Chapter N (please do not write anything in this line. Editors will annotate the chapter number)

Comparison of the antioxidant component in durum wheat pasta and wholewheat pasta by multivariate analysis

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**Abstract.** Whole products have achieved remarkable success over the years for the growing attention by consumers for the health effects of these foods. In the present study, a multivariate model was developed to characterize durum wheat pasta and whole wheat pasta based on the carotenoid content and to assess the possible influence of the drying process used in pasta manufacturing on the total carotenoid amount. 96 pasta samples were analyzed in HPLC-UV/Vis following an accelerated solvent extraction phase (ASE system) previously optimized using an experimental design approach (CCD, central composite design). A PLS-DA (Partial Least Squares-Discriminant Analysis) method was used for data processing. The resulting classification model showed excellent results (100 % of correct classification for both pasta categories) discriminating the samples based on the semolina used for the production (refined durum wheat vs whole wheat). The findings also highlighted that lutein was present in much larger amounts compared to the other bioactive components in all investigated samples, suggesting that it might be used as a product marker of total carotenoids in pasta.

**Keywords.** Whole wheat pasta, carotenoids, lutein, central composite design, PLS-DA.

**N.1 Introduction**

According to the International Pasta Organization, Italy confirms its leadership in the global market in 2021, accounting for about a quarter of the entire world’s production and 67% of that of Europe. The pasta was also the most exported product during the Covid-19 pandemic in 2020, growing by 16% in the countertrend with Made in Italy overall. In recent years, due to the increasing demand by consumers for foods that contribute to wellness and health via a balanced nutritional composition, the whole wheat pasta has gained success on the market. The wholegrain products, besides having a lower glycemic index compared to their non-wholegrain counterpart, contribute to reducing the cardiovascular disease risk (Anderson et al., 2000), type 2 diabetes (Liu et al., 2000) and some types of cancer (Jacobs et al., 1995) as evidenced by numerous scientific studies. According to the Healthgrain Consortium of the European Union, “*whole grains shall consist of the intact, ground, cracked or flaked kernel after the removal of inedible parts such as the hull and husk. The principal anatomical components, the starchy endosperm, germ and bran are present in the same relative proportions as they exist in the intact kernel*”. Other definitions were recommended by Regulation (EU) No. 1308/2013; EFSA Panel on Dietetic Products, Nutrition, and Allergies (2010); FDA Consumer Health Information; and various national agencies. Although wholegrain pasta seems to show particular interest for the modern consumer due to its content in fiber and bioactive components with health benefits effects, the different texture, aroma, and color compared to refined pasta still limits its widespread acceptance. In this context, the current challenge for producers is to manufacture whole grain products with organoleptic characteristics most acceptable to customers, since their market penetration for nutritional contribution is already widely visible (Liu, 2007).

In wholewheat pasta, the bran and germ used for its production are high in fibre, minerals, vitamins, and antioxidant substances such as carotenoids, flavonoids, phenols and tocols, which have a positive impact on several biological activities (Gardner, 1988; Giacco et al., 2016). Among the different classes of bioactive components in pasta, this study focused on the carotenoid determination. Carotenoids are yellow-orange pigments classified into two groups (depending on the presence or absence of oxygen in their molecular structure): xanthophylls such as lutein and carotenes (with provitamin A activity) such as beta-carotene. In durum wheat, lutein accounts for 86-94% of the total carotenoids (Panfili et al., 2004; Fratianni et al., 2005).

Carotenoids class continues to receive scientific interest, not only because it contributes to the brilliant yellow color of pasta (e.g., lutein can be used as an indicator of the quality color) - an aspect widely appreciated by consumers - but because it is an important source of vitamin A. This aspect is particularly significant since vitamin A insufficiency is one of the leading causes of death among children in third-world areas (WHO, 2009). In addition, their antioxidant capacity is the most relevant property in terms of human health.

Due to their low stability, carotenoids are particularly susceptible to oxidation reactions; however, the presence of proteins in the food matrix increases their stability. This is also true for thermal degradation, as these molecules can undergo degradation to relatively low temperatures (40-50 °C) if isolated (Ornelas-Paz & Yahia, 2010). Two competing effects can occur in cereal-based product manufacturing: on one side, the mixing phase can lead to the breakdown of the protein-carotenoid complexes, increasing their bioavailability; on the other side, the drying process temperatures can lead to a decrease in carotenoids levels due to their thermolability. Therefore, in pasta produced with LT-Lt (Low Temperature-Long time) methods, it is possible to expect a decrease in the carotenoid content less than those subjected to the HT/VHT-St (High Temperature/Very High Temperature-Short time) methods (more drastic temperature conditions). During pasta manufacturing, physical processes also can affect the total content of carotenoids in the finished product (Behsnilian et al., 2009).

In this study, a multivariate classification model was developed to characterize durum wheat pasta and whole durum wheat pasta based on the carotenoid profiles. Particularly attention was paid to lutein to investigate its potential as a product marker. One further statistical model to assess the possible influence of the temperature used in the pasta drying process on the total carotenoid content was also constructed.

**N.2 Analytical procedure and statistical analysis**

A set of 96 short pasta samples (64 durum wheat pasta and 32 whole wheat pasta) was analyzed. An ASE (accelerated solvent extraction) procedure for the carotenoid extraction from the samples was developed. ASE parameters were optimized using an experimental design and the optimal conditions obtained were used for the analysis. 5 g of pasta samples were ground and extracted under the following conditions: three static cycles of 6 min each, a temperature of 100 °C, and an extraction mixture of ethanol/acetone/hexane (27.6:22.4:50) v/v/v. The extraction pressure was kept constant at 1500 psi, with a flush volume of 60%. A rotary evaporation system kept at 40 °C was employed for extract evaporation. Before HPLC-UV/Vis analysis, the extracts were dissolved in 1 mL of ethanol. A Kinetex® C18 LC column (5 μm 250 x 4.6 mm ID) maintained at 25 °C was used for chromatographic separation. The mobile phase was a binary solvent system consisting of phase A water/acetonitrile (90:10) v/v and phase B 100 % acetonitrile, both containing 0.1 % formic acid. The elution gradient was programmed as follows: 2 min. at 25% B, 2-12 min. at 100% B, 12-22 min. at 100% B, and 22-30 min. at 25% B. The flow rate was 1 mL/min, and the wavelength was set at 450 nm. Data were collected by Chromeleon 7 software. To optimize the main ASE parameters used for the carotenoid extraction from pasta samples, an experimental design was performed. This statistical approach enables the planning of the minimum number of experiments to be performed, resulting in increased cost-effectiveness of the analysis and lower consumption of solvents.Considering that there might be a non-linear relationship between the values ​​of the three factors and the response to be optimized, a Central Composite Design (CCD) was used. The discriminant classification method PLS-DA (*Partial Least Squares-Discriminant Analysis*) was used for data processing with the goal of characterizing the two pasta typologies (durum wheat pasta and whole wheat pasta) in relation to carotenoid content.

**N.3 Results and discussions**

**N.3.1 Experimental design**

In this study, a three-factor face-centred central composite design (CCD) was performed to optimize the principal ASE parameters: extraction temperature, time of each static cycle, and volume ratio of extraction solvents. The limits of the experimental domain were identified with the following values: ​​40 and 100 °C for the temperature, 2 and 6 minutes for static time, and (25:25:50) v/v/v and (33:17:50) v/v/v for the mixture of ethanol/acetone/hexane. Based on these conditions, 15 experiments were required overall from the experimental design. Each experiment was carried out in duplicate to evaluate the accuracy of the measurements and randomly performed to avoid spurious correlations. The chromatographic data were then imported into Matlab, pretreated using the iCofish algorithm to eliminate the slight peaks misalignment, and statistically processed. Two different approaches were performed to define the responses to be optimized: a targeted approach in which the peak areas of lutein (the compound of interest) were assessed and an untargeted approach where the whole chromatographic profile was considered.

Multiple linear regression was used for the targeted strategy, which showed the best response (highest lutein peak area) for T = 100 ° C, static time of 6 min, and intermediate ratio for the extraction solvent combination (27.6 % ethanol, 22.4 % acetone, 50 % hexane). For the untargeted approach, required to evaluate the influence of the various parameters on the total recovery of the compounds of interest, the analysis of the main components (PCA) was performed. The obtained model confirmed that the optimal conditions with this strategy match those obtained with the targeted approach. The 96 samples of pasta were analyzed once the conditions that maximized the desired result, i.e., optimal conditions for the extraction of lutein and total carotenoids, were obtained.

**N.3.2 PLS-DA classification**

For data processing, the PLS-DA method was used to highlight possible differences in terms of carotenoid content between samples of durum wheat pasta and samples of whole wheat pasta. Before the classification model construction, the data were processed with the iCofish algorithm to reduce peak misalignment.

The first performed classification was focused on the type of durum wheat semolina used in pasta manufacturing (refined and whole wheat). Due to the different number of analyzed samples for the two pasta categories (64 conventional and 32 whole wheat), the classification percentage error was previously studied. Despite the disparity in the number of samples between the two classes, the average between the errors made for both classes and the total classification error was almost coincident. The minimum relative error, corresponding to 10 latent variables, was 1 %; so, it is reasonable to assume that the two classes are predicted in a comparable way. This preliminary cross-validation output revealed the possibility to carry out a classification like that. The classification model was then built and validated with an external set of samples using the Duplex algorithm. The training set used to develop the model was constructed with 60 samples (40 conventional and 20 whole wheat) and the test set (i.e., the validation set with which the predictive power of the model is tested) with 36 samples (24 conventional and 12 whole wheat). The classification error in cross-validation on the training set model with 10 latent variables was 1.7 %. The model developed using the training set has been applied to the missing data, i.e., those in the test set. It was thus feasible to assess its prediction capacity, which was shown to be 100 % correct classification for both types of samples, i.e., all traditional pasta samples are included in class 1 and all whole wheat pasta samples are included in class 2.

The second classification, on the other hand, was designed to investigate the temperature (LT and HT) effect of the drying process on the overall carotenoid content. According to the pack label, 26 of the 96 samples analyzed were LT-Lt (class 1) and 70 HT-St (class 2). The Duplex algorithm is also used in this case including 66 samples in the training set (16 LT-Lt and 50 HT-St) and 30 in the test set (10 LT-Lt and 20 HT-St). Unlike the first classification, this PLS-DA model with 8 latent variables (minimal classification error) does not provide excellent results since the sensitivity for class 1 is 50 % and for class 2 is 70 % (63.3 % accuracy). Nevertheless, a preliminary data analysis suggests that these values may be influenced by the fact that the sample set includes indiscriminately both refined and wholewheat pasta. For this reason, the same classification was performed using only the durum wheat pasta samples to avoid any contributing effects resulting from the different treatments of durum wheat, but the sensitivity of the model stayed at 50% for class 1 and 73% for class 2. However, a more in-depth assessment reveals that the reasons why the findings of carotenoids chromatographic analysis do not lead to the correct models for this classification may be found in more than one reason. On the one side, the drying process may have no effect on the carotenoid content in pasta, or the samples analyzed are insufficient or unrepresentative of the context in concern. As already reported in the Introduction section, according to several studies rising temperatures cause the destruction of carotenoids due to their low thermal resistance on the one hand, and the release of these substances on the other due to the breakdown of connections between carotenoids and other macronutrients. As a result, it could be reasonable that separation based on carotenoids cannot be performed due to the series of “contrasting” reactions that occur in the matrix during the whole manufacturing process.

**N.4 Conclusions and future perspectives**

The classification based on refined and whole wheat semolina obtained from the 96 pasta samples investigate produced excellent results with a capacity prediction of the model equal to 100%. In consideration of the numerous scientific studies that have amply demonstrated the beneficial effects of carotenoids on human health, it is reasonable to conclude that consuming whole-wheat pasta contributes positively to the supply of antioxidant substances to the body. The same can be stated for lutein levels found in whole wheat pasta when compared to refined pasta samples, resulting in a clearly higher antioxidant capacity of the whole meal product, which is accompanied by a lower loss of nutritional components due to the use of germ and bran during grinding. The results also revealed that lutein was present in significantly higher amounts than any of the other components in all the samples studied, and hence might act as a marker product of the total amounts of carotenoids in pasta.

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