis of experimental wines obtained from inoculated fermentations showed significant differences between the wines. The main variables considered in the strain differentiation were the residual sugars and the production of acetic acid, which ranged from 148.64 to 3.44 g/l and from 0.20 to 0.60 g/l, respectively. As a consequence, strain variability should be considered as a relevant resource to select suitable starter cultures in order to improve or characterize wines with a close bond to the geographic region.

*Keywords:* *Saccharomyces cerevisiae*, yeast biodiversity, indigenous starter, interdelta PCR, selection

## 1. INTRODUCTION

Wine production is an ancient tradition that has been carried out for centuries through the spontaneous fermentation of grape juice, which takes place due to the presence of indigenous yeasts from different genera and species (FLEET, 2003; PRETORIUS, 2000; ROMANO *et al.*, 2003). The number of species and their presence during fermentation depends on several factors (PRETORIUS *et al.*, 1999), which lead to subsequent wine quality variations from region to region, but also from one year to another, and all this makes the outcome of spontaneous fermentation difficult to predict (PRETORIUS, 2000). In an attempt to address this issue, many winemakers have used pure commercial *Saccharomyces cerevisiae* cultures inoculated into the must (PRETORIUS, 2000). However, it has been suggested that native *S. cerevisiae* strains are better suited to the micro-area climatic conditions of the wine production region (LOPES *et al.*, 2002) and can therefore more easily dominate the natural biota (LA JEUNE *et al.*, 2006).

*S. cerevisiae*, the most relevant species in winemaking, is usually chosen as the wine yeast, and the particular strain is chosen according to a set of physiological features that are indicative of their potential usefulness for industrial wine production. In addition to the primary end products of the glycolytic fermentation of glucose and fructose, certain oenological criteria must be considered in order to select yeast strains that show desirable characteristics, including: tolerance and high ethanol production, exhaustion of the sugar in must and high fermentation activity, growth at high sugar concentrations, high glycerol production, resistance and low sulphur dioxide production, good enzymatic profile (high  $\beta$ -glucosidase and proteolytic activities) and low acetic acid formation (ESTEVE-ZARZOSO *et al.*, 2000).

At present, there is increasing interest, in the wine community, in the use of indigenous *S. cerevisiae* strains that may contribute to the overall sensorial quality of wine and reflect the characteristics of a given region, even in guided fermentations using selected *S. cerevisiae* starter cultures (CAPECE *et al.*, 2010; SUZZI *et al.*, 2012). Recently, in an attempt to respond to these aspects coupled with the current emphasis on the preservation of all forms of genetic biodiversity, some research groups have focused on the selection of yeasts from restricted areas (SETTANNI *et al.*, 2012; FRANCESCA *et al.*, 2009; ORLIC *et al.*, 2007; LOPES *et al.*, 2007).

We have previously extensively studied the indigenous mycobiota originating from the Barbera grapes from the Monferrato area, Piedmont region, North-West Italy (ALESSANDRIA *et al.*, 2015). Barbera grapes produce a ruby-red coloured wine with berry, cherry, plum and spicy flavours, depending on the clone, as well as the location and the age of the plant (BOSSO *et al.*, 2011). In this study, *S. cerevisiae* isolates were characterized to establish their genetic and technological variability in order to contribute to the preservation of the *S. cerevisiae* genetic resources of the Barbera of Monferrato *terroir*. Interdelta-PCR was used to help establish the genetic diversity of the isolates. Physiological tests, which focused on the production of extracellular hydrolytic enzymes and on their growth in different ethanol and total SO<sub>2</sub> concentrations, were then conducted. Finally, selected genotypes were used to ferment Barbera must, during micro-fermentation trials, in order to evaluate their fermentation potential.

## 2. MATERIALS AND METHODS

### 2.1. Fermentation set up and yeast isolation

Spontaneous fermentations were conducted using *Vitis Vinifera* L. Barbera grapes obtained from fifteen different vineyards (Fig. 1), located in five areas in the Asti and Alessandria

districts of the Piedmont region (ALESSANDRIA *et al.*, 2015). The vineyards, which were identified on the basis of their geographical locations, were: 1 (Murisengo), 2 (San Martino Alfieri), 3 (Costigliole d'Asti), 4 (Isola d'Asti), 5 (Montegrosso d'Asti), 6 (Agliaio Terme), 7 (Vinchio), 8 (Nizza Monferrato), 9 (Incisa Scapaccino), 10 (Loazzolo), 11 (Ricaldone), 12 (Alice bel colle), 13 (Acqui -Terme Crocera south west zone), 14 (Acqui Terme - Crocera south est zone) and 15 (Acqui Terme - Dannonna zone).

Approximately 25 kg of healthy grapes, without signs of bird damage or *Botryotinia fuckeliana* infection, were harvested. The grapes were crushed in the laboratory and the obtained juice (about 15 L volume) was transferred to sterile jugs where it underwent spontaneous fermentation at room temperature (between 22 and 25°C). Yeasts were isolated from each container at the beginning of the fermentation (after 1 day and 3 days), in the middle (after 7 days) and when alcoholic fermentation was completed. The alcoholic fermentation was monitored with a densitometer. Aliquots (0.1 mL each) of several decimal dilutions, in a 0.1% ringer solution (Oxoid, Milan, Italy), were plated on Wallerstein Laboratory Nutrient (WLN) medium (Oxoid) (Pallmann *et al.*, 2001). The plates were incubated at 28°C for 5 days. WLN allows the presumptive identification of the yeast species according to the colony morphology and colour (URSO *et al.*, 2008). At least 10 colonies were selected from each sample and at each fermentation stage and were isolated on WLN; priority was given to putative colonies of *Saccharomyces* spp. The isolates were stored at -80 °C in YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose, all obtained from Oxoid) after the addition of glycerol (30%, v/v) (Sigma-Aldrich, Milan, Italy).

## 2.2. Yeast identification

The putative *Saccharomyces* spp. isolates were subsequently identified and simultaneously differentiated at a strain level on the basis of a PCR interdelta element analysis ( $\delta$ -PCR). In order to conduct the  $\delta$ -PCR analysis, the total DNA was extracted from 1 millilitre of an overnight culture in YPD broth, according to COCOLIN *et al.* (2000), quantified using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy) and standardized at 100 ng/ $\mu$ L. The delta12 (5'-TCAACAATGGAATCCCAAC-3') and delta21 (5'-CATCTTAACACCGTATATGA-3') oligonucleotide primers were used to amplify regions between the repeated interspersed delta sequences (Legras and Karst, 2003). Amplification reactions were performed with a PTC-200 DNA Engine MJ Research thermal cycler (Biorad, Milan, Italy) using the following programme: initial denaturation at 95°C (5 min), 35 cycles of denaturing at 94°C (1 min), annealing at 50°C (1 min), extension at 72°C (1 min) and a final extension at 72°C (10 min). The PCR products were separated in 1.5% agarose gels and stained with ethidium bromide. The resulting fingerprints were analyzed by means of the BioNumerics V4.0 software package (Applied-Maths, Sint-Martens-Latem, Belgium). Similarity among the digitized profiles was calculated using the Pearson correlation, and an average linkage (UPGMA) dendrogram was derived from the profiles. A coefficient of correlation of 85% was arbitrarily selected to distinguish the clusters. The yeasts that were not amplified with  $\delta$ -PCR, were subsequently identified by using the RFLP of the ribosomal region method as described in ALESSANDRIA *et al.* (2015).

## 2.3. Physiological characterization

### 2.3.1 Hydrogen sulphite production

The ability to produce hydrogen sulphite was determined by streaking single colonies onto Biggy agar (Oxoid) and incubating them at 25°C for 48-72 h. Colony colour was observed and scored as being white, pale hazel, hazel, dark hazel or black.

### 2.3.2 Enzymatic activities

The esterase, protease and  $\beta$ -glucosidase activities of the isolates were screened as described by ENGLEZOS *et al.* (2015).

### 2.3.3. Ethanol and SO<sub>2</sub> tolerance assays

Ethanol tolerance and SO<sub>2</sub> tolerance were determined in microplates, according to the method proposed by ARROYO-LOPEZ *et al.* (2010) and TOFALO *et al.* (2012), with some modifications. Yeast Nitrogen Base with amino acids (YNB, 6.7 g/L, [Remel, Lenexa, KS, USA]) and pH 5.5 was supplemented with 20 g/L of glucose and sterilized by filtration using a 0.2  $\mu$ m membrane filter (VWR, Milan, Italy). The medium was supplemented with different concentrations of ethanol (Sigma) (final concentrations of 0, 12, 14 and 16% v/v) in order to test for ethanol tolerance, while, in order to establish SO<sub>2</sub> resistance, different amounts of total SO<sub>2</sub> were added (after adjustment to pH 3.0) until final concentrations of 0, 50, 100 and 150 mg/L. Cells for the inoculation were prepared from an overnight culture in 1 mL of YPD medium, centrifuged at 9000 rpm for 10 min. The obtained pellet was washed twice in a sterile salt solution (8 g/L NaCl) and then re-suspended in the same solution to obtain a concentration of about 10<sup>6</sup> cells/mL. The diluted cells (20  $\mu$ L) were mixed with 180  $\mu$ L of YNB, prepared as above. The microplates were incubated at 25 °C and the optical density (OD) was measured at 600 nm using a microtiter plate reader (Savatec Instruments, Torino, Italy) at 24 and 48 hours after an orbital shaking of 30 s, in order to re-suspend the cells in the medium before the measurement. YNB without ethanol or SO<sub>2</sub> was used as the control. Cell growth was determined on the basis of the ratio (%) of OD values obtained in medium with and without ethanol or SO<sub>2</sub> for the specific incubation times. Tests were carried out in triplicate. Isolates with a percentage of growth < 10% were considered sensitive.

## 2.4. Microfermentations

The fermentation potential of the different genotypes was evaluated in microfermentation trials. These were carried out in duplicate for 14 days, in 50 mL tubes containing 25 mL of Barbera grape must (120 g/L glucose, 124 g/L fructose, 5.25 titratable acidity as g/L of tartaric acid, pH 3.20 and 184 mg/L yeast available nitrogen (YAN)). Before the inoculation, the must was thermally treated at 60 °C for 50 min, and the absence of viable populations was evaluated by plating 100  $\mu$ L of the must after the treatment on the WLN medium, followed by incubation at 28 °C for 5 days. Pre-cultures were prepared in must at 25 °C for 24 h, and then used to inoculate each fermentation with a cell concentration of 10<sup>6</sup> per mL, which was determined through a microscopical cell count. The fermentations were carried out under static conditions at 25 °C.

## 2.5. Chemical analysis

After 14 days of fermentation, the sugar consumption (glucose and fructose) and the ethanol, glycerol and acetic acid production were evaluated directly by means of HPLC, according to the method proposed by ROLLE *et al.* (2012). YAN was measured following the protocols reported in ENGLEZOS *et al.*, 2016).

## 2.6. Data analysis

The results of the chemical composition of the wines obtained from the micro-fermentation trials were subjected to a Principal Component Analysis (PCA), in order to evaluate the intraspecific biodiversity of *S. cerevisiae* isolates. Statistical analyses were performed using the IBM SPSS Statistics software package (version 19.0, IBM Corp., Armonk, NY, USA).

## 3. RESULTS AND DISCUSSIONS

The possible association between territory and yeasts is being actively investigated in recent years and is believed to have a positive impact and influence purchase decision-making by the consumer. The use of indigenous selected yeasts could represent a useful alternative to spontaneous fermentation in order to optimize the typical attributes of the grape variety (CLEMENTE JIMENEZ *et al.*, 2004; REMENTERIA *et al.*, 2003; ROMANO *et al.*, 2008). The goal of this study was to isolate and characterize indigenous *S. cerevisiae* yeasts present on Barbera grapes in the vineyards of the Monferrato area.

Fifteen vineyards, located in the Piedmont region (Fig. 1) and cultivated with the Barbera grape variety, were studied during the 2012 harvest season and the collected grapes were crushed to obtain 15 spontaneous alcoholic fermentations.

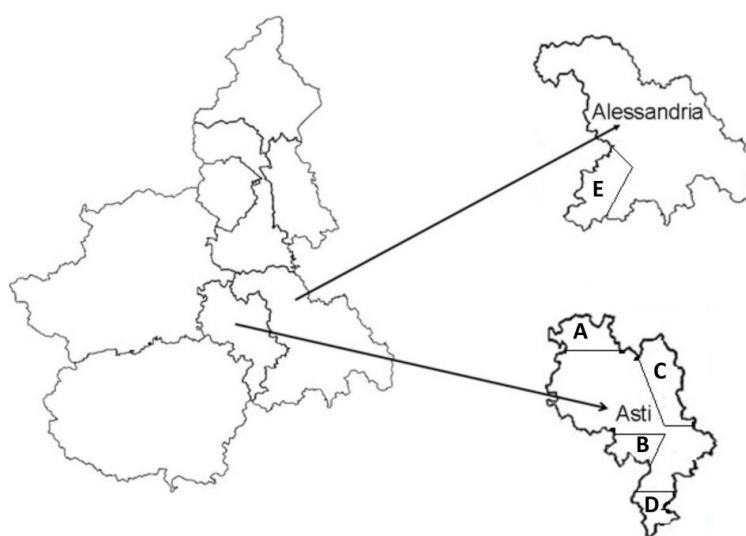


**Figure 1.** Geographic location of the fifteen vineyards in the Monferrato region (Asti and Alessandria) with indication of the 5 areas considered. The distribution of the vineyards in the five areas was as follows: A (Murisengo), B (Montegrosso d'Asti, Costigliole d'Asti, San Martino Alfieri, Agliano Terme, Isola d'Asti), C (Vinchio, Nizza Monferrato, Incisa Scapaccino), D (Loazzolo) and E (Ricaldone, Aquis Terme, Alice bel colle).

Overall, 943 yeast colonies were isolated during the fermentations, and after molecular identification, most of them (636 isolates) were identified, through the use of  $\delta$ -PCR, as *S. cerevisiae*. Other species, namely *Hanseniaspora uvarum* (248 isolates), *Starmerella bacillaris* (synonym *Candida zemplinina*) (11 isolates), *Pichia anomala* (7 isolates) and *Torulaspora delbruecki* (7 isolates) were also isolated, mainly at the beginning and middle of the fermentations.

The PCR amplification of the  $\delta$  interspersed sequences was also used to identify the genetic differences among the *S. cerevisiae* isolated during the fermentations. The molecular fingerprinting analysis, using a coefficient of similarity of 85%, allowed numerous strains among the isolates to be distinguished. The dendrogram resulting from the analysis of 636 *S. cerevisiae* isolates highlighted the presence of 62 clusters and 37 strains, which were unique and did not cluster with any other isolate. The most numerous clusters were: XVII and XLI with 60 and 44 isolates, respectively (Table 1). It is interesting to notice the different level of heterogeneity of *S. cerevisiae* isolated from the different vineyards. For example, most of the isolates from vineyard 1 grouped in one single cluster (XLI), while in other cases (vineyards 12, 13 and 14) a high level of diversity was observed. Only 37 *S. cerevisiae*  $\delta$ -PCR patterns were unique, demonstrating a feeble biodiversity of indigenous *S. cerevisiae* strains in Monferrato area. Considering the ratio between the number of *S. cerevisiae* isolated and the number of observed patterns, as an approximate biodiversity estimation, our results showed similar values to those found in Portugal (SCHULLER *et al.*, 2005) and in France (VALERO *et al.*, 2007).

In order to investigate further the *S. cerevisiae* diversity, 96 strains were selected on the basis of the cluster analysis, and screened for desirable oenological characteristics (ESTEVE-ZARZOSO *et al.*, 2000) such as: a low production of hydrogen sulphide and tolerance to a final concentration of 150 mg/L total sulphur dioxide and 16% (v/v) of ethanol (Table 2). All the strains exhibited a medium hydrogen sulphide production level; 5% of them appeared to be pale hazel on Biggy agar, while the others were hazel. Concerning the results of the tolerance to SO<sub>2</sub>, the selected strains were able to grow in the presence of 50 and 100 mg/L of SO<sub>2</sub> (83% and 60% of the isolates, respectively), while only a few isolates (32%) grew at 150 mg/L of SO<sub>2</sub> within 24 h. Extending the incubation time to 48 h, the number of the isolates that were able to grow at 150 mg/L of SO<sub>2</sub> increased to 63%. Only one strain (ScBa20) was totally inhibited by SO<sub>2</sub>. As far as ethanol tolerance is concerned, 60% of the strains grew at 14% v/v within 24 h. Ethanol mainly affected yeast growth by increasing the lag phase, and this evidence explains why after increasing the incubation time to 48 h, 95% of the strains were able to grow in all the ethanol concentrations (Table 2).  $\beta$ -glucosidase activity was found in only 2.7% of the strains, thus indicating possible production and activity during the fermentation. Protease activity was detected in 37.8% of the tested *S. cerevisiae*, while 21.6% were able to hydrolyse esters (data not shown).

In order to extend the information on the 96 *S. cerevisiae* strains of the Monferrato area, alcoholic fermentations were carried out in Barbera grape must. The experimental wines obtained were analyzed to establish the content of some by-products correlated to the organoleptic quality of the wine and the obtained results are reported in Table 3. The values of the residual sugars ranged from 3.44 to 148.64 g/L. Only seven isolates (ScBa4, ScBa5, ScBa13, ScBa26, ScBa44, ScBa60 and ScBa63) were able to leave less than 5 g/L of residual sugars after 14 days of fermentation. All the strains, except ScBa12, ScBa47, ScBa48 and ScBa57, were capable of consuming almost all the glucose of the must, confirming the glucophylic character of this species. All the yeast strains, that completed the fermentation, formed low amounts of acetic acid in the wines (less than 0.6 g/L). Glycerol production was relatively low, ranging from 5.20 to 7.86 g/L. The fermentation purity was high; most strains had a low ratio between the produced acetic acid and ethanol (range 0.01-0.09) and only three strains showed values above 0.04. As regards ethanol production, 52% of the strains produced more than 13%, and 6% of them managed to develop more than 14%.

**Table 1.** Clusters obtained from a comparison of the different fingerprinting profiles of the *S. cerevisiae* isolates examined in this study by means of the molecular technique. The arbitrarily selected coefficient of similarity was 85%. The table shows their composition according to the geographical locations (vineyard) from which the isolates were obtained.

| Cluster | Number of strains in the cluster | Monferrato's vineyards |    |   |   |    |   |   |   |   |    |    |    |    |    |    |
|---------|----------------------------------|------------------------|----|---|---|----|---|---|---|---|----|----|----|----|----|----|
|         |                                  | 1                      | 2  | 3 | 4 | 5  | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| I       | 5                                | /                      | /  | / | / | /  | / | / | / | / | /  | 5  | /  | /  | /  | /  |
| II      | 15                               | /                      | /  | / | / | 11 | / | 4 | / | / | /  | /  | /  | /  | /  | /  |
| III     | 14                               | /                      | /  | 6 | / | /  | / | 4 | / | / | /  | 2  | /  | 2  | /  | /  |
| IV      | 6                                | /                      | /  | 6 | / | /  | / | / | / | / | /  | /  | /  | /  | /  | /  |
| V       | 3                                | /                      | 3  | / | / | /  | / | / | / | / | /  | /  | /  | /  | /  | /  |
| VI      | 6                                | /                      | /  | / | / | /  | / | / | / | / | 2  | /  | /  | /  | 3  | 1  |
| VII     | 2                                | /                      | /  | / | / | /  | / | / | / | / | /  | /  | /  | /  | /  | 2  |
| VIII    | 24                               | /                      | 14 | 5 | / | /  | / | / | 2 | / | /  | /  | /  | 1  | /  | 2  |
| IX      | 4                                | /                      | 4  | / | / | /  | / | / | / | / | /  | /  | /  | /  | /  | /  |
| X       | 7                                | /                      | /  | / | / | 3  | / | / | / | / | /  | 4  | /  | /  | /  | /  |
| XI      | 10                               | /                      | /  | / | / | /  | / | / | / | 3 | /  | /  | /  | 7  | /  | /  |
| XII     | 8                                | /                      | /  | / | / | /  | / | / | / | 4 | /  | /  | /  | 4  | /  | /  |
| XIII    | 7                                | /                      | 7  | / | / | /  | / | / | / | / | /  | /  | /  | /  | /  | /  |
| XIV     | 18                               | /                      | 4  | / | / | 10 | / | / | / | / | /  | 1  | /  | 2  | 1  | /  |
| XV      | 4                                | /                      | /  | / | / | /  | 2 | / | / | / | /  | /  | /  | 2  | /  | /  |
| XVI     | 2                                | /                      | /  | / | / | /  | / | / | / | / | 1  | /  | /  | /  | 1  | /  |
| XVII    | 60                               | 6                      | 4  | 3 | 2 | 1  | / | 3 | 8 | / | 2  | /  | 2  | 5  | 9  | 15 |
| XVIII   | 6                                | /                      | /  | / | / | /  | / | 2 | 2 | / | /  | /  | /  |    | 2  | /  |
| XIX     | 10                               | /                      | /  | 4 | / | /  | / | / | / | / | /  | /  | /  | 4  | /  | 2  |
| XX      | 15                               | 1                      | /  | / | 9 | 2  | / | / | / | / | /  | /  | /  | /  | 2  | 1  |
| XXI     | 13                               | /                      | /  | / | 2 | 2  | / | / | 2 | / | 2  | /  | /  | 2  | 2  | 1  |
| XXII    | 28                               | 2                      | /  | 1 | / | /  | / | / | 1 | / | /  | /  | 1  | 1  | 4  | 18 |
| XXIII   | 2                                | /                      | /  | 1 | / | /  | / | / | / | / | /  | /  | /  | 1  | /  | /  |
| XXIV    | 3                                | /                      | /  | / | / | /  | / | / | / | / | 3  | /  | /  | /  | /  | /  |
| XXV     | 3                                | /                      | /  | / | / | /  | / | / | / | / | 3  | /  | /  | /  | /  | /  |
| XXVI    | 4                                | /                      | /  | / | / | /  | / | / | / | / | /  | /  | /  | /  | /  | 4  |
| XXVII   | 5                                | /                      | /  | 3 | / | /  | / | / | / | / | /  | /  | /  | /  | 1  | 1  |
| XVIII   | 2                                | /                      | /  | / | / | /  | / | / | / | / | /  | /  | /  | 2  | /  | /  |
| XXIX    | 10                               | /                      | /  | / | / | /  | / | / | / | / | /  | /  | /  | 10 | /  | /  |
| XXX     | 6                                | /                      | /  | / | / | /  | / | / | / | / | /  | /  | /  | 3  | 3  | /  |
| XXXI    | 7                                | /                      | /  | / | / | /  | / | / | / | / | 3  | /  | /  | 3  | /  | 1  |
| XXXII   | 3                                | 1                      | /  | / | / | /  | / | / | / | / | 2  | /  | /  | /  | /  | /  |
| XXXIII  | 6                                | /                      | /  | / | / | /  | / | / | / | / | /  | /  | 5  | 1  | /  | /  |
| XXXIV   | 5                                | /                      | /  | / | / | /  | / | / | / | / | /  | /  | 5  | /  | /  | /  |
| XXXV    | 3                                | /                      | /  | / | 1 | /  | / | / | / | / | /  | /  | /  | 2  | /  | /  |
| XXXVI   | 5                                | /                      | /  | / | / | /  | 5 | / | / | / | /  | /  | /  | /  | /  | /  |
| XXXVII  | 9                                | /                      | /  | / | 2 | /  | / | / | 2 | / | 4  | /  | /  | /  | /  | 1  |
| XXXVIII | 4                                | /                      | /  | / | / | /  | / | / | / | / | /  | /  | /  | /  | /  | 4  |

**Table 1.** Continues.

| Cluster | Number of strains<br>in the cluster | Monferrato's vineyards |   |   |   |   |    |    |   |   |    |    |    |    |    |    |
|---------|-------------------------------------|------------------------|---|---|---|---|----|----|---|---|----|----|----|----|----|----|
|         |                                     | 1                      | 2 | 3 | 4 | 5 | 6  | 7  | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| XXXIX   | 2                                   | /                      | / | / | / | / | /  | /  | 2 | / | /  | /  | /  | /  | /  | /  |
| XL      | 7                                   | /                      | / | / | / | / | /  | /  | / | / | /  | /  | /  | 7  | /  | /  |
| XLI     | 44                                  | 38                     | / | / | / | / | /  | /  | / | / | /  | /  | 3  | 3  | /  | /  |
| XLII    | 7                                   | /                      | / | / | / | / | 2  | /  | 1 | 4 | /  | /  | /  | /  | /  | /  |
| XLIII   | 9                                   | /                      | / | 5 | / | / | /  | /  | / | 1 | /  | /  | 2  | /  | 1  | /  |
| XLIV    | 7                                   | /                      | / | / | / | / | /  | /  | 3 | 3 | 1  | /  | /  | /  | /  | /  |
| XLV     | 6                                   | /                      | / | / | / | / | /  | /  | / | / | /  | /  | /  | 6  | /  | /  |
| XLVI    | 5                                   | /                      | / | / | / | / | /  | 5  | / | / | /  | /  | /  | /  | /  | /  |
| XLVII   | 10                                  | /                      | / | / | 9 | / | /  | /  | / | / | /  | /  | /  | 1  | /  | /  |
| XLVIII  | 15                                  | /                      | / | / | / | / | /  | /  | / | / | /  | 13 | 2  | /  | /  | /  |
| XLIX    | 6                                   | /                      | / | / | / | 2 | 4  | /  | / | / | /  | /  | /  | /  | /  | /  |
| L       | 10                                  | /                      | 3 | / | / | / | /  | /  | 6 | / | 1  | /  | /  | /  | /  | /  |
| LI      | 4                                   | /                      | / | / | / | / | /  | /  | / | 3 | /  | 1  | /  | /  | /  | /  |
| LII     | 30                                  | /                      | / | / | / | 2 | 16 | 10 | / | / | /  | /  | /  | 2  | /  | /  |
| LIII    | 12                                  | /                      | / | / | / | 2 | /  | 7  | / | / | /  | /  | 1  | /  | 2  | /  |
| LIV     | 2                                   | /                      | / | / | / | / | /  | /  | / | / | /  | /  | /  | /  | 2  | /  |
| LV      | 10                                  | /                      | / | / | / | / | /  | /  | / | / | /  | /  | /  | /  | /  | 10 |
| LVI     | 5                                   | /                      | / | / | / | 3 | 2  | /  | / | / | /  | /  | /  | /  | /  | /  |
| LVII    | 19                                  | 2                      | / | / | / | 2 | /  | /  | 1 | / | /  | /  | /  | 6  | 8  | /  |
| LVIII   | 15                                  | /                      | / | / | / | 3 | /  | /  | / | / | 2  | /  | 10 | /  | /  | /  |
| LIX     | 25                                  | /                      | / | / | / | / | 5  | 7  | / | / | /  | /  | 1  | /  | 12 | /  |
| LX      | 22                                  | /                      | / | / | / | / | /  | /  | 1 | / | 7  | /  | /  | 12 | 1  | 1  |
| LXI     | 3                                   | /                      | / | / | / | / | /  | /  | / | / | 1  | /  | /  | 2  | /  | /  |
| LXII    | 17                                  | /                      | 3 | / | / | / | /  | 1  | / | / | /  | 4  | 8  | /  | 1  | /  |

**Table 2.** Results of the resistance to ethanol and SO<sub>2</sub> of the tested strains. The values presented are the ratio between the OD of the isolates in broth with and without ethanol or SO<sub>2</sub> times 100 at the specific incubation times. The values are the means of triplicate experiments.

| Strains | SO <sub>2</sub> growth (mg/L) |     |     |                        |     |     | Ethanol growth (% vol.) |    |     |                        |     |     |
|---------|-------------------------------|-----|-----|------------------------|-----|-----|-------------------------|----|-----|------------------------|-----|-----|
|         | 24 hours of incubation        |     |     | 48 hours of incubation |     |     | 24 hours of incubation  |    |     | 48 hours of incubation |     |     |
|         | 50                            | 100 | 150 | 50                     | 100 | 150 | 12                      | 14 | 16  | 12                     | 14  | 16  |
| ScBa1   | 36                            | 6   | 14  | 81                     | 74  | 81  | 0                       | 0  | 0   | 64                     | 0   | 0   |
| ScBa2   | 82                            | 87  | 71  | 93                     | 99  | 89  | 100                     | 93 | 100 | 100                    | 100 | 100 |
| ScBa3   | 81                            | 46  | 36  | 100                    | 91  | 100 | 36                      | 6  | 7   | 97                     | 47  | 0   |
| ScBa4   | 34                            | 3   | 4   | 100                    | 72  | 36  | 2                       | 1  | 1   | 17                     | 0   | 0   |
| ScBa5   | 81                            | 46  | 36  | 98                     | 99  | 96  | 36                      | 6  | 7   | 98                     | 73  | 4   |
| ScBa6   | 44                            | 46  | 16  | 87                     | 67  | 52  | 11                      | 1  | 1   | 13                     | 1   | 1   |
| ScBa7   | 58                            | 48  | 42  | 95                     | 88  | 61  | 9                       | 4  | 4   | 90                     | 6   | 5   |
| ScBa8   | 76                            | 49  | 42  | 98                     | 94  | 93  | 8                       | 4  | 5   | 85                     | 7   | 5   |
| ScBa9   | 70                            | 42  | 43  | 96                     | 92  | 94  | 5                       | 4  | 5   | 72                     | 4   | 5   |
| ScBa10  | 26                            | 15  | 9   | 70                     | 69  | 60  | 19                      | 1  | 0   | 70                     | 0   | 0   |
| ScBa11  | 22                            | 6   | 6   | 87                     | 83  | 10  | 2                       | 1  | 1   | 5                      | 0   | 0   |
| ScBa12  | 9                             | 6   | 2   | 44                     | 7   | 7   | 4                       | 2  | 2   | 3                      | 1   | 1   |
| ScBa13  | 60                            | 32  | 32  | 99                     | 93  | 83  | 9                       | 3  | 3   | 97                     | 3   | 3   |
| ScBa14  | 38                            | 2   | 1   | 85                     | 55  | 16  | 51                      | 1  | 0   | 61                     | 0   | 0   |
| ScBa15  | 26                            | 3   | 3   | 72                     | 30  | 14  | 49                      | 0  | 0   | 58                     | 0   | 0   |
| ScBa16  | 2                             | 4   | 23  | 6                      | 40  | 77  | 51                      | 0  | 0   | 61                     | 0   | 0   |
| ScBa17  | 12                            | 0   | 0   | 91                     | 54  | 17  | 37                      | 0  | 0   | 76                     | 0   | 1   |
| ScBa18  | 47                            | 0   | 0   | 55                     | 9   | 0   | 1                       | 1  | 0   | 38                     | 0   | 0   |
| ScBa19  | 87                            | 58  | 52  | 85                     | 84  | 75  | 40                      | 0  | 0   | 77                     | 0   | 0   |
| ScBa20  | 63                            | 0   | 0   | 64                     | 2   | 0   | 0                       | 0  | 0   | 13                     | 0   | 0   |
| ScBa21  | 52                            | 31  | 46  | 60                     | 59  | 50  | 55                      | 0  | 0   | 83                     | 0   | 0   |
| ScBa22  | 96                            | 91  | 83  | 100                    | 97  | 96  | 100                     | 95 | 97  | 98                     | 93  | 99  |
| ScBa23  | 67                            | 63  | 54  | 96                     | 66  | 40  | 96                      | 70 | 71  | 100                    | 92  | 100 |
| ScBa24  | 28                            | 16  | 8   | 85                     | 87  | 78  | 100                     | 69 | 75  | 100                    | 71  | 84  |
| ScBa25  | 76                            | 76  | 75  | 95                     | 82  | 58  | 73                      | 71 | 52  | 81                     | 67  | 57  |
| ScBa26  | 39                            | 31  | 21  | 74                     | 40  | 22  | 79                      | 17 | 8   | 69                     | 65  | 66  |
| ScBa27  | 14                            | 11  | 10  | 74                     | 40  | 22  | 67                      | 59 | 59  | 69                     | 65  | 66  |
| ScBa28  | 83                            | 65  | 62  | 88                     | 86  | 76  | 92                      | 83 | 71  | 100                    | 94  | 90  |
| ScBa29  | 95                            | 81  | 65  | 100                    | 98  | 89  | 89                      | 77 | 58  | 100                    | 92  | 83  |
| ScBa30  | 44                            | 33  | 7   | 88                     | 91  | 38  | 69                      | 55 | 64  | 71                     | 63  | 68  |
| ScBa31  | 100                           | 90  | 86  | 100                    | 98  | 99  | 80                      | 52 | 1   | 90                     | 78  | 0   |
| ScBa32  | 100                           | 84  | 83  | 100                    | 97  | 98  | 99                      | 90 | 5   | 100                    | 98  | 7   |
| ScBa33  | 100                           | 81  | 75  | 100                    | 98  | 97  | 98                      | 90 | 5   | 99                     | 97  | 1   |

|        |     |    |     |     |     |     |     |     |     |     |     |     |
|--------|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ScBa34 | 45  | 37 | 10  | 97  | 87  | 67  | 77  | 50  | 1   | 79  | 63  | 0   |
| ScBa35 | 36  | 22 | 28  | 50  | 39  | 30  | 44  | 32  | 27  | 87  | 83  | 67  |
| ScBa36 | 65  | 62 | 33  | 100 | 95  | 84  | 96  | 83  | 86  | 88  | 84  | 87  |
| ScBa37 | 66  | 32 | 23  | 100 | 68  | 65  | 100 | 95  | 97  | 100 | 100 | 100 |
| ScBa38 | 70  | 43 | 29  | 100 | 70  | 66  | 100 | 98  | 100 | 100 | 98  | 100 |
| ScBa39 | 60  | 34 | 28  | 100 | 96  | 98  | 97  | 86  | 73  | 82  | 67  | 44  |
| ScBa40 | 27  | 9  | 14  | 100 | 99  | 98  | 92  | 87  | 78  | 100 | 96  | 98  |
| ScBa41 | 25  | 8  | 7   | 72  | 30  | 14  | 51  | 28  | 0   | 87  | 44  | 1   |
| ScBa42 | 24  | 14 | 20  | 100 | 97  | 97  | 96  | 91  | 74  | 99  | 96  | 90  |
| ScBa43 | 43  | 18 | 21  | 100 | 98  | 86  | 80  | 62  | 13  | 99  | 92  | 75  |
| ScBa44 | 66  | 55 | 38  | 89  | 86  | 83  | 100 | 96  | 99  | 100 | 98  | 100 |
| ScBa45 | 52  | 35 | 25  | 88  | 89  | 77  | 99  | 91  | 85  | 100 | 93  | 91  |
| ScBa46 | 40  | 26 | 22  | 98  | 90  | 76  | 76  | 77  | 86  | 93  | 88  | 94  |
| ScBa47 | 29  | 28 | 9   | 100 | 64  | 70  | 83  | 0   | 0   | 100 | 100 | 100 |
| ScBa48 | 14  | 4  | 6   | 69  | 63  | 46  | 90  | 27  | 4   | 100 | 100 | 100 |
| ScBa49 | 59  | 53 | 52  | 90  | 85  | 76  | 98  | 89  | 85  | 97  | 88  | 86  |
| ScBa50 | 57  | 50 | 44  | 77  | 86  | 88  | 95  | 94  | 89  | 95  | 96  | 95  |
| ScBa51 | 58  | 51 | 55  | 80  | 83  | 70  | 100 | 91  | 85  | 100 | 89  | 87  |
| ScBa52 | 100 | 96 | 100 | 99  | 96  | 99  | 100 | 99  | 100 | 100 | 98  | 100 |
| ScBa53 | 91  | 91 | 87  | 90  | 91  | 87  | 91  | 88  | 91  | 92  | 93  | 93  |
| ScBa54 | 100 | 98 | 100 | 100 | 97  | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| ScBa55 | 100 | 98 | 99  | 100 | 99  | 99  | 100 | 100 | 100 | 100 | 100 | 100 |
| ScBa56 | 98  | 73 | 90  | 100 | 95  | 99  | 100 | 100 | 99  | 100 | 100 | 99  |
| ScBa57 | 20  | 12 | 13  | 70  | 16  | 11  | 100 | 93  | 63  | 100 | 100 | 100 |
| ScBa58 | 72  | 19 | 12  | 100 | 88  | 71  | 100 | 98  | 92  | 100 | 100 | 96  |
| ScBa59 | 80  | 45 | 27  | 100 | 98  | 99  | 100 | 98  | 96  | 100 | 98  | 96  |
| ScBa60 | 70  | 76 | 53  | 100 | 100 | 96  | 97  | 77  | 92  | 100 | 100 | 100 |
| ScBa61 | 86  | 87 | 62  | 99  | 97  | 98  | 100 | 95  | 81  | 100 | 100 | 91  |
| ScBa62 | 88  | 69 | 73  | 100 | 98  | 100 | 96  | 94  | 90  | 100 | 94  | 87  |
| ScBa63 | 80  | 54 | 49  | 100 | 100 | 97  | 98  | 98  | 92  | 100 | 99  | 93  |
| ScBa64 | 86  | 49 | 31  | 100 | 100 | 98  | 96  | 96  | 91  | 98  | 96  | 90  |
| ScBa65 | 100 | 93 | 94  | 100 | 100 | 100 | 100 | 95  | 100 | 100 | 96  | 99  |
| ScBa66 | 35  | 12 | 15  | 67  | 50  | 61  | 95  | 92  | 72  | 95  | 95  | 88  |
| ScBa67 | 30  | 7  | 15  | 70  | 70  | 66  | 95  | 83  | 61  | 100 | 97  | 92  |
| ScBa68 | 36  | 6  | 8   | 72  | 68  | 65  | 99  | 99  | 97  | 100 | 100 | 100 |
| ScBa69 | 25  | 7  | 8   | 79  | 70  | 73  | 100 | 100 | 100 | 100 | 100 | 100 |
| ScBa70 | 65  | 79 | 42  | 79  | 82  | 61  | 100 | 97  | 83  | 100 | 99  | 85  |
| ScBa71 | 18  | 35 | 18  | 70  | 74  | 71  | 97  | 81  | 78  | 100 | 95  | 88  |
| ScBa72 | 80  | 77 | 42  | 100 | 93  | 78  | 100 | 96  | 96  | 100 | 100 | 99  |

|        |     |     |     |     |     |     |     |     |     |     |     |     |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ScBa73 | 54  | 59  | 32  | 83  | 81  | 73  | 97  | 96  | 85  | 100 | 100 | 100 |
| ScBa74 | 93  | 86  | 68  | 100 | 98  | 91  | 100 | 95  | 84  | 100 | 97  | 100 |
| ScBa75 | 57  | 59  | 59  | 89  | 55  | 56  | 66  | 66  | 51  | 66  | 67  | 53  |
| ScBa76 | 88  | 85  | 86  | 86  | 83  | 84  | 97  | 97  | 74  | 97  | 99  | 79  |
| ScBa77 | 87  | 85  | 85  | 84  | 82  | 86  | 95  | 88  | 71  | 100 | 92  | 76  |
| ScBa78 | 100 | 99  | 95  | 99  | 97  | 97  | 100 | 99  | 99  | 100 | 100 | 100 |
| ScBa79 | 100 | 100 | 99  | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| ScBa80 | 91  | 92  | 88  | 90  | 91  | 88  | 83  | 70  | 65  | 88  | 71  | 66  |
| ScBa81 | 81  | 72  | 43  | 100 | 97  | 98  | 100 | 96  | 93  | 100 | 100 | 100 |
| ScBa82 | 74  | 79  | 63  | 100 | 97  | 100 | 100 | 92  | 98  | 100 | 97  | 100 |
| ScBa83 | 100 | 99  | 99  | 100 | 99  | 99  | 100 | 100 | 100 | 100 | 100 | 100 |
| ScBa84 | 84  | 84  | 86  | 84  | 84  | 86  | 87  | 69  | 62  | 93  | 72  | 63  |
| ScBa85 | 92  | 90  | 91  | 93  | 91  | 92  | 99  | 86  | 75  | 100 | 97  | 93  |
| ScBa86 | 91  | 93  | 93  | 91  | 92  | 93  | 100 | 100 | 100 | 100 | 100 | 100 |
| ScBa87 | 92  | 91  | 92  | 89  | 87  | 88  | 86  | 71  | 64  | 91  | 70  | 62  |
| ScBa88 | 91  | 86  | 90  | 91  | 86  | 90  | 99  | 92  | 83  | 100 | 96  | 89  |
| ScBa89 | 93  | 83  | 89  | 100 | 96  | 88  | 100 | 97  | 97  | 100 | 100 | 100 |
| ScBa90 | 97  | 95  | 99  | 99  | 96  | 100 | 100 | 96  | 90  | 100 | 100 | 92  |
| ScBa91 | 80  | 84  | 63  | 93  | 95  | 86  | 100 | 100 | 92  | 100 | 100 | 100 |
| ScBa92 | 99  | 90  | 100 | 99  | 96  | 100 | 100 | 99  | 100 | 100 | 100 | 100 |
| ScBa93 | 100 | 79  | 72  | 100 | 75  | 68  | 100 | 98  | 99  | 100 | 100 | 100 |
| ScBa94 | 100 | 100 | 100 | 100 | 100 | 99  | 100 | 100 | 100 | 100 | 100 | 99  |
| ScBa95 | 100 | 98  | 94  | 100 | 99  | 95  | 100 | 85  | 86  | 100 | 86  | 88  |
| ScBa96 | 100 | 90  | 65  | 100 | 90  | 66  | 100 | 97  | 97  | 100 | 98  | 99  |

**Table 3.** Chemical analysis of the wines obtained from the fermentation of the pure indigenous *S. cerevisiae* cultures. The data are means±standard deviations. With \* were reported strains which were unique and did not cluster with any other isolate.

| Strains | Vineyard | Cluster | Residual glucose (g/L) | Residual fructose (g/L) | Glycerol (g/L) | Acetic acid (g/L) | Ethanol (%v/v) | Fermentation purity <sup>a</sup> | Ethanol yield <sup>b</sup> | Glycerol yield <sup>c</sup> | Acetic acid yield <sup>d</sup> |
|---------|----------|---------|------------------------|-------------------------|----------------|-------------------|----------------|----------------------------------|----------------------------|-----------------------------|--------------------------------|
| ScBa1   | 11       | I       | 2.18±0.54              | 24.23±2.14              | 7.00±0.15      | 0.45±0.00         | 12.57±0.19     | 0.036±0.001                      | 0.058±0.001                | 0.032±0.001                 | 0.0359±0.0008                  |
| ScBa2   | 13       | *       | 0.88±0.07              | 8.68±0.80               | 6.86±0.05      | 0.51±0.02         | 13.77±0.05     | 0.037±0.001                      | 0.059±0.001                | 0.029±0.001                 | 0.0367±0.0012                  |
| ScBa3   | 5        | II      | 0.65±0.08              | 4.43±1.04               | 7.01±0.01      | 0.56±0.01         | 14.11±0.11     | 0.039±0.001                      | 0.059±0.001                | 0.029±0.001                 | 0.0395±0.0005                  |
| ScBa4   | 7        | *       | 0.68±0.01              | 2.77±1.47               | 7.42±0.82      | 0.29±0.03         | 14.14±0.11     | 0.021±0.002                      | 0.059±0.001                | 0.031±0.003                 | 0.0208±0.0023                  |
| ScBa5   | 7        | III     | 0.38±0.27              | 3.08±1.84               | 6.89±0.10      | 0.30±0.05         | 14.27±0.01     | 0.021±0.004                      | 0.059±0.001                | 0.029±0.001                 | 0.0207±0.0037                  |
| ScBa6   | 7        | *       | 1.14±0.37              | 6.57±2.71               | 7.08±0.03      | 0.31±0.07         | 13.89±0.35     | 0.022±0.006                      | 0.059±0.001                | 0.026±0.001                 | 0.0224±0.0056                  |
| ScBa7   | 3        | IV      | 1.58±0.73              | 12.15±1.93              | 6.92±0.16      | 0.33±0.00         | 13.82±0.03     | 0.024±0.001                      | 0.060±0.001                | 0.030±0.001                 | 0.0240±0.001                   |
| ScBa8   | 2        | V       | 2.98±3.16              | 16.78±18.98             | 6.54±0.43      | 0.47±0.34         | 12.60±0.90     | 0.037±0.025                      | 0.056±0.002                | 0.029±0.005                 | 0.0367±0.0247                  |
| ScBa9   | 5        | *       | 0.69±0.25              | 5.68±4.83               | 7.07±0.01      | 0.29±0.02         | 13.73±0.20     | 0.021±0.001                      | 0.058±0.002                | 0.030±0.001                 | 0.0215±0.0014                  |
| ScBa10  | 10       | VI      | 1.65±0.49              | 15.33±2.45              | 6.81±0.14      | 0.41±0.04         | 13.46±0.13     | 0.031±0.002                      | 0.059±0.001                | 0.030±0.001                 | 0.0307±0.0025                  |
| ScBa11  | 15       | VII     | 1.11±0.96              | 11.44±10.36             | 7.44±0.08      | 0.35±0.01         | 13.46±0.58     | 0.026±0.001                      | 0.058±0.001                | 0.032±0.001                 | 0.026±0.0007                   |
| ScBa12  | 2        | VIII    | 78.13±0.19             | 70.52±0.15              | 5.20±0.10      | 0.43±0.01         | 4.90±0.09      | 0.088±0.001                      | 0.051±0.001                | 0.054±0.001                 | 0.0879±0.0001                  |
| ScBa13  | 2        | IX      | 0.82±0.25              | 2.80±0.68               | 6.63±0.08      | 0.20±0.02         | 13.87±0.28     | 0.014±0.002                      | 0.058±0.001                | 0.028±0.001                 | 0.0144±0.0015                  |
| ScBa14  | 11       | X       | 1.14±0.72              | 13.37±5.12              | 6.99±0.07      | 0.29±0.03         | 13.59±0.45     | 0.021±0.003                      | 0.059±0.001                | 0.030±0.001                 | 0.0213±0.0033                  |
| ScBa15  | 14       | XI      | 2.88±0.61              | 28.44±1.79              | 7.10±0.03      | 0.44±0.01         | 12.38±0.05     | 0.035±0.001                      | 0.058±0.001                | 0.033±0.001                 | 0.0352±0.0008                  |
| ScBa16  | 14       | XII     | 3.71±0.58              | 28.71±1.54              | 6.82±0.22      | 0.38±0.04         | 12.04±0.14     | 0.032±0.003                      | 0.057±0.001                | 0.032±0.001                 | 0.0319±0.0028                  |
| ScBa17  | 2        | XIII    | 1.09±0.07              | 6.93±0.34               | 7.37±0.01      | 0.39±0.01         | 14.03±0.03     | 0.028±0.001                      | 0.059±0.001                | 0.031±0.001                 | 0.0275±0.0004                  |
| ScBa18  | 2        | *       | 0.89±0.37              | 11.64±5.35              | 6.46±0.65      | 0.41±0.18         | 13.00±0.32     | 0.031±0.013                      | 0.056±0.001                | 0.028±0.004                 | 0.0314±0.0129                  |
| ScBa19  | 5        | XIV     | 0.83±0.23              | 8.09±5.27               | 6.85±0.09      | 0.36±0.05         | 13.34±0.71     | 0.028±0.006                      | 0.056±0.004                | 0.029±0.001                 | 0.0275±0.0055                  |
| ScBa20  | 14       | XV      | 0.63±0.24              | 7.80±2.79               | 7.26±0.02      | 0.41±0.00         | 13.89±0.07     | 0.029±0.001                      | 0.060±0.001                | 0.031±0.001                 | 0.0292±0.0004                  |
| ScBa21  | 10       | XVI     | 1.58±0.21              | 10.27±2.32              | 6.72±0.04      | 0.44±0.06         | 13.40±0.15     | 0.030±0.001                      | 0.0059±0.001               | 0.030±0.001                 | 0.0234±0.003                   |
| ScBa22  | 15       | XVII    | 1.54±0.62              | 17.88±4.13              | 7.88±0.12      | 0.38±0.04         | 12.88±0.14     | 0.030±0.004                      | 0.057±0.001                | 0.035±0.001                 | 0.0297±0.0037                  |
| ScBa23  | 13       | *       | 1.04±0.65              | 13.68±5.92              | 7.35±0.04      | 0.34±0.01         | 13.21±0.26     | 0.026±0.001                      | 0.058±0.001                | 0.032±0.001                 | 0.026±0.0012                   |
| ScBa24  | 14       | *       | 1.18±0.04              | 15.75±0.21              | 7.13±0.20      | 0.34±0.01         | 13.14±0.19     | 0.026±0.001                      | 0.058±0.001                | 0.031±0.001                 | 0.0261±0.0007                  |
| ScBa25  | 7        | *       | 3.86±1.76              | 22.9±5.06               | 6.81±0.17      | 0.26±0.01         | 12.82±0.71     | 0.021±0.001                      | 0.059±0.001                | 0.031±0.001                 | 0.0206±0.0006                  |

|        |    |         |            |            |           |           |            |             |             |             |               |
|--------|----|---------|------------|------------|-----------|-----------|------------|-------------|-------------|-------------|---------------|
| ScBa26 | 7  | XVIII   | 0.69±0.08  | 4.20±1.12  | 7.13±0.21 | 0.25±0.04 | 13.96±0.22 | 0.018±0.003 | 0.058±0.001 | 0.030±0.001 | 0.0181±0.0035 |
| ScBa27 | 14 | XIX     | 0.73±0.48  | 9.18±6.55  | 6.88±0.13 | 0.24±0.04 | 13.72±0.64 | 0.017±0.004 | 0.059±0.001 | 0.029±0.001 | 0.0174±0.0037 |
| ScBa28 | 4  | XX      | 0.75±0.10  | 7.31±2.50  | 6.65±0.12 | 0.32±0.07 | 13.68±0.30 | 0.024±0.006 | 0.058±0.001 | 0.028±0.001 | 0.0237±0.0058 |
| ScBa29 | 5  | XXI     | 1.84±0.13  | 16.38±0.98 | 6.80±0.11 | 0.41±0.05 | 13.32±0.20 | 0.031±0.004 | 0.059±0.001 | 0.030±0.001 | 0.0307±0.0043 |
| ScBa30 | 15 | XXII    | 5.12±2.03  | 31.28±5.30 | 6.65±0.03 | 0.44±0.01 | 11.8±0.54  | 0.037±0.001 | 0.057±0.001 | 0.032±0.001 | 0.037±0.0008  |
| ScBa31 | 2  | XXIII   | 1.15±0.44  | 11.07±4.09 | 6.59±0.01 | 0.40±0.06 | 13.56±0.41 | 0.029±0.005 | 0.058±0.001 | 0.028±0.001 | 0.0293±0.0051 |
| ScBa32 | 10 | XXIV    | 0.96±0.47  | 6.09±0.38  | 7.03±0.84 | 0.37±0.02 | 13.67±0.17 | 0.027±0.002 | 0.058±0.001 | 0.03±0.004  | 0.0269±0.0015 |
| ScBa33 | 10 | XXV     | 0.65±0.17  | 5.69±1.43  | 6.42±0.17 | 0.44±0.03 | 13.97±0.27 | 0.031±0.003 | 0.059±0.002 | 0.027±0.001 | 0.0313±0.003  |
| ScBa34 | 13 | *       | 1.13±0.54  | 18.34±1.89 | 7.78±0.05 | 0.32±0.00 | 12.82±0.01 | 0.025±0.001 | 0.057±0.001 | 0.035±0.001 | 0.0249±0.0001 |
| ScBa35 | 7  | *       | 0.62±0.20  | 5.13±1.56  | 7.44±0.03 | 0.52±0.02 | 13.93±0.20 | 0.037±0.002 | 0.058±0.001 | 0.031±0.001 | 0.0372±0.0019 |
| ScBa36 | 15 | XXVI    | 1.14±0.42  | 15.81±2.76 | 6.69±0.08 | 0.40±0.06 | 12.99±0.12 | 0.031±0.004 | 0.057±0.001 | 0.029±0.001 | 0.0306±0.004  |
| ScBa37 | 3  | XXVII   | 0.57±0.13  | 5.55±2.26  | 6.59±0.07 | 0.33±0.01 | 13.87±0.12 | 0.024±0.001 | 0.058±0.001 | 0.028±0.001 | 0.0239±0.0012 |
| ScBa38 | 10 | *       | 0.79±0.02  | 7.81±1.69  | 6.99±0.32 | 0.41±0.02 | 13.23±0.50 | 0.031±0.002 | 0.056±0.003 | 0.030±0.001 | 0.0310±0.0002 |
| ScBa39 | 14 | XXVIII  | 1.58±0.35  | 17.36±1.76 | 7.86±0.10 | 0.31±0.01 | 12.99±0.23 | 0.024±0.001 | 0.058±0.001 | 0.035±0.001 | 0.0240±0.0010 |
| ScBa40 | 14 | XXIX    | 2.68±0.47  | 24.44±0.38 | 7.49±0.68 | 0.42±0.05 | 12.24±0.90 | 0.036±0.007 | 0.054±0.004 | 0.034±0.003 | 0.0356±0.0068 |
| ScBa41 | 12 | *       | 1.40±0.86  | 19.33±4.79 | 7.12±0.16 | 0.39±0.02 | 13.01±0.28 | 0.03±0.002  | 0.058±0.001 | 0.032±0.001 | 0.0297±0.0025 |
| ScBa42 | 13 | XXX     | 2.52±0.62  | 25.2±1.99  | 6.98±0.23 | 0.36±0.00 | 12.6±0.17  | 0.029±0.001 | 0.058±0.001 | 0.032±0.001 | 0.0287±0.0003 |
| ScBa43 | 15 | XXXI    | 3.07±1.42  | 26.09±4.50 | 6.91±0.20 | 0.36±0.02 | 12.50±0.68 | 0.029±0.001 | 0.058±0.002 | 0.032±0.001 | 0.0289±0.0001 |
| ScBa44 | 1  | XXXII   | 0.62±0.01  | 3.63±0.59  | 6.99±0.08 | 0.27±0.01 | 14.05±0.04 | 0.019±0.001 | 0.058±0.001 | 0.029±0.001 | 0.0189±0.0003 |
| ScBa45 | 12 | XXXIII  | 3.88±0.02  | 30.01±0.48 | 6.86±0.11 | 0.41±0.03 | 12.11±0.02 | 0.034±0.002 | 0.058±0.001 | 0.033±0.001 | 0.0338±0.0020 |
| ScBa46 | 12 | XXXIV   | 3.32±0.14  | 24.06±0.06 | 7.69±0.27 | 0.39±0.00 | 12.43±0.22 | 0.031±0.001 | 0.057±0.001 | 0.035±0.001 | 0.0313±0.0003 |
| ScBa47 | 4  | XXXV    | 61.26±8.62 | 48.21±7.22 | 5.65±0.44 | 0.52±0.02 | 7.42±0.89  | 0.071±0.006 | 0.055±0.001 | 0.042±0.002 | 0.0708±0.0058 |
| ScBa48 | 6  | XXXVI   | 68.47±0.49 | 53.25±1.42 | 5.51±0.29 | 0.54±0.02 | 6.86±0.28  | 0.079±0.001 | 0.056±0.002 | 0.045±0.002 | 0.0786±0.0003 |
| ScBa49 | 15 | XXXVII  | 1.39±0.90  | 17.01±3.35 | 6.94±0.05 | 0.37±0.00 | 12.88±0.40 | 0.029±0.001 | 0.057±0.001 | 0.031±0.001 | 0.0288±0.0012 |
| ScBa50 | 9  | *       | 2.78±0.19  | 25.25±0.23 | 6.75±0.15 | 0.39±0.01 | 12.6±0.08  | 0.031±0.001 | 0.058±0.001 | 0.031±0.001 | 0.0311±0.0003 |
| ScBa51 | 15 | XXXVIII | 2.48±0.03  | 24.83±0.26 | 6.81±0.18 | 0.36±0.01 | 12.49±0.22 | 0.029±0.001 | 0.058±0.001 | 0.031±0.001 | 0.0291±0.0001 |
| ScBa52 | 8  | XXXIX   | 0.71±0.09  | 8.32±0.62  | 6.67±0.11 | 0.43±0.02 | 13.73±0.08 | 0.031±0.001 | 0.058±0.001 | 0.028±0.001 | 0.0312±0.0010 |
| ScBa53 | 11 | *       | 1.78±1.01  | 21.99±5.78 | 6.80±0.04 | 0.42±0.03 | 12.60±0.14 | 0.033±0.002 | 0.057±0.001 | 0.031±0.001 | 0.0333±0.0019 |
| ScBa54 | 14 | XL      | 1.72±1.39  | 15.94±7.53 | 7.06±0.01 | 0.31±0.00 | 13.24±0.42 | 0.023±0.001 | 0.058±0.001 | 0.031±0.001 | 0.0233±0.0011 |
| ScBa55 | 1  | XLI     | 2.11±1.08  | 17.06±5.14 | 6.70±0.06 | 0.24±0.01 | 13.18±0.48 | 0.018±0.001 | 0.059±0.001 | 0.030±0.001 | 0.0181±0.0015 |
| ScBa56 | 6  | XLII    | 1.85±0.39  | 15.86±1.64 | 6.78±0.01 | 0.32±0.01 | 13.35±0.16 | 0.024±0.001 | 0.059±0.001 | 0.030±0.001 | 0.0238±0.0007 |

|        |    |        |           |             |           |           |            |             |             |             |               |
|--------|----|--------|-----------|-------------|-----------|-----------|------------|-------------|-------------|-------------|---------------|
| ScBa57 | 3  | XLIII  | 68.19±0.5 | 45.76±2.71  | 5.21±0.13 | 0.60±0.00 | 7.39±0.17  | 0.081±0.002 | 0.057±0.001 | 0.040±0.002 | 0.0811±0.0024 |
| ScBa58 | 8  | XLIV   | 4.84±3.73 | 30.43±10.18 | 6.53±0.20 | 0.43±0.01 | 11.73±1.22 | 0.038±0.004 | 0.055±0.002 | 0.031±0.003 | 0.0375±0.0045 |
| ScBa59 | 1  | *      | 3.60±1.45 | 28.12±4.22  | 6.84±0.01 | 0.42±0.00 | 12.20±0.46 | 0.034±0.001 | 0.057±0.001 | 0.032±0.001 | 0.0341±0.0011 |
| ScBa60 | 10 | XLIV   | 0.69±0.20 | 4.12±2.80   | 7.81±0.20 | 0.30±0.02 | 14.08±0.03 | 0.022±0.001 | 0.059±0.001 | 0.033±0.001 | 0.0216±0.0013 |
| ScBa61 | 14 | XLV    | 0.56±0.05 | 7.10±0.59   | 6.96±0.19 | 0.42±0.00 | 13.79±0.18 | 0.030±0.001 | 0.058±0.001 | 0.029±0.001 | 0.0305±0.0007 |
| ScBa62 | 13 | *      | 0.98±0.08 | 13.73±0.37  | 6.43±0.17 | 0.32±0.01 | 13.21±0.28 | 0.024±0.001 | 0.058±0.001 | 0.028±0.001 | 0.0239±0.0001 |
| ScBa63 | 14 | *      | 0.39±0.15 | 3.85±1.71   | 7.20±0.07 | 0.50±0.14 | 13.58±0.48 | 0.038±0.012 | 0.056±0.002 | 0.030±0.001 | 0.0377±0.0116 |
| ScBa64 | 14 | *      | 0.98±0.54 | 7.81±1.64   | 6.87±0.14 | 0.32±0.09 | 13.33±0.61 | 0.025±0.008 | 0.056±0.002 | 0.029±0.001 | 0.0248±0.0079 |
| ScBa65 | 7  | XLVI   | 1.07±0.51 | 9.91±4.72   | 6.46±0.09 | 0.23±0.01 | 13.42±0.50 | 0.017±0.001 | 0.057±0.001 | 0.028±0.001 | 0.0173±0.0013 |
| ScBa66 | 4  | XLVII  | 3.00±0.12 | 25.73±0.44  | 6.76±0.01 | 0.39±0.01 | 12.15±0.07 | 0.032±0.001 | 0.056±0.001 | 0.031±0.001 | 0.0319±0.0005 |
| ScBa67 | 4  | *      | 3.17±1.05 | 25.26±2.74  | 7.72±1.37 | 0.37±0.01 | 12.71±0.56 | 0.029±0.001 | 0.059±0.004 | 0.036±0.007 | 0.0292±0.0002 |
| ScBa68 | 4  | *      | 0.89±0.22 | 9.31±2.35   | 6.60±0.22 | 0.22±0.03 | 13.46±0.31 | 0.016±0.002 | 0.057±0.001 | 0.028±0.001 | 0.0162±0.0017 |
| ScBa69 | 4  | *      | 0.85±0.42 | 8.44±4.20   | 6.54±0.03 | 0.24±0.02 | 13.41±0.27 | 0.018±0.002 | 0.057±0.001 | 0.028±0.001 | 0.0182±0.0021 |
| ScBa70 | 12 | XLVIII | 4.27±1.33 | 31.23±3.89  | 6.88±0.02 | 0.40±0.03 | 12.27±0.27 | 0.033±0.001 | 0.059±0.003 | 0.033±0.001 | 0.0330±0.0014 |
| ScBa71 | 11 | *      | 0.72±0.33 | 4.54±2.92   | 6.72±0.09 | 0.30±0.01 | 13.88±0.39 | 0.021±0.001 | 0.058±0.001 | 0.028±0.001 | 0.0214±0.0001 |
| ScBa72 | 6  | XLIX   | 0.66±0.09 | 7.05±0.76   | 6.84±0.01 | 0.40±0.01 | 13.78±0.08 | 0.029±0.001 | 0.058±0.001 | 0.029±0.001 | 0.0291±0.0009 |
| ScBa73 | 14 | *      | 0.78±0.04 | 7.81±0.64   | 7.10±0.04 | 0.42±0.00 | 13.82±0.02 | 0.031±0.001 | 0.059±0.001 | 0.030±0.001 | 0.0307±0.0002 |
| ScBa74 | 8  | L      | 0.93±0.28 | 9.96±2.72   | 6.64±0.01 | 0.40±0.01 | 13.49±0.11 | 0.030±0.001 | 0.058±0.001 | 0.028±0.001 | 0.0300±0.0007 |
| ScBa75 | 9  | LI     | 3.57±2.41 | 27.49±8.01  | 6.79±0.13 | 0.38±0.01 | 12.35±0.66 | 0.031±0.002 | 0.058±0.001 | 0.032±0.001 | 0.0309±0.0022 |
| ScBa76 | 9  | *      | 3.63±1.97 | 30.74±4.86  | 6.58±0.05 | 0.37±0.00 | 12.01±0.29 | 0.031±0.001 | 0.057±0.001 | 0.031±0.001 | 0.0308±0.0008 |
| ScBa77 | 9  | *      | 3.96±1.15 | 30.12±3.88  | 6.61±0.04 | 0.37±0.03 | 11.93±0.41 | 0.031±0.001 | 0.057±0.001 | 0.031±0.001 | 0.0314±0.0011 |
| ScBa78 | 7  | *      | 1.02±0.22 | 9.71±2.42   | 6.15±0.11 | 0.31±0.04 | 13.42±0.11 | 0.023±0.003 | 0.057±0.001 | 0.026±0.001 | 0.0228±0.0033 |
| ScBa79 | 11 | *      | 1.23±0.37 | 12.73±2.91  | 6.52±0.13 | 0.23±0.01 | 13.37±0.06 | 0.017±0.001 | 0.058±0.001 | 0.028±0.001 | 0.0169±0.0007 |
| ScBa80 | 8  | *      | 6.79±2.11 | 36.88±3.98  | 6.57±0.00 | 0.41±0.02 | 11.22±0.36 | 0.037±0.003 | 0.056±0.001 | 0.033±0.001 | 0.0366±0.0032 |
| ScBa81 | 7  | LII    | 1.96±1.25 | 15.41±6.55  | 6.96±0.03 | 0.40±0.01 | 13.30±0.38 | 0.030±0.002 | 0.059±0.001 | 0.031±0.001 | 0.0299±0.0017 |
| ScBa82 | 6  | LIII   | 1.00±0.06 | 10.89±0.92  | 6.65±0.04 | 0.21±0.00 | 13.36±0.07 | 0.016±0.001 | 0.057±0.001 | 0.029±0.001 | 0.0157±0.0001 |
| ScBa83 | 5  | LIV    | 2.43±1.29 | 17.35±5.92  | 6.66±0.15 | 0.38±0.01 | 12.82±0.45 | 0.030±0.002 | 0.057±0.001 | 0.030±0.001 | 0.0298±0.0017 |
| ScBa84 | 15 | LV     | 2.14±0.86 | 23.56±3.33  | 6.68±0.05 | 0.40±0.02 | 12.47±0.29 | 0.032±0.002 | 0.057±0.001 | 0.031±0.001 | 0.0318±0.0022 |
| ScBa85 | 14 | *      | 3.92±1.20 | 29.25±3.40  | 6.70±0.02 | 0.38±0.00 | 12.10±0.16 | 0.031±0.001 | 0.057±0.001 | 0.032±0.001 | 0.0314±0.0008 |
| ScBa86 | 6  | LVI    | 1.06±0.15 | 10.25±1.61  | 6.73±0.13 | 0.42±0.01 | 13.60±0.20 | 0.031±0.001 | 0.058±0.002 | 0.029±0.001 | 0.0307±0.0011 |
| ScBa87 | 1  | LVII   | 1.74±0.96 | 21.21±4.94  | 6.85±0.07 | 0.39±0.02 | 12.54±0.20 | 0.031±0.002 | 0.057±0.001 | 0.031±0.001 | 0.0311±0.0021 |

|        |    |       |           |            |           |           |            |             |             |             |               |
|--------|----|-------|-----------|------------|-----------|-----------|------------|-------------|-------------|-------------|---------------|
| ScBa88 | 12 | LVIII | 2.85±2.21 | 25.68±9.50 | 6.64±0.24 | 0.37±0.02 | 12.39±0.02 | 0.029±0.002 | 0.058±0.003 | 0.031±0.003 | 0.0295±0.0016 |
| ScBa89 | 13 | *     | 2.24±0.24 | 19.4±0.70  | 7.12±0.07 | 0.33±0.02 | 13.07±0.18 | 0.025±0.001 | 0.059±0.001 | 0.032±0.001 | 0.0253±0.0009 |
| ScBa90 | 13 | LIX   | 2.55±0.28 | 24.87±0.73 | 7.43±0.20 | 0.32±0.01 | 12.29±0.13 | 0.026±0.001 | 0.057±0.002 | 0.034±0.001 | 0.0258±0.0011 |
| ScBa91 | 10 | LX    | 2.64±0.41 | 19.53±1.64 | 6.36±0.17 | 0.37±0.01 | 12.64±0.11 | 0.029±0.001 | 0.057±0.001 | 0.029±0.001 | 0.0292±0.0007 |
| ScBa92 | 14 | LXI   | 3.43±2.51 | 26.05±8.16 | 6.49±0.02 | 0.36±0.01 | 12.06±0.33 | 0.030±0.001 | 0.056±0.001 | 0.030±0.002 | 0.0301±0.0001 |
| ScBa93 | 6  | LXII  | 0.67±0.31 | 6.69±5.00  | 6.46±0.18 | 0.21±0.01 | 13.55±0.34 | 0.016±0.001 | 0.057±0.002 | 0.027±0.001 | 0.0156±0.0001 |
| ScBa94 | 5  | XX    | 0.83±0.05 | 10.16±0.53 | 6.59±0.05 | 0.28±0.00 | 13.46±0.05 | 0.021±0.001 | 0.058±0.001 | 0.028±0.001 | 0.0205±0.0003 |
| ScBa95 | 15 | LXII  | 2.42±0.73 | 24.31±2.48 | 6.55±0.17 | 0.37±0.01 | 12.31±0.24 | 0.030±0.002 | 0.057±0.002 | 0.030±0.001 | 0.0301±0.0017 |
| ScBa96 | 12 | LIX   | 3.18±1.87 | 25.13±5.26 | 6.67±0.06 | 0.40±0.02 | 12.12±0.28 | 0.033±0.001 | 0.056±0.001 | 0.031±0.001 | 0.0329±0.0007 |

<sup>a</sup> Fermentation purity: acetic acid (g/L)/ethanol % (v/v), <sup>b</sup> Ethanol yield: ethanol % (v/v)/sugar consumption (g/L), <sup>c</sup> Glycerol yield: glycerol (g/L)/sugar consumption (g/L), <sup>d</sup> Acetic acid: acetic acid (g/L)/sugar consumption(g/L).

The physiological data from the growth tests at 50 mg/L of SO<sub>2</sub> after 24 h (enological conditions, variable: “Ability to grow”), the presence or absence of enzymatic activities (esterase and protease activity), H<sub>2</sub>S production and the chemical composition (glucose, fructose, malic and acetic acids, glycerol, and ethanol) of the wines obtained after 14 days of fermentation were used to evaluate the technological diversity of the strains. A Principal Component Analysis (PCA) was carried out and the outcome is presented in Fig. 2, including the loadings plot (Fig. 2a) and the scatter plot (Fig. 2b).

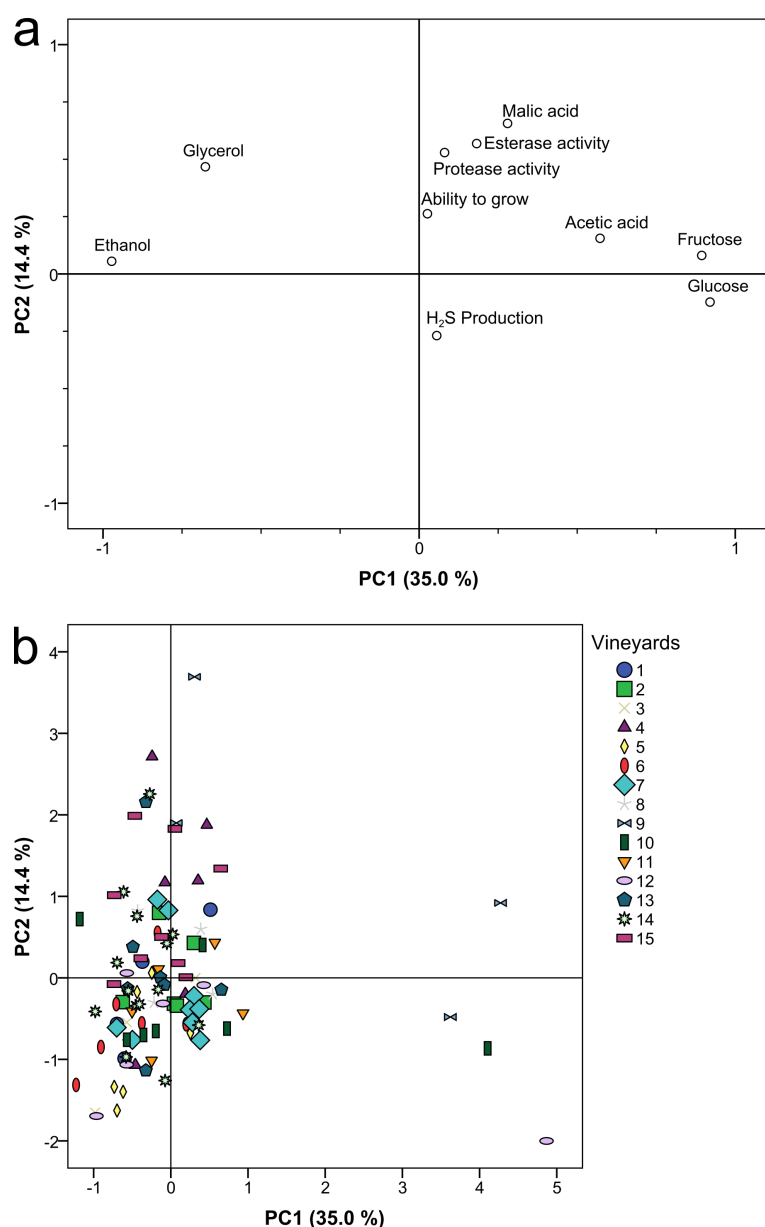


**Figure 2.** Principal component analysis of the *S. cerevisiae* strains from the fifteen vineyards (as reported in Figure 1), according to the wine chemical composition: loadings plot (a) and scatter plot (b). The fifteen vineyard considered were: 1 (Murisengo), 2 (San Martino Alfieri), 3 (Costigliole d'Asti), 4 (Isola d'Asti), 5 (Montegrosso d'Asti), 6 (Agliaio Terme), 7 (Vinchio), 8 (Nizza Monferrato), 9 (Incisa Scapaccino), 10 (Loazzolo), 11 (Ricaldone), 12 (Alice bel colle), 13 (Acqui Terme Crocera south west), 14 (Acqui Terme Crocera south est) and 15 (Acqui Terme Dannonna).

PC1 (35.0 % of variance explained) was better correlated with the strains that left high level of residual sugars present in the wine and that produced high amount of acetic acid, while PC2 (14.4 % of variance explained) was mainly correlated to the high production of glycerol and low degradation of malic acid (Fig. 2a). Two groups may be differentiated according to the scatter plot (PC1 or PC2 with values close or higher than 2, respectively). Some strains from vineyards 4, 13, 14, and 15 produced high glycerol amounts and preserved malic acid contents, and two isolates from vineyards 10 and 12 were unsatisfactory due to their sugars degradation. In addition, all the four isolates from vineyard 9 were present either in the first or in the second group (Fig. 2b).

#### 4. CONCLUSIONS

The present study investigated the genetic and technological diversity of autochthonous *S. cerevisiae* in the North-West of Italy, in the Monferrato area. It was possible to genetically and phenotypically differentiate the strains. In order to investigate the ability of the isolated strains to properly ferment Barbera must, at pilot scale level first and in industrial settings after, relevant technological characteristics, such as sugar consumption and acetic acid production, should be taken into consideration. All the data presented here were obtained from pasteurized natural must, and the ability of the selected strains to dominate the natural grape and must mycobiota should therefore be determined throughout the fermentation process. In parallel, the ability to complete the fermentation in the competitive environment of a natural grape should also be confirmed. Finally, the production of compounds, such as alcohols, esters, carbonyl compounds and fatty acids that have an impact on the sensory characteristics of wine should also be evaluated.

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## NUTRITIONAL QUALITY OF DIFFERENT FISH SPECIES FARMED IN THE ADRIATIC SEA

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### ABSTRACT

We investigated the nutritional quality of commercially important farmed fish species: sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), dentex (*Dentex dentex*), and turbot (*Scophthalmus maximus*). The omega-3 fatty acid content of dentex was twice as high as that of any other fish species, and its eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid contents were 2-4 times higher. The recommended n-3/n-6 ratio was present in all the fish species, but the recommended polyunsaturated/saturated fatty acid ratio was not present in the turbot. All the fish species, except turbot, met the recommended atherogenic index, thrombogenic index, and ratio of hypocholesterolaemic to hypercholesterolaemic fatty acids, whereas the highest flesh lipid quality value was observed in the dentex.

**Keywords:** nutritional quality, farmed fish, Adriatic Sea, basic chemical composition, mineral, fatty acid

## 1. INTRODUCTION

Since 1970, fish farming (aquaculture) has been the world's fastest growing sector in the production of foods of animal origin. Because wild fisheries are stagnating and the human population is growing, aquaculture is expected to fill the gap in supplying fish intended for human consumption, because the demand has continued to increase (FAO, 2016). Consumer habits are also changing continuously, and issues such as convenience, health, ethics, variety, value for money, sustainability, and safety are becoming more important. Health and well-being increasingly influence our decisions about what we consume. Fish have a particularly prominent role in this context because mounting evidence confirms the health benefits of consuming fish. A moderate-to-high fish intake is associated with a reduced prevalence of the chronic diseases that go hand in hand with obesity, such as cardiovascular diseases, diabetes, and some cancers (OEHLENSCHLÄGER, 2012).

Fish is also considered an integral component of a well-balanced diet, providing a healthy source of energy, high-quality proteins, vitamins (D, A, E and B12), essential minerals and particularly n-3 long-chain polyunsaturated fatty acids (LC PUFA), mainly eicosapentaenoic acid (20:5 n-3 EPA) and docosahexaenoic acid, (22:6 n-3 DHA), whose pleiotropic effects on health promotion and disease prevention are well recognized (GIL and GIL, 2015). In fresh fish, the muscle composition is considered to be the most important aspect of quality, whereas its sensory properties and nutritional value, together with freshness, are also important quality parameters in terms of consumer acceptability (GRIGORAKIS, 2007). Fish proteins are easily digested (because the proportion of collagen is low). They are also a good source of essential amino acids. The nutritional value of fish meat is also linked to its lipid composition, as well as its constituent proteins (OEHLENSCHLÄGER, 2012).

Lipid contents are highly variable both between and within fish species. Many factors contribute to this variability, including feed, farm location, fish size and stage of maturity, biological variations, tissue sampled, and starvation (RUEDA *et al.*, 2001). Fish lipids contain a high proportion of unsaturated fatty acids (60%-84%), especially long-chain n-3 fatty acids. The fatty acids (FAs) in fish, especially EPA and DHA, have important effects on the health of the human body and differ from those of meat. The well-known hypotriglyceridaemic effect of n-3 LC PUFA may be beneficial in terms of reducing the percentage of pro-atherogenic small low-density lipoprotein (LDL) particles, and perhaps by ameliorating the inflammatory processes associated with the metabolic syndrome seen in patients with diabetes mellitus or cardiovascular disease (LOPEZ-HUERTAS, 2012). In recent decades, fish nutrition research has devoted much effort to the development of sustainable fish feeds with an optimal FA composition so that the fish provide adequate levels of n-3 FAs for human nutrition (IZQUIERDO *et al.*, 2005; KRIS-ETHERTON *et al.*, 2003; PRATOOMYOT *et al.*, 2010). The minerals in raw farmed fish meat, which occur at overall levels of 0.6-1.5 g/100 g, are also very important in the human diet (ERKAN and ÖZDEN, 2007). According to the literature, the origins of fish and their feeding patterns have no effect on their mineral composition, other than their calcium content (FUENTES *et al.*, 2010). The nutritional value of fish is affected by a low-energy value and high levels of fat-soluble vitamins, especially A and D (MAHAN and ESCOTT-STUMP, 2004).

Because the world's fish stocks are limited, it is now suggested that farmed fish provide an alternative for consumers. Farmed sea-foods have an advantage over captured fishery products because they are produced and harvested under controlled conditions, which allow consumption-related risks to be minimized. In recent years, the Mediterranean aquaculture industry has become interested in farming new species to overcome the problems arising from the overproduction of two main species, the gilthead sea bream (*Sparus aurata*) and the European sea bass (*Dicentrarchus labrax*) and to diversify their

products. The common dentex (*Dentex dentex*) is a fast-growing sparid, and a candidate species for fish farming in the Mediterranean (RIGOS *et al.*, 2012). Alternatively, the commercial farming of turbot (*Scophthalmus maximus*), a high-value flat fish, along the Adriatic coast is still in its infancy, and the turbot is mainly farmed along the Atlantic coasts of France and Spain (SÉROT *et al.*, 1998; MANTHEY-KARL *et al.*, 2016). Although the production of this species has achieved high quality and efficiency, very little is known about its nutritional quality relative to our knowledge of other fish species.

Given that the demand for fish is increasing globally and fish consumption is generally recommended by dieticians, it is surprising that there are no data on the FA profiles and FA contents of the different fish species farmed in the Adriatic Sea, especially the turbot and dentex. Therefore, in this study, we investigated the quality parameters of four farmed white fish species sampled from different farms along the Adriatic Coast: the European sea bass (*D. labrax*), gilthead sea bream (*Sp. aurata*), turbot (*S. maximus*), and common dentex (*D. dentex*). The basic chemical parameters, minerals, FA compositions, and health-related lipid indices: atherogenic index [AI], thrombogenic index [TI], the ratio of hypocholesterolaemic to hypercholesterolaemic FAs [HH], and flesh lipid quality [FLQ]) were determined and compared across the four species analyzed.

## 2. MATERIALS AND METHODS

### 2.1. Fish farming and sampling conditions

Sea bass and sea bream are the main marine fish species commercially farmed in Croatian sea farms, whereas the cultivation of dentex, and especially turbot, is far less common in these regions. Fish are cultivated in a similar manner at each farming location, consistent with the rules of Mediterranean fish farming, which are as follows: cultivation density of 5-12 kg per cubic meter (for turbot > 20 kg per cubic meter) in 22-28 months; the use of cages; and feeding regimens adjusted to bodyweight, sea temperature, photoperiod, oxygen saturation, etc. The main difference between the northern part of the Adriatic and its middle part is the greater temperature differential between the winter and summer months in the north, whereas in the middle part, the temperature never falls below 12 °C in winter or exceeds 25 °C in summer. The turbot-farming technology has been sufficiently mastered in some geographic areas, but is still in the experimental stage in the Adriatic Sea. The biggest challenge is the extremely high temperatures in the Adriatic Sea, and the most effective modes of manipulation are yet to be determined. Fry are released from different commercial hatcheries in France, Italy, and Greece to on-grow, are cultivated in floating net cages of different sizes, and are fed a commercially available fish feed with different nutritional contents, as shown in Table 1.

**Table 1.** Declared compositions of feeds used in the production of cultivated fish species.

| Parameter (%)   | Commercial name of the food/fish species |                        |                       |
|-----------------|------------------------------------------|------------------------|-----------------------|
|                 | Efico Sigma 870/Turbot/Dentex            | Efico YM 854/Sea Bream | Efico YM 868/Sea Bass |
| Crude proteins  | 54.0                                     | 41.0                   | 40.0                  |
| Crude lipids    | 20.0                                     | 18.0                   | 23.0                  |
| Crude cellulose | 0.4                                      | 4.0                    | 3.0                   |
| Ash             | 10.2                                     | 6.5                    | 6.6                   |

In this study, samples of sea bass, sea bream, and dentex were collected from different farms situated in the northern (Istria) and middle parts of the Adriatic Sea, whereas turbot was only sampled from the northern part of the Adriatic Sea (Istria). The fish were sampled in March-May 2016 (three groups of samples were collected per species). In total, 72 pieces of fish (18 pieces of each species) were transported to our laboratory in Styrofoam boxes on ice. Before the edible part of the fish flesh was eviscerated and filleted for analysis, the bodyweight and length were measured to the closest g (280-300 g for sea bass, sea bream, and dentex) and cm (28.5-30.5 g for sea bass, sea bream, and dentex), respectively. For the turbot, these parameters were 260-320 g and 24.5-31.0 cm, respectively. Sample preparation involved cleaning the body surfaces, descaling and beheading each specimen, and removing the vertebrae and viscera (not the skin). The fillets and skin of each of the 72 fish samples were homogenized with a laboratory homogenizer (Grindomix GM 200, Retsch, Haam, Germany). The samples were analyzed immediately, first for their basic chemical parameters and then for their FA profiles.

## **2.2. Determination of basic chemical composition and mineral content**

The water content was determined with a gravimetric analysis (ISO 1442:1997) using an Epsa 2000 thermostat (Ba-Ri, Velika Gorica, Croatia). The total protein content was determined by the Kjeldahl method given by International Organization for Standardization (ISO) (ISO 937:1978) using a Digestion Unit 8-Basic (Foss, Höganäs, Sweden) and a Kjelttec 8400 automated distillation and titration device (Foss). The total fat content was determined with the Soxhlet method (ISO 1443:1973), in which the samples are digested with acid hydrolysis and the fats are then extracted with petroleum ether using a Soxtherm 2000 automated device (Gerhardt, Munich, Germany). The ash content was determined according to ISO 936:1998 using a Nobertherm LV9/11/P320 furnace (Lilienthal, Germany). The phosphorus content was determined according to ISO 13730:1996 with a DR/4000U spectrophotometer (Hach, Düsseldorf, Germany). We used the procedures described by PETROVIĆ *et al.* (2015) to determine the calcium and sodium contents. For the basic chemical composition and mineral content analyses, two samples of each of the 72 fish samples (18 samples for each species) were tested in parallel, and the mean values were analysed statistically for percentage weight (%), with an accuracy of 0.01%.

## **2.3. Fatty acid analysis**

The preparation of samples for the analysis of FA methyl esters has been described previously by PLEADIN *et al.* (2015). The FA methyl esters were analysed with gas chromatography (GC) according to ISO 12966-4:2015 and EN ISO 12966-4:2015. A 7890BA gas chromatographer, equipped with a flame ionization detector (FID) and a 60 m DB-23 capillary column with an internal capillary diameter of 0.25 mm and a stationary phase thickness of 0.25 µm (Agilent Technologies, Santa Clara, CA< USA), was used. The components were detected with a FID at a temperature of 280 °C, a hydrogen flow of 40 mL/min, and an airflow of 450 mL/min. Nitrogen was used as the make-up gas at a flow rate of 25 mL/min. The initial column temperature was 130 °C; after 1 min, it was increased by 6.5 °C/min until it reached 170 °C. The temperature was further increased by 2.75 °C/min until it reached 215 °C, where it was maintained for 12 min. The temperature was then increased further by 40 °C/min until the final column temperature reached 230 °C, which was maintained for 3 min. Each sample (1 µL) was injected into a split-splitless injector at a temperature of 270 °C with a split ratio of 1:50. The carrier gas was helium (99.9999%), flowing at the constant rate of 43 cm/s. The FA methyl esters were

identified by comparing their retention times with those of FA methyl esters in the standard mixture, as described previously by PLEADIN *et al.* (2015). The FA composition was determined for each of the 72 fish samples (18 pieces of each species). The mean values per species are expressed as the percentage (%) of a particular FA in the total FAs, with an accuracy of 0.01%. The FA methyl ester values were converted into FA values per 100 g of edible part (EP), according to the FAO/INFOODS Guidelines for Converting Units, Denominators and Expressions (2012).

## 2.4. Determination of lipid quality

The lipid quality indices AI, TI, HH, and FLQ were calculated from the FA compositions. AI indicates the relationship between the total major saturated fats, which are considered pro-atherogenic agents (because they facilitate the adhesion of lipids to the cells of the immune and circulatory systems), and the total major unsaturated fats, which are considered anti-atherogenic agents (because they inhibit the formation of plaques and reduce the levels of esterified FAs, cholesterol, and phospholipids, and therefore prevent micro- and macro-coronary diseases) (ULBRITCTH and SOUTHGATE, 1991). This parameter was calculated as:

$$AI = ([12:0 + (4 \times 14:0) + 16:0]) / (\text{total monounsaturated fatty acids [MUFA]} + \text{PUFA n-6} + \text{PUFA n-3})$$

TI indicates the tendency for blood to clot in blood vessels. It is defined as the relationship between pro-thrombogenic (saturated) and anti-thrombogenic FAs (MUFA, PUFA n-6, and PUFA n-3) (ULBRITCTH and SOUTHGATE, 1991). The index is calculated as:

$$TI = (14:0 + 16:0 + 18:0) / ([0.5 \times \text{total MUFA} + 0.5 \times \text{PUFA n-6} + 3 \times \text{PUFA n-3}] + [\text{PUFA n-3} / \text{PUFA n-6}])$$

The ratio between the hypocholesterolaemic and hypercholesterolaemic FAs (HH) takes into account the known effects of certain FAs on cholesterol metabolism (SANTOS-SILVA *et al.*, 2002). It is calculated as:

$$HH = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3) / (C14:0 + C16:0)$$

FLQ indicates the proportion of the main PUFAs n-3 (EPA and DHA) in the muscles relative to the total lipid content. The higher this index, the higher the quality of the dietary lipid source (ABRAMI *et al.*, 1992; SENSO *et al.*, 2007). The index is calculated as:

$$FLQ = (\text{EPA} + \text{DHA in g FAs} / 100 \text{ g EP}) / (\text{total lipids in g} / 100 \text{ g EP}) \times 100$$

## 2.5. Statistical analysis

The statistical analysis was performed with SPSS Statistics Software 22.0 (IBM SPSS Statistics 2013, NY, USA). The results were tested for the normality of their distribution ( $p > 0.05$ ) using the Shapiro-Wilks test. To determine the statistical significance of the differences in the chemical and FA compositions between the fish species analysed, one-way ANOVA and the robust Brown-Forsythe test were used. To establish the homogeneity of variance, the Scheffe *post hoc* test or Tamhane's T2 *post hoc* were used. Decisions on statistical relevance were made at the significance level of  $p < 0.05$ .

### 3. RESULTS AND DISCUSSIONS

The results presented in this study provide nutritional profiles of four fish species (sea bream, sea bass, turbot, and dentex), with particular emphasis on their FA components and health-related lipid indices. All the fish species are farmed in the Adriatic Sea. The basic chemical and mineral compositions of these fish species are shown in Table 2.

**Table 2.** Basic chemical and mineral compositions of four species of farmed fish.

| Parameter   | Sea bass                | Dentex                    | Turbot                      | Sea bream               |
|-------------|-------------------------|---------------------------|-----------------------------|-------------------------|
| Water (%)   | 70.81±3.28 <sup>c</sup> | 69.12±0.39 <sup>c</sup>   | 77.88±1.31 <sup>a,b,d</sup> | 70.16±2.50 <sup>c</sup> |
| Ash (%)     | 1.21±0.02 <sup>b</sup>  | 1.31±0.03 <sup>a,c</sup>  | 1.16±0.05 <sup>b,d</sup>    | 1.24±0.07 <sup>c</sup>  |
| Fat (%)     | 9.11±3.06 <sup>c</sup>  | 10.46±0.40 <sup>c</sup>   | 4.00±1.20 <sup>a,b,d</sup>  | 10.48±3.08 <sup>c</sup> |
| Protein (%) | 19.22±1.46 <sup>c</sup> | 18.92±0.43 <sup>c</sup>   | 17.59±1.15 <sup>a,b,d</sup> | 19.09±0.33 <sup>c</sup> |
| Na (mg/kg)  | 706±277 <sup>c</sup>    | 465±43.9 <sup>c,d</sup>   | 1212±292 <sup>a,b</sup>     | 968±184 <sup>b</sup>    |
| Ca (mg/kg)  | 729±189 <sup>b</sup>    | 1300±128 <sup>a,c,d</sup> | 656±151 <sup>b</sup>        | 540±152 <sup>b</sup>    |
| P (mg/kg)   | 2214±122 <sup>b,c</sup> | 2740±195 <sup>a,c,d</sup> | 1811±127 <sup>a,b,d</sup>   | 2180±164 <sup>b,c</sup> |

Results are expressed as mean values (18 samples per species, each of which was analysed in duplicate) ± standard deviations. Significant differences ( $p < 0.05$ ): <sup>a</sup>vs. sea bass; <sup>b</sup>vs. dentex; <sup>c</sup>vs. turbot; <sup>d</sup>vs. sea bream.

Analysis of the basic chemical and mineral parameters showed that the turbot had a statistically significantly ( $p < 0.05$ ) higher water content and lower fat and protein contents than the other fish analysed. In the remaining three fish species, the basic quality parameters were similar to those presented in earlier studies (ÖZDEN and ERKAN, 2008; ERKAN and ÖZDEN, 2007; KYRANA and LOUGOVOIS, 2002). The descriptive data for the basic chemical fish composition did not clearly correlate with the composition of the feed with which the fish were fed (Table 1), because it is not the only parameter that affects the nutritional composition of a fish species. Nutritional composition may be affected by a number of factors, including age, sex, and environmental factors, such as temperature, salinity, etc. (GRIGORAKIS, 2007). Therefore, although the dentex and turbot were fed the same type of feed, their basic chemical and mineral contents differed significantly.

In a study by ÖZDEN and ERKAN (2008) that compared the properties of sea bream, sea bass, and dentex, the water, ash, protein, and fat contents were in the ranges 69.68%-76.42%, 1.49%-1.95%, 18.21%-21.70%, and 2.29%-8.10%, respectively. In a study of the composition of turbot, these parameters were in the ranges 78.7%-80.2%, 1.0%-1.3%, 18.9%-20.3%, and 1.0%-2.0%, respectively (MANTHEY-KARL *et al.*, 2016). In general, research has shown that the basic chemical compositions of fish vary with the season of cultivation. Because fish are ectothermic poikilotherms, the fat content in a certain period of the year can be attributed to the ongoing physiological process of conserving the fat stock, which occurs in autumn after intense feeding, or the process of spending the fat stock, which occurs during winter. In early summer, when environmental conditions change, primarily as an increase in temperature, the fish metabolism accelerates and the energy taken from food is used for fish growth. PETROVIĆ *et al.* (2015) showed similar proportions of fat and moisture in the sea bass and sea bream, with fat contents of 3.2%-12.3% in the sea bass and 4.2%-15.0% in the sea bream, which depended on and varied strongly across the farming seasons, as explained previously. The fat content is inversely

related to the water content, and these parameters together account for approximately 80% of the total fish meat composition.

The dentex had a significantly higher ( $p < 0.05$ ) proportion of calcium and phosphorus than the other fish species analysed, whereas the turbot had the lowest proportion of phosphorus (significantly lower than in the other fish species,  $p < 0.05$ ). The dentex had a significantly lower sodium content than the turbot and sea bream, and the sodium content of the turbot was significantly higher than that of the common dentex or sea bass. As in earlier studies, the calcium and phosphorus contents were higher in the sea bass than in the sea bream (ERKAN and ÖZDEN, 2007; PETROVIĆ *et al.*, 2015). However, the calcium content determined in this study was significantly higher than the results previously published for the sea bream (ORBAN *et al.*, 2003; PETROVIĆ *et al.*, 2015) and turbot (MANTHEY-KARL *et al.*, 2016), whereas our results for the sea bass were similar to previous results (PETROVIĆ *et al.*, 2015).

The FA compositions, expressed as mean values and standard deviations relative to the total FAs for each species, are shown in Table 3.

The results show that the most strongly represented FA in all four species analysed was oleic acid (C18:1n-9, OA), followed by linoleic acid (C18:2n-6, LA) and palmitic acid (C16:0, PA). The results of this study are consistent with earlier research that demonstrated an increase in C18 FAs, such as OA, LA, and ALA, in farmed fish, in response to the use of vegetable oils in their feed (STROBEL *et al.*, 2012). Among the unsaturated FAs, OA and LA contribute most significantly to the enrichment of aromatic components (ELMORE *et al.*, 1999) and are considered to be of high nutritional value because they protect against cardiovascular disease (HORNSTRA, 1999).

Statistically significant differences in the FA profiles were observed among the fish species analysed ( $p < 0.05$ ). No FA analysis of the commercial feed used was available. The proportion of saturated FAs (SFAs; C14:0, C15:0, and C16:0) was the highest in the turbot. The sea bass and sea bream had significantly higher proportions of MUFA than the dentex or turbot, which is attributable to their oleic acid contents. A significantly lower proportion of PUFA (C18:2n-6c, C18:3n-3) was found in the turbot. In the study by ÖZDEN and ERKAN (2008), the FAs detected in the sea bass, sea bream, and dentex were 28.01%-32.41% SFA, 25.88%-28.62% MUFA, and 24.75%-27.42% PUFA. Compared with these values, our study found significantly higher proportions of MUFA in all the fish species investigated, whereas our SFA and PUFA values were similar to previously reported values, except in the turbot. The samples of turbot analysed in this study contained more SFA and MUFA, and at the same time, less n-3 and less PUFA than previously reported levels, resulting in a less favorable n-3/n-6 ratio than obtained by other researchers (SEROT *et al.*, 1998; MANTHEY-KARL *et al.*, 2016). The n-3/n-6 ratios obtained for turbot in these studies were 3-7-fold higher than that obtained in the present study.

The protective role of fish consumption against coronary heart disease has been widely demonstrated and is mainly attributed to the effects of n-3 FAs and their cardioprotective action (KRIS-ETHERTON *et al.*, 2003; PSOTA *et al.*, 2006). Earlier studies suggested that the n-3/n-6 ratio is a reliable index for interspecies comparisons of relative nutritional values (PIGGOT and TUCKER, 1990). According to SARGENT (1997), the optimum n-3/n-6 PUFA ratio should be 1:5 (0.2). The amounts of the FAs (ALA, EPA, DHA, AA, and LA,) present in the four fish species in our study, as determined by converting the proportion of an individual FA in the total FAs to the amount of the individual FA per 100 g of fish EP, are shown in Table 4.

**Table 3.** Fatty acid compositions (% of total FAs) of four species of farmed fish.

| Fatty acids    | Relative FA amount (% of FA)<br>mean±standard deviation |                             |                             |                           |
|----------------|---------------------------------------------------------|-----------------------------|-----------------------------|---------------------------|
|                | Sea bass                                                | Dentex                      | Turbot                      | Sea bream                 |
| C12:0          | ND                                                      | 0.06±0.00                   | 0.17±0.09                   | 0.06±0.05                 |
| C14:0          | 2.65±0.52 <sup>b,c</sup>                                | 4.13±0.18 <sup>a,c,d</sup>  | 9.66±0.81 <sup>a,b,d</sup>  | 2.69±0.78 <sup>b,c</sup>  |
| C15:0          | 0.33±0.05 <sup>b</sup>                                  | 0.45±0.02 <sup>c</sup>      | 0.75±0.11 <sup>a,b,d</sup>  | 0.28±0.11 <sup>c</sup>    |
| C16:0(PA)      | 16.17±1.43 <sup>c</sup>                                 | 17.95±0.39 <sup>c,d</sup>   | 25.36±2.02 <sup>a,b,d</sup> | 14.18±1.74 <sup>b,c</sup> |
| C17:0          | 0.39±0.08                                               | 0.51±0.01                   | 0.49±0.25                   | 0.26±0.11                 |
| C18:0          | 3.97±0.41 <sup>b</sup>                                  | 5.50±0.12 <sup>a,d</sup>    | 4.72±1.37                   | 3.30±0.59 <sup>b</sup>    |
| C20:0          | 0.33±0.05                                               | 0.30±0.15                   | 0.36±0.04                   | 0.35±0.07                 |
| C22:0          | 0.08±0.08                                               | 0.10±0.01                   | ND                          | 0.13±0.11                 |
| C14:1          | ND                                                      | 0.08±0.00                   | 0.08±0.09                   | ND                        |
| C16:1n-7t      | 0.45±0.03                                               | 0.44±0.01                   | 0.51±0.07                   | 0.45±0.03                 |
| C16:1n-7c      | 3.46±0.58 <sup>b,c</sup>                                | 5.82±0.11 <sup>a,c</sup>    | 11.37±1.53 <sup>a,b,d</sup> | 4.11±1.25 <sup>c</sup>    |
| C17:1          | 0.24±0.06                                               | 0.36±0.01                   | 0.17±0.19                   | 0.20±0.07                 |
| C18:1n-9t      | 0.34±0.25                                               | 0.07±0.07                   | ND                          | 0.27±0.11                 |
| C18:1n-9c (OA) | 38.85±3.98 <sup>b,c</sup>                               | 28.10±1.16 <sup>a,c,d</sup> | 20.63±2.18 <sup>a,b,d</sup> | 42.07±6.23 <sup>b,c</sup> |
| C18:1n-7       | 3.16±0.22 <sup>c</sup>                                  | 3.41±0.06 <sup>c</sup>      | 4.66±0.15 <sup>a,b,d</sup>  | 3.07±0.18 <sup>c</sup>    |
| C20:1n-9       | 3.10±0.45 <sup>c</sup>                                  | 2.53±0.05 <sup>c</sup>      | 3.87±0.37 <sup>a,b,d</sup>  | 2.41±0.71 <sup>c</sup>    |
| C22:1n-11      | 1.42±0.77 <sup>c</sup>                                  | 1.15±0.04 <sup>c</sup>      | 3.35±0.47 <sup>a,b,d</sup>  | 1.01±0.42 <sup>c</sup>    |
| C22:1n-9       | 0.49±0.09                                               | 0.49±0.02                   | 0.89±0.45                   | 0.62±0.18                 |
| C24:1n-9       | 0.31±0.06                                               | 0.40±0.23                   | 0.89±0.86                   | 0.46±0.16                 |
| C18:2n-6t      | 0.08±0.06                                               | 0.13±0.01                   | 0.09±0.10                   | 0.05±0.05                 |
| C18:2n-6c (LA) | 14.60±2.00 <sup>b,c</sup>                               | 10.98±0.02 <sup>a,c,d</sup> | 6.08±0.84 <sup>a,b,d</sup>  | 15.71±0.62 <sup>b,c</sup> |
| C18:3n-6       | 0.11±0.06                                               | 0.13±0.00                   | ND                          | 0.12±0.13                 |
| C20:2n-6       | 0.68±0.23 <sup>b</sup>                                  | 0.40±0.02 <sup>a</sup>      | 0.37±0.23                   | 0.68±0.21                 |
| C20:3n-6       | ND                                                      | 0.13±0.01                   | ND                          | 0.11±0.10                 |
| C20:4n-6 (AA)  | 0.26±0.05 <sup>b</sup>                                  | 0.57±0.05 <sup>a,c,d</sup>  | 0.27±0.15 <sup>b</sup>      | 0.20±0.10 <sup>b</sup>    |
| C18:3n-3 (ALA) | 2.92±0.46 <sup>b,c</sup>                                | 1.78±0.04 <sup>a,c</sup>    | 0.52±0.31 <sup>a,b,d</sup>  | 3.06±0.99 <sup>c</sup>    |
| C18:4n-3       | 0.39±0.13 <sup>b</sup>                                  | 0.82±0.06 <sup>a</sup>      | 0.50±0.30                   | 0.33±0.11                 |
| C20:3n-3       | 0.07±0.07 <sup>d</sup>                                  | 0.14±0.01                   | ND                          | 0.22±0.05 <sup>a</sup>    |
| C20:4n-3       | 0.23±0.07 <sup>b</sup>                                  | 0.46±0.02 <sup>a</sup>      | 0.23±0.19                   | 0.32±0.15                 |
| C20:5n-3 (EPA) | 1.89±0.53 <sup>b</sup>                                  | 4.23±0.42 <sup>a,c,d</sup>  | 2.01±0.87 <sup>b</sup>      | 1.12±0.58 <sup>b</sup>    |
| C22:6n-3 (DHA) | 3.01±0.89 <sup>b</sup>                                  | 8.32±1.12 <sup>a,c,d</sup>  | 2.06±1.47 <sup>b</sup>      | 2.19±1.15 <sup>b</sup>    |
| SFA            | 23.93±2.13 <sup>b,c</sup>                               | 29.06±0.60 <sup>a,c</sup>   | 41.45±3.04 <sup>a,b,d</sup> | 21.24±3.25 <sup>c</sup>   |
| MUFA           | 51.82±2.45 <sup>b,c</sup>                               | 42.85±1.38 <sup>a,d</sup>   | 46.43±2.64 <sup>a,d</sup>   | 54.66±3.74 <sup>b,c</sup> |
| PUFA           | 24.25±2.30 <sup>c</sup>                                 | 28.09±1.73 <sup>c</sup>     | 12.12±3.72 <sup>a,b,d</sup> | 24.10±1.38 <sup>c</sup>   |
| Total n-6      | 15.74±2.06 <sup>b,c</sup>                               | 12.34±0.07 <sup>a,c,d</sup> | 6.81±1.12 <sup>a,b,d</sup>  | 16.87±0.37 <sup>b,c</sup> |
| Total n-3      | 8.51±1.29 <sup>b</sup>                                  | 15.75±1.66 <sup>a,b,c</sup> | 5.31±2.61 <sup>b</sup>      | 7.23±1.48 <sup>b</sup>    |

Results are expressed as mean values (18 samples per species) ± standard deviations; ND, not detected; LOD, the limit of detection of 0.05%; PA, palmitic acid; OA, oleic acid; LA, linoleic acid; ALA,  $\alpha$ -linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Significant differences ( $p < 0.05$ ): <sup>a</sup>*vs.* sea bass; <sup>b</sup>*vs.* dentex; <sup>c</sup>*vs.* turbot; <sup>d</sup>*vs.* sea bream.

**Table 4.** Absolute quantification of fatty acids (mg FA/100 g edible muscle part) ALA, EPA, DHA, AA, and LA.

| Fatty acids (mg/100 g) | Sea bass                      | Dentex                          | Turbot                        | Sea bream                      |
|------------------------|-------------------------------|---------------------------------|-------------------------------|--------------------------------|
| ALA                    | 245.01±38.71 <sup>b,c</sup>   | 172.28±4.34 <sup>a,c</sup>      | 18.59±11.01 <sup>a,b,d</sup>  | 295.29±95.63 <sup>c</sup>      |
| EPA                    | 158.79±44.66 <sup>b,c</sup>   | 410.40±40.68 <sup>a,c,d</sup>   | 72.74±31.67 <sup>a,b</sup>    | 108.27±56.02 <sup>b</sup>      |
| DHA                    | 254.06±75.55 <sup>b,c</sup>   | 810.32±109.05 <sup>a,c,d</sup>  | 74.99±53.53 <sup>a,b</sup>    | 219.90±112.20 <sup>b</sup>     |
| EPA + DHA              | 418.25±113.42 <sup>b,c</sup>  | 1220.72±149.60 <sup>a,c,d</sup> | 147.73±69.83 <sup>a,b</sup>   | 321.16±162.62 <sup>b</sup>     |
| AA                     | 22.20±4.29 <sup>b</sup>       | 55.54±4.48 <sup>a,c,d</sup>     | 9.69±5.37 <sup>b</sup>        | 19.57±9.43 <sup>b</sup>        |
| LA                     | 1223.79±167.09 <sup>c,d</sup> | 1062.69±2.47 <sup>c,d</sup>     | 219.33±30.40 <sup>a,b,d</sup> | 1517.76±57.98 <sup>a,b,c</sup> |

Results are expressed as mean values (18 samples per species) ± standard deviations; ALA,  $\alpha$ -linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; LA, linoleic acid.

The contents of LA and ALA should depend on the fish's dietary intake because fish lack the *delta* 12 and *delta* 15 desaturase enzymes responsible for the conversion of OA to LA and ALA (RUIZ-LOPEZ *et al.*, 2012). The higher LA and ALA contents presumably indicate the presence of vegetable oils in the feed, such as sunflower, soybean, rapeseed, and linseed oil. LA and ALA were significantly ( $p < 0.05$ ) lower in the turbot, whereas LA was significantly higher ( $p < 0.05$ ) in the sea bream than in the other species (Table 4).

There are various recommendations for both the consumption of fish and the intake of n-3 LCPUFA (primarily EPA and DHA). To meet the recommended EPA and DHA dietary requirements for human nutrition, the American Heart Association (2015) recommends the consumption of two servings of fish (particularly oily fish) twice a week. In terms of the fat content, the German Nutrition Society recommends a smaller serving of oily fish (i.e., 70 g) and a larger serving of lean fish (80-150 g) once a week (BERGLAITER, 2012).

The majority of organizations recommend two servings of fish (approximately 140 g per meal) per week. This would provide approximately 500 mg of EPA and DHA combine a day, which is the intake most countries and organizations recommend (WHO, 2003; KRIS-ETHERTON and INNIS, 2007) for optimal overall health and a reduced cardiovascular risk. However, the lowest daily value set for adults by the European Food Safety Authority (EFSA) is 250 mg of EPA plus DHA (EFSA, 2009). The absolute EPA and DHA contents were significantly higher ( $p < 0.05$ ) in the dentex than in other species analysed, with values of 1220 mg/100 g EP (Table 4). Therefore, to fulfil the lowest-level recommendation set by the EFSA, the amounts of sea bass, dentex, turbot, and sea bream that must be consumed on a daily basis are 61 g, 21 g, 167 g, and 78 g, respectively.

The dietetic value of fish meat is also determined by its lipid quality indices, which reflect the relative proportions of constituent saturated and unsaturated FAs. These indices indicate the global dietetic quality of the lipids and their potential impact on the development of coronary heart disease (ULBRITCTH and SOUTHGATE, 1991). The nutritional quality indices determined for different fish species in this study are shown in Table 5.

The sea bass, sea bream, and turbot had significantly higher proportions of n-6 FAs than the dentex, whereas the dentex had a significantly higher proportion of n-3 FAs (C18:4n-3, C20:4n-3, EPA, DHA) (Table 2). Consequently, the n-3/n-6 ratio was significantly higher for the dentex (Table 5). RIGOS *et al.* (2012) also confirmed the high proportion of n-3 FAs in farmed dentex. They also found a significantly higher n-3/n-6 ratio in farmed dentex than in wild dentex. The n-3 FAs are lower in modern aquaculture products than in wild, naturally caught fish, and the high levels of terrestrial-plant-originating C18:2 n-6 present in feedstuff affect the n-3/n-6 ratio in the EP of farmed fish (GRIGORAKIS, 2007).

**Table 5.** Nutritional quality indices determined for four species of farmed fish.

| Parameter | Sea bass                 | Dentex                      | Turbot                     | Sea bream                |
|-----------|--------------------------|-----------------------------|----------------------------|--------------------------|
| n-3/n-6   | 0.55±0.11 <sup>b</sup>   | 1.28±0.13 <sup>a,b,c</sup>  | 0.74±0.30 <sup>b</sup>     | 0.43±0.09 <sup>b</sup>   |
| PUFA/SFA  | 1.02±0.17 <sup>c</sup>   | 0.97±0.07 <sup>c</sup>      | 0.30±0.10 <sup>a,b,d</sup> | 1.15±0.19 <sup>c</sup>   |
| AI        | 0.35±0.05 <sup>c</sup>   | 0.49±0.02 <sup>c</sup>      | 1.10±0.13 <sup>a,b,d</sup> | 0.32±0.07 <sup>c</sup>   |
| TI        | 0.38±0.04 <sup>c</sup>   | 0.36±0.03 <sup>c</sup>      | 0.97±0.33 <sup>a,b,d</sup> | 0.35±0.06 <sup>c</sup>   |
| HH        | 3.34±0.55 <sup>b,c</sup> | 2.46±0.08 <sup>a,c,d</sup>  | 0.91±0.13 <sup>a,b,d</sup> | 3.94±1.03 <sup>b,c</sup> |
| FLQ       | 4.53±1.25 <sup>b</sup>   | 11.67±1.43 <sup>a,c,d</sup> | 3.69±1.75 <sup>b</sup>     | 3.06±1.55 <sup>b</sup>   |

Results are expressed as mean values (18 samples per species) ± standard deviations; n-3, omega-3 fatty acids; n-6, omega-6 fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AI, atherogenic index; TI thrombogenic index; HH hypocholesterolaemic/hypercholesterolaemic ratio; FLQ, flesh lipid quality. Significant differences ( $p < 0.05$ ): <sup>a</sup>*vs.* sea bass; <sup>b</sup>*vs.* dentex; <sup>c</sup>*vs.* turbot; <sup>d</sup>*vs.* sea bream.

PUFA/SFA values above 0.4-0.5 and n-3/n-6 < 0.25 are required if a diet is to combat various 'lifestyle diseases', such as coronary heart disease and cancer (SIMOPOULOS, 2002). The higher the n-3/n-6 ratio, the more the body is able to use n-3 fats (WOOD *et al.*, 2008). In this study, the recommended n-3/n-6 ratio was met by all the fish species, but the recommended PUFA/SFA ratio was not met by the turbot, which had the lowest PUFA/SFA ratio (0.30 ± 0.10). The n-3/n-6 ratio is suggested to be a good parameter for comparing the relative nutritional values of different species (PIGGOT and TUCKER, 1990). However, this index is of limited value if the FA composition is unknown. Generally, C20 and C22 FAs are more valuable from a nutritional standpoint than C18 FAs (ARTS *et al.*, 2001). Because they are quantitatively predominant, the two FAs EPA and DHA are largely responsible for differences in the n-3/n-6 ratio, a reliable indicator of relative nutritive value of lipids, as was also confirmed in our study (Table 5).

However, some researchers consider that an index such as PUFA/SFA may be inadequate for evaluating the nutritional value of fats, because some SFAs do not increase plasma cholesterol and because the ratio ignores the effects of MUFA (ORELLANA *et al.*, 2009). A recent study suggested that C12:0 and C14:0 more effectively increase total cholesterol than C16:0, whereas C18:0 has no effect on the concentration of total serum cholesterol, and no apparent effect on either LDLs or high-density lipoproteins (MENSINK and KATAN, 1992; DALEY *et al.*, 2010). Therefore, the C12:0, C14:0, and C16:0 FAs present in human diets are associated with increased plasma cholesterol. This association is strongest for C14:0, which has a potentially 4-6 times higher capacity to increase cholesterol concentrations than C16:0 (ULBRITCH and SOUTHGATE, 1991; MENSINK and KATAN, 1992; BRESSAN *et al.*, 2011).

Based on the discussion presented above, another indicator of nutritional quality, HH, was determined to gain insight into the effects of FAs on blood cholesterol levels. A higher value for the HH index is preferable (SANTOS-SILVA *et al.*, 2002; TESTI *et al.*, 2006). In this study, the HH values ranged from 0.91 ± 0.13 in the turbot to 3.94 ± 1.03 in the sea bream. The HH ratios were significantly higher for the sea bass and sea bream than for the turbot because these two species contain significantly lower proportions of SFA.

Two other indices, AI and TI, were investigated because their effects on the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation, differ from those of single FAs. As expected, AI and TI were always highest for the most atherogenic and thrombogenic dietary components. It is assumed that lipids with AI < 1 and TI < 1 are beneficial to human health. TONIAL *et al.* (2014) suggested that MUFA and PUFA have more profound health benefits because they prevent coronary disease. The AI and TI

values determined in this study were lower than 1, except those for turbot, which had significantly higher ( $p < 0.05$ ) AI and TI values than the other fish species because it has a higher proportion of SFA. In the study conducted by VALFRÈ and co-authors (2003), an AI of 0.45 and a TI of 0.25 were determined for the sea bass, whereas DE FRANCESCO *et al.* (2007), in a study of the sea bream, found AIs of 1.545-1.565 and TIs of 0.196-0.430.

Our results are consistent with previously published data. FLQ was significantly higher in the dentex ( $p < 0.05$ ) than in the other three species. Because this index is related to the proportions of DHA and EPA in the total fish lipid content, it is expected to be highest in the dentex, because this species is the richest source of these two n-3 FAs. However, the lipid quality indices AI and FLQ for the dentex were lower in this study than in another study (SUAREZ and CERVERA, 2010), whereas the TI values were quite similar. However, FLQ for the other species in this study were lower than those reported previously for the gilthead sea bass (SENSO *et al.*, 2007).

#### 4. CONCLUSIONS

This study demonstrates the high variability in the basic chemical and mineral contents of the species studied. The proportion of omega-3 FAs was twice as high in the dentex than in the sea bream, sea bass, or turbot, and the proportions of EPA and DHA are 2-4 times higher in the dentex. The recommended n-3/n-6 ratio was present in all the fish species, but the recommended PUFA/SFA ratio was not present in the turbot, which had the lowest PUFA/SFA ratio. The HH ratio was significantly higher for the sea bass and sea bream than for the turbot. Because the fish species analysed vary in their nutritional quality, the consumption of diverse fish species is advisable. Based on established lipid quality indicators, the dentex, a fish species underexploited in aquaculture, is a highly recommended and important source of the FAs required for human health.

#### ABBREVIATIONS

AA, arachidonic acid (C20:4 n-6); AI, atherogenic index; DHA, docosahexaenoic acid (C22:6 n-3); EP, edible part; EPA, eicosapentaenoic acid (C20:5 n-3); FAs, fatty acids; FLQ, flesh lipid quality; HDL, high-density lipoprotein; HH, ratio of hypocholesterolaemic to hypercholesterolaemic fatty acids; LA, linoleic acid, C18:2 n-6; LC PUFA, long-chain polyunsaturated fatty acids; LDL, low-density lipoproteins; MUFA, monounsaturated fatty acids; OA, oleic acid, C18:1 n-9; PA, palmitic acid, C16:0; SFA, saturated fatty acids; TI, thrombogenic index.

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## EVALUATION OF THE SAFETY OF *MILANO*-TYPE DRY FERMENTED SAUSAGES PRODUCED BY A FAST DRYING TECHNOLOGY

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### ABSTRACT

A challenge test based on the inoculum of a multi-strain cocktail of *Listeria innocua* and *Salmonella enterica* viable cells was carried out to evaluate the capacity of an accelerated manufacturing technique (including conventional fermentation of the meat batter followed by freezing, slicing and drying) to guarantee the safety of *Milano*-type fermented sausages. The counts of *S. enterica* decreased by 1.5 and 1.6 log CFU/g in the sausages inoculated with 2 and 4 log CFU/g, respectively, while a less notable reduction (0.4 log CFU/g) was recorded for *L. innocua*, independently from the inoculum load. The comparison between the main microbiological and physico-chemical features of non-inoculated fermented sausages, produced through either the accelerated or the traditional process, highlighted significant differences in the percent R.H. and  $a_w$  values, as well as pH. In both cases, the absence of *Salmonella* spp. and *Listeria monocytogenes* was ascertained. These outcomes encourage further investigation on the fate of these foodborne pathogens during a shelf-life challenge test. No differences were highlighted for the main sensory parameters analyzed.

*Keywords:* accelerated drying, challenge test, dry fermented sausages, food safety, *Milano*-type fermented sausages

## 1. INTRODUCTION

Meat fermentation combined with salting, drying, and sometimes smoking dates back to very ancient times; therefore, in most European Countries the production of fermented dry sausages is largely carried out by following traditional procedures (AQUILANTI *et al.*, 2016). As ready-to-eat (RTE) meat products, dry (fermented) sausages must be stable and safe at the end of the production process; therefore, particular attention must be devoted to the control of pathogenic microorganisms such as *Listeria monocytogenes* and *Salmonella* spp. that are more likely to survive during manufacturing (PETRUZZELLI *et al.*, 2010), thus constituting a risk for consumer health. Safety of fermented dry sausages, as well as stability towards alterations caused by spoilage microorganisms are generally assured by the interaction of various hurdles that include water activity ( $a_w$ ), acidity (pH), redox potential (Eh), preservatives (e.g., nitrate/nitrite, sorbate, sulfite), and competitive microorganisms, such as lactic acid bacteria. Among these hurdles, low values of  $a_w$  and pH are considered the most important for the inhibition and inactivation of pathogenic bacteria, moreover, pH decrease and the water loss occurring during ripening also exerts a fundamental effect on the textural properties (FEINER, 2006; DALZINI *et al.*, 2015). The drying/ripening phase is therefore regarded as crucial in fermented dry sausage production, where it also represents the most time-consuming step. For this reason, in 2004, Comaposada *et al.* (COMAPOSADA *et al.*, 2004) proposed a new technique called Quick-Dry-Slice “QDS process®”, which is aimed at accelerating the drying/ripening phase of dry sausages. With such a method, sausages are fermented to the desired pH and then frozen, sliced, and dried using a continuous system based on the application of convective air (COMAPOSADA *et al.*, 2008; STOLLEWERK *et al.*, 2011).

In the present study a challenge test based on the inoculum of *Listeria* and *Salmonella* viable cells was carried out in order to evaluate the capacity of an accelerated manufacturing technique, similar to the QDS process®, to guarantee the safety of *Milano*-type sausages. The main microbiological and physico-chemical features of the fermented sausages produced through either the accelerated or the traditional process were compared as well.

## 2. MATERIALS AND METHODS

### 2.1. Experimental manufacturing

Two independent manufacturing trials were performed according to the traditional procedure originally described for the production of *Salame Milano* and the modified procedure, including accelerated drying (Fig. 1). In both cases the same recipe was used, including pork shoulder (75%) and belly (25%), minced with a 4-mm plate, and the following ingredients (expressed as g/100 kg of meat): NaCl (2,300), black pepper powder (200), white pepper powder (100), and granulated garlic (20). The recipe also included 100 g of Nitritec 10/90 Sa, 1,000 g of Saltec Whitec-16+Niko Sa, and 400 g of Protec Pork PR 70 Nat Sa (all purchased by Tec-Al, Traversetolo, Italy) that supplied a defined amount of NaCl (1,500 g/100 kg of meat), sodium nitrite (E252), sodium ascorbate (E301), dextrose, and saccharose. The addition of *Lactobacillus sakei* starter cultures (Lyocarni BOM-13-Clerici-Sacco Group, Cadorago, Italy) was carried out according to the manufacturer's directions.

The meat batter destined for the production of *Milano*-type sausages through the accelerated process was divided into three separate batches (1a, 1b, and 1c) in order to carry out the challenge test. Two batches (1a and 1b) were inoculated with the pathogens

under study at two different contamination levels, whereas the third batch (1c) was processed without pathogens. This last batch was taken as the negative control of the challenge test and the final product obtained from it was compared with *Milano*-type fermented sausages produced through the traditional process.

All three meat batter batches were stuffed into 100-mm-diameter collagen casings and subjected to fermentation at 20-25 °C and 60-70% relative humidity (R.H.) for 48 hours. Subsequently, the sausages were placed at -20 °C, and the frozen sausages were sliced.

The slides were placed on a grid and a final drying was achieved in a ripening room with forced ventilation at a temperature of 20-25°C and 50-60% R.H., for approximately 50 minutes; a decrease in humidity of approximately 40% in the product was obtained.

For each batch, six samples weighing 500 g each were produced.

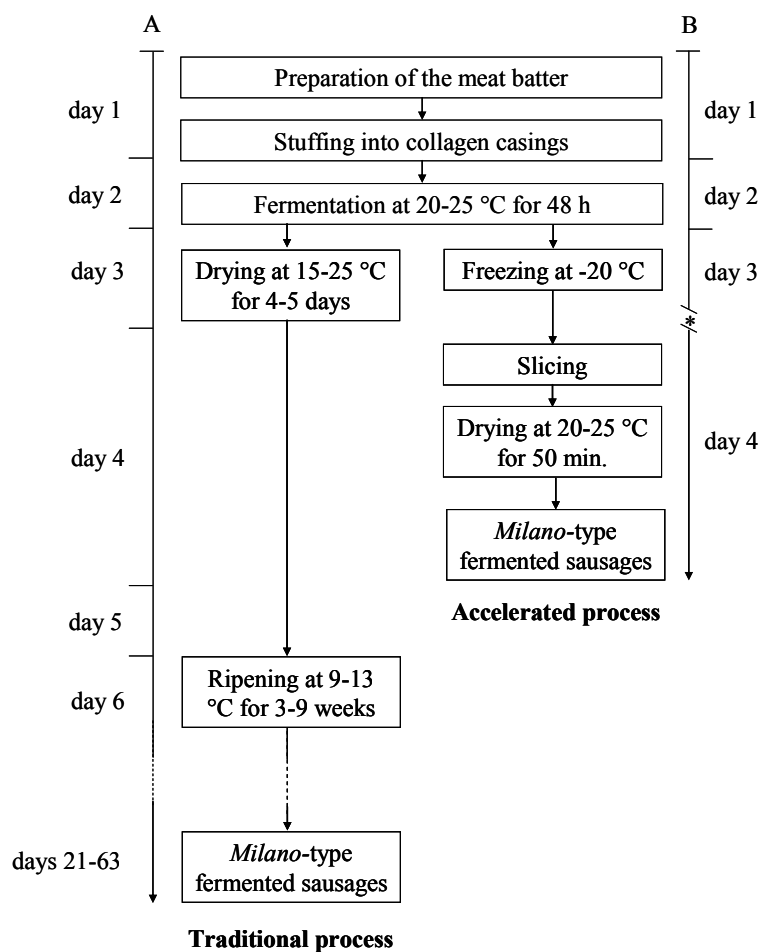


**Figure 1.** Flow chart of the traditional (A) and accelerated (B) process for manufacturing of *Milano*-type fermented sausages.

\*The frozen product is further processed upon market request.

## 2.2. Challenge test

Four strains were used in the challenge test: the *Listeria innocua* ATCC 33090 reference strain was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA), whereas the three *Salmonella enterica* serovars, namely, *Salmonella* Pomona IZSUM 76/13, *Salmonella* Derby IZSUM 31/13, and *Salmonella* Agona IZSUM 39/13, previously isolated from meat products, were obtained from the culture collection of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (Perugia, Italy). All of the strains were stored at -80 °C.

The strain of *Listeria innocua* (as a surrogate of *L. monocytogenes*) and the three low-pathogenicity serovars of *Salmonella enterica* were chosen to allow the challenge test to be safely carried out at the factory. Before being inoculated into the meat batter, each strain was individually subcultured twice in Brain Heart Infusion (BHI, Biokar, Beauvais, France) at 37 °C for 24 h and grown overnight in the same substrate, harvested by centrifugation, washed twice, and resuspended in 0.85% NaCl. Two batches (1a and 1b) were inoculated with the strain cocktail at 2 and 4 Log colony forming units (CFU)/g, respectively. The third non-inoculated batch (1c) was taken as a negative control. Triplicate samples were withdrawn from the three batches along the production timeline: after meat batter preparation (T0), after fermentation and freezing (T1), and after slicing and drying (T2). Counting of *Listeria innocua* was carried out in accordance with the UNI EN ISO 11290-2: 2005 standard method, whereas *Salmonella* spp. viable counts were carried out in CHROMagar *Salmonella* plus (Sharlab, Barcelona, Spain) with incubation at 37 °C for 48 h. The pH values were determined according to ISO 2917:1999 using a portable pH meter (Seven Multi, Mettler Toledo);  $a_w$  was determined in accordance with the ISO 21807:2004 standard method using an Aqualab 3TE device (Decagon Devices, Inc. Pullaman, Washington, USA).

## 2.3. Microbiological analyses

Viable counts were carried out on triplicate samples as follows: total mesophilic aerobes in accordance with UNI EN ISO 4833-1:2013; lactic acid bacteria in accordance with ISO 15214:1998; *Enterobacteriaceae* in accordance with AFNOR BIO 12/21-12/06 (TEMPO, BioMérieux); yeasts on Wallerstein Laboratory Nutrient (WLN) agar (OSIMANI *et al.*, 2009); moulds on Rose-Bengal Agar with Chloramphenicol Selective Supplement (Oxoid) at 22 °C for 3-5 days. The presence of *Listeria monocytogenes* and *Salmonella* spp. was assessed in accordance with AFNOR BIO 12/11-03/04 and AFNOR BIO 12/16-09/05 standard methods, respectively.

## 2.4. Physico-chemical analyses

Physico-chemical analyses were carried out on triplicate samples, as follows: moisture as percent R.H., in accordance with AOAC Official Method 950.46; percent ashes, in accordance with AOAC Official Method 935.42; nitrates and nitrites, via high-performance anion exchange chromatography, using Dionex DX-500 chromatography system (Dionex, Sunnyvale, CA, USA).

## 2.5. Sensory analyses

A preliminary acceptance test to compare the end products obtained through the accelerated technique and the traditional process was carried out. To this aim, eight panelists familiar with the taste/consumption of fermented dry sausages were chosen

among the employees of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche of Perugia (Italy). The following sensory parameters were assessed: odour (meat, animal, spicy, other); aroma (meat, animal, spicy, other); salinity (from low to high); sourness (from low to high); bitterness (from low to high); spicy (from low to high); colour intensity (from pink to dark red); colour uniformity; consistency (from low to high); elasticity (from low to high); hardness (from low to high); moisture (from low to high); chewiness (from low to high). The parameters ranked from low to high were assessed using a 8-point hedonic scale, ranging from 0 (low) to 7 (high).

## 2.6. Statistical analyses

The data collected were subjected to one-way analysis of variance (ANOVA) using JMP statistical software version 11.0.0 (SAS Institute Inc., Cary, NC, USA), and differences were considered non-significant at  $P > 0.05$ .

## 3. RESULTS AND DISCUSSION

*Salame Milano* is a dry fermented sausage originally produced in the geographical area around the city of Milan (Italy). This denomination is included on the official list of Italian traditional products published yearly by the Italian Ministry of Agriculture and Forestry (G.U. *Repubblica Italiana* no. 147, 27/06/2013 Suppl. Ord. No. 52). Today, *Milano*-type fermented sausages are produced in various Italian areas according to the traditional process for *Salame Milano*. Both products are characterized by a bright red colour, a homogeneous "grain of rice" (compact but not elastic) texture, and a sweet-delicate flavour. All of these features are obtained after a long ripening period that varies from 3 to 9 weeks, depending on the product size (20-60 cm of length and 6-11 cm of width). However, recent studies (ARNAU *et al.*, 2007; STOLLEWERK *et al.*, 2011) suggested the possibility of shortening the drying period of fermented sausages, thus reducing the cost of drying facilities (capital and labour) and increasing both profit margin and product competitiveness. Conversely, safety concerns are always to be faced when food processes are shortened. Accordingly, in this study, a challenge test was carried out to investigate the capacity of pathogens to survive during an accelerated production of *Milano*-type dry fermented sausages. A comparison between the main microbiological and physico-chemical features of the dry fermented sausages produced through the traditional protocol for *Salame Milano* and the accelerated technique was carried out as well.

The results of the challenge test during the production of the *Milano*-type fermented sausages are reported in Fig. 2.

It is worth noticing that the samples withdrawn after fermentation and freezing (T1) had microbial counts of *Salmonella* spp. that showed a significant decrease of 1.5 or 1.6 log CFU/g in the sausages inoculated with 2 and 4 log CFU/g, respectively. Afterwards, in both cases, the viable counts did not change significantly, and values of  $0.7 \pm 0.04$  and  $2.7 \pm 0.04$  log CFU/g were recorded in the final samples withdrawn after slicing and drying (T2). A similar behaviour was highlighted with regard to the inoculated *L. innocua* cells, although the decrease recorded from the T0 to the T1 samples (0.4 log CFU/g) was less notable and the final (T2) counts ( $1.4 \pm 0.60$  and  $3.7 \pm 0.01$  log CFU/g) were higher than those measured for *Salmonella*. The non-inoculated control batch (1c) was always negative for the presence of both *Salmonella* and *Listeria* viable cells.

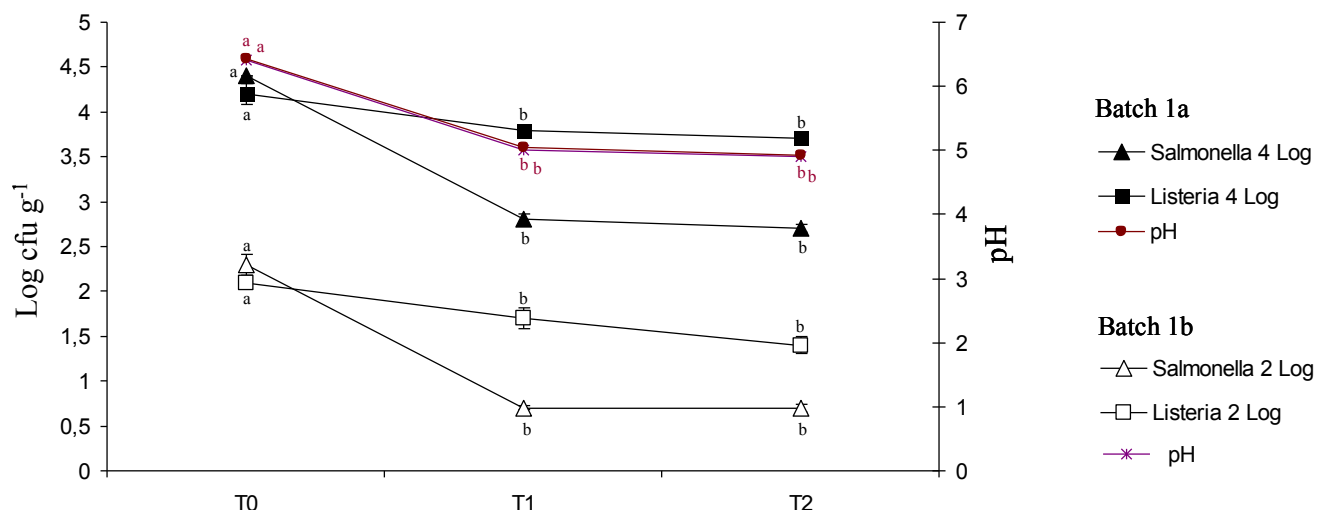


**Figure 2.** Viable counts of inoculated *Listeria innocua* and *Salmonella* spp. strains and pH values measured during the challenge test carried out along the accelerated process for manufacturing of *Milano*-type fermented sausages.

Process steps: after meat batter preparation (T0), after fermentation and freezing (T1, from day 1 to day 3), after slicing and drying (T2, day 4).

Two batches (1a and 1b) were inoculated with the strain cocktail (*Listeria innocua* and *Salmonella* spp.) at 2 and 4 Log colony forming units (cfu) g<sup>-1</sup>, respectively, at T0.

Within each curve, means with different letters are significantly different  $P < 0.05$ , data observed are represented with standard deviation as error bar.

As reported by STOLLEWERK *et al.* (2011), the persistence of *Salmonella* could be related to its adaptation towards acidic environments. Indeed, it is well known that *Salmonella* acid-adapted cells may show increased resistance to organic acids such as those produced by lactic acid bacteria in fermented dairy products. These cells are capable of surviving better than non-adapted ones during fermentation of dairy products stored at 5 °C, possibly as a consequence of adaptive responses to other stresses, including heat shock, oxidative stress, and osmotic stress (LEYER *et al.*, 1992). Conversely, different authors (COLE *et al.*, 1990; SHABALA *et al.*, 2001) have reported that *L. monocytogenes* can survive in stressful environments, such as those characterized by low temperature and high acidity and salt contents. On this topic, MATARAGAS *et al.* (2015) recently found that environmental conditions that prevail during sausage manufacturing may stimulate the expression of general- and/or specific-stress response genes and the intensiveness of these stresses may have an impact on their expression. According to the previous statements, the results of the present challenge test revealed the capacity of both inoculated *Salmonella enterica* serovars and *L. innocua* cells to survive during the accelerated process for the manufacturing of the *Milano*-type fermented sausages. However, the results of our test were more encouraging (especially for *Listeria*) if compared to those obtained by DALZINI *et al.* (2015) in a challenge test during a conventional process for the production of semi-dry low-fat salami. This finding was likely due to the pH ( $4.91 \pm 0.02$  and  $4.93 \pm 0.05$  at 2 and 4 log CFU/g, respectively) and the  $a_w$  ( $0.90 \pm 0.0$ ) values reached in our experimental conditions, which were much lower when compared to those attained by DALZINI *et al.* (2015). Conversely, the final  $a_w$  values obtained through the accelerated process were similar to those reported by ZANARDI *et al.* (2002) for *Milano*-type sausage (0.89) produced in a traditional way.

The main microbiological and physico-chemical features of the *Milano*-type sausages produced through the accelerated technique (batch 1c) were analysed in comparison with the corresponding product obtained (after 3 weeks of ripening) through the traditional process, and the results are reported in Table 1.

The counts of total mesophilic aerobes and lactic acid bacteria were similar in both types of products, independently of the manufacturing process adopted. Because lactic acid bacteria possibly represent a significant fraction of the total mesophilic population, this finding suggests that the accelerated technique does not interfere with the growth of the pro-technological bacteria that was added as starter cultures in either the traditional or the accelerated process. In both cases, the values recorded for LAB counts were in line with those (8 Log CFU/g) reported by REBECCHI *et al.* (1998) for the same type of fermented dry sausages obtained through a traditional process.

By contrast, the absence of a conventional ripening phase was responsible for the significantly lower values found for yeasts and moulds in the samples of sausages obtained through the accelerated process because a conventional, long-lasting ripening is required to allow these “cosmetic” microorganisms to grow on the sausages surface (AQUILANTI *et al.*, 2007). Similarly, when the *Milano*-type sausages were produced through the accelerated technique, the counts of *Enterobacteriaceae* were significantly higher because the conventional ripening is usually responsible for the abatement of such an acid-sensitive microbial population. As is well known, the load of *Enterobacteriaceae* is currently used as an index of enteric contamination in meat (PETRUZZELLI *et al.*, 2016) and may also imply the presence of pathogens (BROWN *et al.*, 2000). However, neither the traditional products nor those obtained through the accelerated technique were found to be positive for *Salmonella* spp. or *Listeria monocytogenes*.

**Table 1.** Microbiological and physico-chemical features of the *Milano*-type fermented sausages produced through either the traditional or the accelerated process.

|                                                            | Traditional process     | Accelerated process     |
|------------------------------------------------------------|-------------------------|-------------------------|
| <b>Microbiological parameters (Log cfu g<sup>-1</sup>)</b> |                         |                         |
| Total mesophilic aerobes                                   | 8.4±0.46 <sup>a</sup>   | 8.4±0.02 <sup>a</sup>   |
| <i>Enterobacteriaceae</i>                                  | < 1                     | 1.4±0.02 <sup>a</sup>   |
| Lactic acid bacteria                                       | 8.4±0.18 <sup>a</sup>   | 8.4±0.01 <sup>a</sup>   |
| Yeasts                                                     | 4.2±0.08 <sup>a</sup>   | 2.2±0.46 <sup>b</sup>   |
| Moulds                                                     | 4.8±0.03 <sup>a</sup>   | 1.7±0.05 <sup>b</sup>   |
| <i>Salmonella</i> spp.                                     | Absent in 25 g          | Absent in 25 g          |
| <i>Listeria monocytogenes</i>                              | Absent in 25 g          | Absent in 25 g          |
| <b>Physico-chemical parameters</b>                         |                         |                         |
| R.H. (%)                                                   | 31.2±0.62 <sup>b</sup>  | 33.0±0.21 <sup>a</sup>  |
| Ashes (%)                                                  | 5.6±0.06 <sup>a</sup>   | 5.0±0.15 <sup>b</sup>   |
| Nitrates (mg/kg)                                           | 178.0±6.00 <sup>a</sup> | 174.0±9.05 <sup>a</sup> |
| Nitrites (mg/kg)                                           | n.d.                    | n.d.                    |
| pH                                                         | 5.6±0.03 <sup>a</sup>   | 5.0±0.05 <sup>b</sup>   |
| a <sub>w</sub>                                             | 0.86±0.01 <sup>b</sup>  | 0.89±0.01 <sup>a</sup>  |

Values are expressed as means ± standard deviation of triplicate samples.

Within each row, means with different letters are significantly different  $P < 0.05$

cfu = colony forming units

n.d. = not detectable

d.m. = dry matter

With regard to the results of the physico-chemical analyses (Table 1), as expected, the *Milano*-type fermented sausages produced through the accelerated drying process showed percent R.H. and  $a_w$  values higher (and percent ash values lower) than those of the products obtained after a traditional ripening/drying phase. However, the recorded value of  $a_w$  ( $0.89 \pm 0.01$ ) was considerably lower than that of *chorizo* obtained through the QDS process® (STOLLEWERK *et al.*, 2011) and was consistent with the values ( $< 0.90$ ) reported for dry sausages manufactured in the Mediterranean area through a ripening period of at least 4 weeks (AQUILANTI *et al.*, 2016). The other significant difference shown by the ANOVA concerns the pH that was higher in the traditionally produced *Milano*-type sausages, most likely due to the more intense oxidative activity of the mould population on lactic acid produced by LAB during carbohydrate fermentation (PAULSEN *et al.*, 2011). Regarding the preliminary sensory analyses carried out to compare the end products obtained through the accelerated technique and the traditional process, no differences were highlighted for odour, texture, aroma, appearance, salinity, bitterness and sourness (data not shown). It is worth noting that, for the meat descriptor, fermented sausages produced through the traditional technique were characterized by a more intense flavour and aroma of ripened meat, whereas a flavour of sour meat was mainly perceived in fermented sausages produced through the accelerated technique.

#### 4. CONCLUSIONS

Safety concerns need always to be faced when food processes are supposed to be shortened or modified. As ready-to-eat (RTE) meat products, dry sausages must be stable and safe at the end of the production process; therefore, particular attention must be devoted to the control of pathogenic microorganisms such as *Listeria monocytogenes* and *Salmonella* spp. that are more likely able to survive during manufacturing, thus constituting a risk for consumer health. Execution of challenge tests represents a key step to evaluate the behaviour of foodborne pathogens when innovative products or processes are implemented, and they are especially required when scarce literature is available on the innovation applied, as in the present case. Overall, the reduction of viable cells of *Salmonella* and *Listeria* recorded in the challenge test carried out in our study and the microbiological and physico-chemical characterization of the products obtained were quite encouraging. Further investigation on the fate of these foodborne pathogens during the shelf-life of the product must be envisaged in order to provide more complete information to food business operators who would manufacture fermented dry sausages using an accelerated production process.

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## RAPID SCREENING METHOD TO ASSESS TANNIN ANTIOXIDANT ACTIVITY IN FOOD-GRADE BOTANICAL EXTRACT

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### ABSTRACT

Fourier transform infrared (FTIR) spectroscopy measurements were used for the prediction of commercial tannins antioxidant capacity, i.e. DPPH, through Partial-least squares (PLS) regression. Plot of the leave-one-out full cross-validated PLS predicted scavenging activity values (DPPH %) with a good correlation ( $r = 0.82$ ), proving FTIR succeeded to rapidly provide information on commercial tannins antioxidant capacity.

*Keywords:* chemometric, DPPH, FTIR, PLS, tannins

## 1. INTRODUCTION

Tannins are naturally occurring polyphenols produced by plants via secondary metabolic processes. Their ability to bind proteins, pigments, and complex metallic ions, together with their flavouring effect are the basis for their extensive use as additives in the food industry. Tannins are commercially available in water suspension liquids, which can be stabilized by the addition of (poly)saccharides, or as lyophilized powders, which can be either a single extract or a blend of two or more tannins, which can have one or more degree of purity (VERSARI *et al.* 2013).

Taking into account their nutritional and technological potentials, the main challenge is their analytical characterization, since suppliers seldom label their composition and the degree of purity. Manufacturers provide general information only regarding the source of the product and its use, whereas additional details such as their antioxidant activity – which makes tannins of great interest in the wine industry, due to a diminished addition of sulphur dioxide in this beverage – are often needed.

In this view, there is an ongoing interest to improve the measurement of the tannin antioxidant activity. It is well known that tannins bind proteins therefore the assays based on reaction catalysed by enzymes for generating radicals are inadequate because they use proteins sensible to oxidation to measure the antioxidant activity. These leads to the disability of tannins to be measured with, since there is the possibility that tannins can interact with the radical generator per se, or sensor, or even to scavenging the radicals themselves. It is important to notice that these both ways of measurements can be seen as antioxidant activity, but it may not be clear whether the tannins are acting or not (HAGERMAN *et al.*, 1998; RIEDL *et al.*, 2002; MILLER *et al.*, 1993; CAO *et al.*, 1993).

Currently there are several antioxidant assays available: ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) cation radical (ABTS•+) scavenging ability), DPPH (2,2-diphenyl-1-picrylhydrazyl radical, DPPH• scavenging capacity), FRAP (ferric reducing antioxidant power), electrochemistry (HAGERMAN *et al.*, 1998; BOUCHET *et al.*, 1998; MUCCILLI *et al.*, 2017; OSZMIANSKI *et al.*, 2007), each of them presenting both advantages and limitations. For example, the ABTS assay needs the time of incubation to be optimized depending on each and every substrate (WALKER and EVERETTE, 2009). Concerning electrochemical analysis, it is necessary to have trained professionals as an expensive apparatus.

Conversely, the application of Fourier transforms infrared spectroscopy (FTIR) analysis in oenology has had a considerable boost in recent times, due to its fastness, reliability and versatility of use. Analytical devices based on the vibrational spectroscopy method are widespread in analytical laboratories of oenological companies, providing the most important quality parameters and supporting oenologists in different process stages. Vibrational spectroscopy enables to disclose the molecular composition of unknown samples, also including complex matrices, and to highlight interactions involved in the molecular arrangements; this latter is especially useful to determine the occurrence of intermolecular interactions.

Therefore, this study aimed to develop a fast analytical approach suitable for screening antioxidant activity of tannins, which can provide reliable information in the wine and food industry to tailor at best the use of commercial tannins.

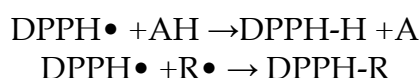
## 2. MATERIALS AND METHODS

### 2.1. Samples

Thirty commercial tannins were provided by suppliers as powder and dissolved in model wine (pH 3.6, 12% EtOH) in a concentration of 1 g L<sup>-1</sup>.

### 2.2. DPPH scavenging activity

The amount of 'bioactive tannins' was calculated with the method of scavenging the DPPH radical (BRAND-WILLIAMS, 1995), which is based on ability of the sample to act as antioxidant agent within the methanolic solution of the free radical 2, 2 diphenyl-1 picryl-hydrazyl (DPPH), which shows a maximum of absorption at 517 nm. Once the antioxidant is added to the solution, absorption diminishes. The chemical reactions that take place are as follows:



DPPH radical reagent was prepared in methanol (25 mg L<sup>-1</sup>). Tannin solutions (100 µL) were mixed with 2.9 mL DPPH radical solution (NIXDORF and HERMOSÍN-GUTIÉRREZ, 2010) and after 60 min, absorbance were measured at 517 nm against methanol. The results are given considering the solution of 2.9 mL of radical and 100 µL as control (100%), and to tannins sample it was considered their ability to neutralize the DPPH radical, in relation to control, according to the following equation:

$$\% \text{ Inhibition} = [(\text{Abs}_{\text{DPPH}} - \text{Abs}_{\text{tannin}}) / \text{Abs}_{\text{DPPH}}] \times 100$$

### 2.3. FTIR Analysis - Spectra acquisition

Fourier-Transform Mid-Infrared (FT-MIR) spectral analysis was performed using a Tensor 27 spectrometer (Bruker Optics) equipped with a horizontal attenuated total reflectance (ATR) zinc selenide (ZnSe) crystal (HATR, PIKE Technologies, Madison, US). A remote-control thermosetting device was used to keep samples (1 mL) at 40 ± 1°C for the whole duration of measurements. Spectra, with a spectral resolution of 4 cm<sup>-1</sup> and 64 scans averaged for each spectrum, were recorded in duplicates from 4000 to 700 cm<sup>-1</sup> for each tannin. The same number of scans was used for background subtraction. Prior to data analysis each spectrum was corrected for the variation in effective path-length using the ATR correction option available in the Spectrum One 5.3.1 software (Perkin-Elmer, Waltham, MA). The spectra were exported in ASCII format to the statistical software for statistical analysis.

### 2.4. Chemometrics and data analysis

Partial Least Square regression (PLS) (Unscrambler 9.7, Camo, Oslo, Norway) was used to model the relationships between the amount of bioactive tannins in commercial samples and the selected wavelengths of FTIR spectra. Multivariate analyses were performed using full cross-validation, i.e. the leave-one-out model, and with x-variables pre-processed as 'Standard Normal Variate' (SNV).

### 3. RESULTS AND DISCUSSION

The tannins were coded by manufacturers (same letter), with info on their chemical classification, if available (Table 1).

**Table 1.** Code, chemical classification and DPPH scavenging activity (%) of commercial tannins.

| Code | Chemical classification                              | DPPH scavenging activity (%)<br>(mean±SD) |
|------|------------------------------------------------------|-------------------------------------------|
| A1   | Not available                                        | 25.7±0.2                                  |
| A2   | Condensed                                            | 44.3±0.7                                  |
| A3   | Hydrolysable / condensed                             | 29.1±0.6                                  |
| A4   | Condensed                                            | 18.7±0.3                                  |
| A5   | Hydrolysable / condensed                             | 26.4±0.01                                 |
| A6   | Hydrolysable / condensed                             | 49.6±2.0                                  |
| A7   | Hydrolysable                                         | 19.3±0.2                                  |
| A8   | Hydrolysable                                         | 25.0±0.3                                  |
| A9   | Hydrolysable / condensed                             | 40.8±0.9                                  |
| A10  | Not available                                        | 66.9±2.0                                  |
| A11  | Hydrolysable                                         | 34.7±1.6                                  |
| A12  | Hydrolysable stabilized with natural polysaccharides | 27.8±0.7                                  |
| A13  | Hydrolysable / condensed                             | 41.9±0.2                                  |
| A14  | Hydrolysable                                         | 36.0±0.4                                  |
| B1   | Hydrolysable                                         | 42.8±0.4                                  |
| B2   | Hydrolysable                                         | 59.4±0.5                                  |
| B3   | Condensed                                            | 30.7±0.6                                  |
| B4   | Hydrolysable                                         | 38.3±0.8                                  |
| B5   | Hydrolysable                                         | 37.9±0.01                                 |
| B6   | Hydrolysable                                         | 29.6±0.7                                  |
| C1   | Hydrolysable                                         | 28.8±0.7                                  |
| C2   | Hydrolysable / condensed                             | 29.8±0.4                                  |
| C3   | Hydrolysable                                         | 34.6±0.1                                  |
| C4   | Condensed                                            | 24.2±1.2                                  |
| C5   | Condensed                                            | 25.4±0.4                                  |
| C6   | Condensed                                            | 32.5±0.3                                  |
| C7   | Condensed                                            | 22.0±0.8                                  |
| D1   | Hydrolysable                                         | 21.3±2.7                                  |
| D2   | Hydrolysable                                         | 36.9±0.2                                  |
| D3   | Hydrolysable                                         | 29.3±2.6                                  |
| D4   | Hydrolysable                                         | 26.2±0.4                                  |

DPPH values ranged between 18.7-49.6%, which can be considered a representative interval suitable for PLS modelling of FTIR spectra. Although the entire IR spectra was acquired, after a preliminary attempt using the whole IR signal (4000 to 700 cm<sup>-1</sup>), a specific region 1750-900 cm<sup>-1</sup> – which is well known as ‘fingerprint’ for polyphenolic compounds – was considered for the PLS modelling, using 6 latent variables explaining up to 77% total X-variance, and 61% total Y-variance. The selection of spectral regions was consistent with

info from the literature (JENSEN *et al.* 2008; RICCI *et al.* 2015), which identified two IR regions, 1485-1425 and 1060-995  $\text{cm}^{-1}$ , as mostly important for tannin quantification.

The fast-molecular screening provided by MIR spectroscopy showed satisfactory correlation with the effective DPPH scavenging activity of commercial tannins ( $r=0.817$ ; slope 0.82) with the root mean square error of full cross-validation (RMSECV) of 6.6 (Fig. 1). This result is consistent with previous finding (VERSARI *et al.*, 2010), therefore confirmed the reliability of FTIR analysis combined with PLS regression as a fast screening method for antioxidant prediction in foodstuffs.

Moreover, it is also known that FTIR associated with chemometric can be useful for the classification of tannin, and fraud disclosure (GRASEL *et al.*, 2016a; GRASEL and FERRAO, 2016b).

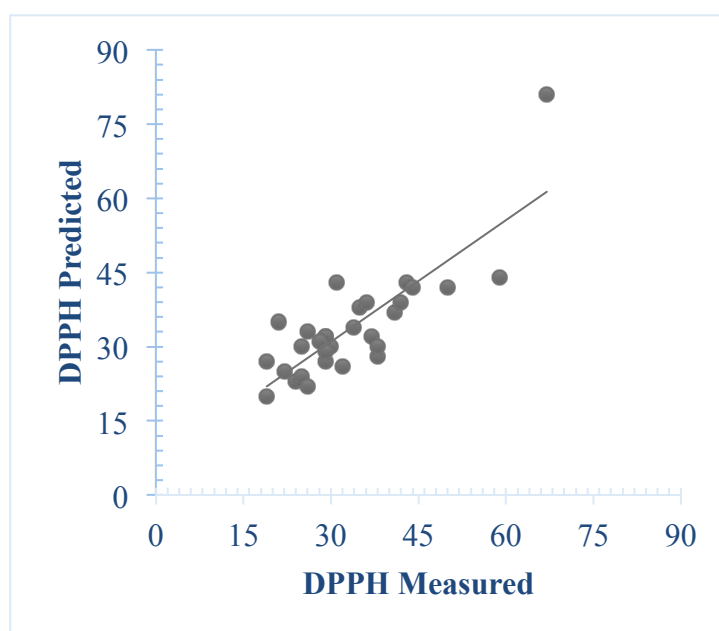


**Figure 1.** Prediction of antioxidant activity (DPPH) of commercial tannins using FTIR and Full Cross PLS Validation.

#### 4. CONCLUSIONS

The preliminary findings of this short communication suggest that FTIR spectroscopy is suitable as a rapid screening tool to provide information on antioxidant activity of commercial tannins. Further FTIR analysis on a larger number of samples is needed to improve the prediction model at best using an independent set of samples.

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