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A novel approach based on untargeted lipidomics reveals differences in the lipid pattern among durum and common wheat

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ABSTRACT

In the present work the possibility of using an untargeted metabolomic strategy to discriminate between common and durum wheat lipidome for an authenticity purpose was explored. A first study was conducted by analyzing 52 samples from two durum and common wheat varieties. Afterwards, an extended and independent sample set (173 samples and five varieties) was used as a confirmatory study to verify the stability and consistency of the models obtained. Putatively identified markers were evaluated applying ROC curves resulting in individual marker AUC > 90% both in preliminary and confirmatory study. In addition, digalactosyl diglyceride (DGDG) 36:4 was shown to be an effective marker differentiating between authentic durum wheat and its adulterated admixture down to 3% adulteration level, which is the maximum contamination level allowed by Italian legislation. The results demonstrated that untargeted lipidomics, in conjunction with chemometric tools has a significant potential for screening and detection of wheat fraud.

1. Introduction

Pasta constitutes a dominant portion of a standard Mediterranean diet, supplying a large fraction of the needs for energy-rich materials, such as considerable amounts of carbohydrates, proteins, fiber or minerals (Pauly, Pareyt, Fierens, & Delcour, 2013; Shewry, 2009). There are many forms to cook pasta, and for this reason, pasta has been regularly voted in the top favorite dishes for many years, for almost everyone. In fact, 14.3 million tons of pasta are produced worldwide according to the survey carried out by the Associations of Pasta Manufacturers of the European Union (UN.A.F.P.A, 2015). In other words, it's clear that pasta is a big business, and where there is big business there is the potential for fraud (Everstine, Spink, & Kennedy, 2013).

The most important wheat species are durum wheat (*Triticum turgidum* spp. *durum*), also called pasta wheat to reflect its major end-use, and common wheat (*Triticum aestivum*), which is usually employed to make bread or other baked goods (Shewry, 2009). In Italy, dried pasta must be exclusively made of durum wheat, allowing a maximum common wheat flour contamination of 3% (Ministero Dell'Interno, 2001), considering that accidental contamination of semolina with bread wheat during harvesting, transport or storage remains possible. Europe's national governments, on the contrary, permit the production of dried pasta using common wheat (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A61985CJ0407).

Durum wheat is the preferred raw material for pasta due to its technological properties. The high level of carotenoids in durum wheat gives pasta its desired yellow color, and the higher protein content is the primary factor associated with superior pasta cooking quality

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(Pauly et al., 2013). However, the price of durum wheat is approx. 25% more expensive compared to common wheat. For this reason, raw material or pasta in industrial food production, which may be adulterated by common wheat, could easily generate more money. This has not been the first time that food was slightly diluted for a purpose of economical profit, to give only two examples, the melamine incidents in 2008 (Gossner et al., 2009) and the horsemeat scandal in 2013 (Abbots & Coles, 2013).

During the last decade, food authenticity has become more and more important and different "omics" techniques have been gradually employed (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009; Cubero-Leon, Peñalver, & Maquet, 2014; Rubert, Zachariasova, & Hajslova, 2015; Sørensen, Khakimov, & Engelsen, 2016). Throughout the biological cascade, durum and common wheat have been verified. Initially, common and durum wheat were authenticated by DNA-based methods (Carloni et al., 2017; Woolfe & Primrose, 2004), taking advantage of the different ploidy levels of common (ABD) and durum wheat (AB). Amplification with end-point PCR of DNA sequences belonging to the DD genome has been also investigated (Arlorio et al., 2003). Nevertheless, DNA degradation may occur during technological processing, generating false negative results. A part from this disadvantage, DNA approaches are relatively expensive and time-consuming. Subsequently, proteins, a step down in the biological cascade, are of great importance, since different genomic structures, such as common and durum wheat, may affect their protein expression. In this frame, the aleurone layer of *Triticum aestivum* and *Triticum durum* were manually dissected and analyzed using two-dimensional gel-based proteomics (Meziani et al., 2012). The comparison between species revealed differences mainly in the globulin type storage proteins, which were involved in carbohydrate metabolism and in stress pathways (Alary, Serin, Duviau, Joudrier, & Gautier, 2002). The absence of the D genome from durum wheat was also investigated by a bottom-up proteomics strategy. In this case, common and durum wheat samples were treated with pepsin and chymotrypsin, and a peptide with a molecular weight of 3909 Da was exclusively found in common wheat samples (Prandi et al., 2012).

Coming to the end of the biological cascade, the study of metabolites is growing up rapidly. Since these small molecules (< 1200 Da) are generated by enzymatic reaction that result from gene expression, the metabolome can be considered the final downstream product of genome, transcriptome and the proteome, linking together genotype and phenotype. Therefore, some most relevant differences in the genetic background (i.e. common and durum wheat) may be detected and amplified investigating differences in the metabolome (Gieger et al., 2008). Up to now, differences in the small molecule composition of common and durum wheat have been scarily reported (Knodler, Most, Schieber, & Carle, 2010; Mattehws et al., 2010). As an example, the alkylresorcinol (AR) composition, and in particular the AR17/AR21 homologues ratio, has been used to estimate the adulteration of durum wheat (Knodler et al., 2010). Unfortunately, alkylresorcinols are present only in the hyaline layer, outer layer, limiting the analysis to whole-grain products. Nevertheless, step-by-step metabolomics emerged as the combination of advanced analytical techniques merged with chemometric pattern recognition, providing a powerful approach for food metabolomics, and it served as a new solution to old problems (Cevallos-Cevallos et al., 2009; Cubero-Leon et al., 2014; Rubert et al., 2015; Sørensen et al., 2016).

The main aim of this research was to investigate common and durum wheat lipidome in order to identify significant markers for wheat verification strategies. A first study was conducted by analyzing 52 samples from two wheat varieties Odisseo (durum wheat) and Blasco (common wheat). Afterwards, the preliminary statistical model was validated by applying two strategies: (i) the analysis of further samples, 173 samples of 5 different wheat varieties (common and durum wheats), and subsequently (ii) the use of statistical tests for a continuous diagnostic markers and the preparation of admixtures at different concentration levels were employed. These novel validation approaches were performed in order to confirm the stability and consistency of the models obtained and the applicability of markers for the authentication purpose.

2. Material and methods

2.1. Chemicals and reagents

Polytetrafluoroethylene (PTFE) 50 mL centrifugation cuvettes were obtained from Merci (Praha, Czech Republic). HPLC grade methanol, dichloromethane and 2-propanol were purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were supplied by Sigma–Aldrich (St. Luis, MO, USA). Water was purified by Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Study design

The experimental design is the plan to perform data-gathering studies in order to provide a realistic strategy, which can catch the variation related to biological observations rather than process variation. This study had to be sufficiently powered to produce meaningful measures of specificity and sensitivity. Thus, also following the guidelines on validation of non-targeted methods recently provided by U.S. Pharmacopeia (U.S. Pharmacopeial Convention, 2016), two complementary studies were carried out (i) the preliminary study and (ii) the confirmatory study.

The preliminary study was initially conducted by analyzing 52 samples from Odisseo (durum) and Blasco (common) wheat lines. In parallel, multivariate data analysis (MVDA) and univariate data analysis (UVDA) strategies (Saccenti, Hoefsloot, Smilde, Westerhuis, & Hendriks, 2014) were performed in order to build unsupervised and supervised statistical models and to discriminate markers. At this point, in order to confirm that the changes observed in Blasco and Odisseo were not attributed to these specific varieties and could be considered as a general change occurring between common and durum wheat, an extended sample set (173 samples and five varieties) was used for the confirmatory study. The confirmatory study was performed repeating in a separate chromatographic run and applying the same analytical and data treatment procedure. In the end, in order to determine the method sensitivity and specificity two approaches were evaluated: (i) receiver operating characteristic (ROC) curves and (ii) admixture samples test. The overall study design scheme is depicted in Fig. 1.

2.3. Plant material

In the first phase of the experimental design, Blasco (common, n = 26) and Odisseo (durum, n = 26) varieties were chosen among genotypes currently cultivated and used for food products in Italy. All samples were cultivated in Parma (Italy).

For the confirmatory study, 173 samples for five varieties of durum (*Triticum durum* Desf.) and common wheat (*Triticum aestivum* L.) were collected (Table 1 supplementary material). Grains were cultivated in two locations in Emilia Romagna region, Parma and Bologna, in plots of 8.25 m^2 with four replications. Samples were grown over two consecutive years (2013/2014 and 2014/2015) under two agricultural conditions: conventional (n = 58) and organic farming (n = 58) in Parma, whereas only conventional farming was applied in Bologna (n = 57). After harvesting, the whole grains were dried at ca. 10% humidity, stored at −20 °C and kept refrigerated until the analysis. Overall, seven wheat varieties were collected resulting in 225 wheat samples, considering both preliminary and confirmatory study.

In order to determine the method sensitivity limit, a set of artificial samples with known content of adulterant were constructed in duplicate. The percentage values of common wheat in the mixtures (15%, 10%, 5%, 3%, 2%, 1%) were calculated on flour wheat weight.