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Authentication of leguminous-based products by targeted biomarkers using high resolution time of flight mass spectrometry

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ABSTRACT

A growing number of health-conscious individuals supplements their diet with protein-rich plant-based products to reduce their meat consumption. Analytical methods are needed to authenticate these new vegetarian products not only for the correct labelling of ingredients according to European legislation but also to discourage food fraud. This paper presents new biomarkers for a targeted proteomics LC-MS/MS work-flow that can simultaneously prove the presence/absence of garden pea, a protein-rich legume, meat and honey and quantify their content in processed vegan food. We show a novel rapid strategy to identify biomarkers for species authentication and the steps for the multi-parameter LC-MS/MS method validation and quantification. A high resolution triple time of flight mass spectrometer (HRMS) with SWATH Acquisition was used for the rapid discovery of all measurable trypsin-digested proteins in the individual ingredients. From these proteins, species-selective biomarkers were identified with BLAST and Skyline. Vicilin and convicilin (UniProt: D3VND9, Q9M3X6) allow pea authentication with regard to other legume species. Myostatin (UniProt: O18831) is a single biomarker for all meat types. For honey, we identified three selective proteins (UniProt: C6K481, C6K482, Q3L6329). The final LC-MS/MS method can identity and quantify these markers simultaneously. Quantification occurs via external matrix calibration.

1. Introduction

The world population is expected to grow from 6.9 billion in 2010 to 9.5 billion by 2050 (United Nations Department of Economic and Social Affairs, 2012). To provide enough food in the form of the essential amino acids only found in proteins, protein-rich plant sources, such as soy and other leguminous seeds but also algae or insect proteins, offer new opportunities and are already a growing supplement option in the human diet (Petrusán, Rawel, & Huschek, 2016; van der Spiegel, Noordam, & van der Fels-Klerx, 2013). For instance, in 2015, there was a 26% growth for vegetarian and vegan products in German retail (IFH, 2016). The estimated annual growth rate for the global market value of these protein-rich sources is 6% in the next five years, reaching a value of $58.49 billion by 2022 (Markets, 2017).

The strong interest in products based on plant proteins is ascribable to consumers who reduce meat consumption for sustainability and health reasons. The reported health benefits of such a diet for metabolic health, blood pressure and reduced risk of type 2 diabetes (Derbyshire, 2016; Kim & Bae, 2015; Rodenas et al., 2011; Turner-McGrievy et al., 2015) will likely increase the number of consumers for plant-based products. The so-called “flexitarians”, including vegetarian and vegan consumers, represent currently 29% and 37% of consumers in Britain and Germany respectively (Derbyshire, 2016; GfK, 2016). Flexitarians primarily focus their buying decisions on nutritional and health relevance and the sustainability of the food supply but also on the transparency of labelling (authenticity of ingredients, regional origin) and on allergen, GMO and synthetic additive free products (Cavaliere, Ricci, & Banterle, 2015; Comission Regulation (EU) No 432/2012, 2012; Regulation (EC) No 1924/2006, 2006).

In April 2016, a first unified legal definition of the terms “vegan” and “vegetarian” for the labelling of food was introduced in Germany in reaction to the demand of clear labelling practices from consumer interest groups and the food industry (European Vegetarian Union, 2016). This definition will be effective until the European Commission issues standardized regulations as stipulated in Article 36(3) (b) of (Regulation (EU) No 1169/2011, 2011). The new definition has two main objectives: (1) absence of meat and (2) description of improved quality attributes as compared to animal-based products.

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The product labelling needs to be combined with an authentication analysis of plant ingredients and additives to increase consumer confidence and to prevent false labelling and adulteration (Primrose, Woolfe, & Rollinson, 2010; Regulation (EU) 2017/625, 2017). The Grocery Manufacturers Association of America reported that up to 10% of the food in the developed world and 20% in the developing world is affected by food fraud (Johnson, 2014). Therefore, food manufactures are required to provide and confirm the authenticity of vegan/vegetarian products with respect to the regulation (EU) No. 1169/2011, (EU) No. 178/2002 and the USA FDA Food Safety Modernization Act signed on January 4, 2011. Reliable analytical methods are thus needed to check the suitability of vegetarian/vegan products as well as the authenticity and quality of ingredients.

Different analytical techniques can test authenticity and detect adulterations of food ingredients, f.i. measurement ratios of stable isotopes (mostly $^{13}C/^{12}C$), spectroscopic, chromatographic, molecular biological, and electrochemical methods. Molecular biological methods such as real-time PCR have been used to identify and quantify food, for example meat species (Laube, Zagon, & Broll, 2007; Montowska, Alexander, Tucker, & Barrett, 2015). A broad variety of analytical methods tried to authenticate honey (for a review see (Trifkovic, Andric, Ristivojevic, Guzelmeric, & Yesilada, 2017)). However, some methods are not suitable for processed food, because processing especially thermal treatment leads to a change in the structure and properties of the targeted analytes. For instance, DNA can undergo heat denaturation and therefore a standard PCR analysis that produces copies of DNA fragments is likely to over- or underestimate the targeted molecular species. Processing also introduces new structures into food, a problem for metabolomics analytical strategies.

The recent developments in proteomics and mass spectrometry (MS) offer new opportunities for the authentication of food ingredients due to the fast and easy sample preparation, high throughput processing, the incorporation of post-translational and processing-dependent modifications and quantification of analytes. Processing also effects the protein structure and properties, but a well-defined proteomics strategy can analyze processed products, when it utilizes thermal stable parts/fragments of the targeted protein during the analysis. MS-based protein biomarkers have successfully been used to differentiate and authenticate ingredients and food additives (Garcia-Canas, Simo, Herrero, Ibanez, & Cifuentes, 2012; Huschek, Bönick, Löwenstein, Sievers, & Rawel, 2016; Orduna, Husby, Yang, Ghosh, & Beaudry, 2015; Primrose et al., 2010). For example, for meat species and meat products, an authentication based on a peptide biomarker analysis protocol has recently been published (Montowska et al., 2015). Similarly, for cereal-based baked products, an analytical method for the differentiation of cereals species has been developed (Bönick, Huschek, & Rawel, 2017).

We add to this field of authentication in proteomics by focusing on the market for plant-based protein–rich processed foods that should be free of meat and/or honey (for vegan products). We developed a targeted-proteomics LC/MS/MS multi-parameter method that can simultaneously detect the presence/absence of garden pea, meat and honey in processed plant-based products and quantify these ingredients. Another novelty aspect addressed is the new biomarkers for these food ingredients that were derived by employing an innovative rapid identification of potential species-selective biomarkers from trypsin-digested proteins using high-resolution mass spectrometry (HRMS) in combination with bioinformatics tools.

2. Materials and methods

2.1. Materials

2.1.1. Raw materials

Different pea protein isolates were purchased from Roquette, France and Emsland-Stärke GmbH, Germany, from production years 2014–2017. Yellow pea flour (*Pisum sativum* L. “Rocket”) was obtained from Norddeutsche Pflanzenzucht, Hans Georg Lembke GmbH (Hohenlieth, Germany) as well as chickpea flour (*Cicer arietinum)* and red lentil flour (*lens culinaris*) from Müller´s Mühle Gelsenkirchen, Germany; soybean flour (*Glycine* max (L.)) from Rapunzel Naturkost GmbH, Legau, Germany; white Lupine (*Lupinus alba)* from Veggie's Delight; Düsseldorf, Germany; peanut (*Arachis hypogaea*); grass pea (*Lathyrus sativus* L.) Agriturismo parco verde, Grumento Nova, Italy; fava bean (*Vicia faba*) Sperli GmbH, Lüneburg, Germany. Honey and meat (an organic chicken breast, a shoulder piece of beef, pork, and horse goulash) were bought in a local supermarket as well as Durum wheat pasta for the matrix calibration.

2.1.2. Reference materials

For the simultaneous detection of multiple ingredients in plant-based processed products, the reference material - vegan pea patties developed at IGV Ltd - for the matrix calibration and quantification are spiked with different concentrations of meat and honey: Raw meat of horse, pig, chicken and cow are homogenized with an Ultra Turrax T25 (Janke & Kunkel – IKA Labortechnik, Staufen, Germany) and spiked in 0.5%, 1%, 3%, 5% to the pea patties. Additionally, the meat is fried in a stainless steel pan for at least 6 min until no raw parts are visible inside and is then spiked in similar concentrations to the pea patties. Honey is spiked in concentrations of 0.5%, 1%, 3%, 5% and 10% to the patties.

For the quantification of the pea content, we use an additional matrix to show the practical suitability of our method for processed food. We use both pea patties and pasta developed at IGV Ltd. For the external matrix calibration, however, pasta made of 100% pea flour is mixed with durum wheat pasta in different concentrations (1%, 5%, 10%, 15% and 20%). During the sample preparation, the pea pasta extract is then diluted 1:5 with extraction buffer.

2.1.3. Chemical compounds

We used ammonium bicarbonate (PubChem CID: 14013), 1,4-Dithiothreitol (DTT) (PubChem CID: 19001), urea (PubChem CID: 1176) for the extraction buffer; acetonitrile LC-MS grade (PubChem CID: 6342), formic acid (PubChem CID: 284) from Carl Roth GmbH, Karlsruhe, Germany, as LC-MS solvents; N-Hexane Picograde (PubChem CID: 8058) from LGC Promochem, Wesel, Germany, for defatting; Iodacetamide (IAA) (PubChem CID: 3727) and Trypsin from bovine pancreas from Sigma Aldrich, St. Louis, Missouri, US, for enzymatic digestion.

2.2. Methods/workflow: identification and quantification of biomarkers

An exemplified workflow for the identification (2.2.1) and quantification (2.2.2) of pea-specific biomarkers is shown in Fig. 1 and described in the following sections. Similarly, biomarkers for honey and meat are identified and later combined into a multi-parameter sMRM method. To rapidly identify potential biomarkers (Scheidweiler, Jarvis, & Huestis, 2015), the presented workflow modifies the experimental proteomics strategy described in (Orduna et al., 2015) by using a SCIEX Triple TOF 5600 with SWATH Acquisition and the ProteinPilot software. This allows to forego time consuming electrophoresis analyses or database searches for potential biomarkers that may later not be measurable. For the quantification, the workflow follows Huschek et al., 2016.

2.2.1. Identification of species-selective biomarkers with SWATH MS/MS, BLAST and skyline

2.2.1.1. Extraction of leguminous proteins and trypsin digestion. 1 g of pre-dried sample is mixed with 10 mL extraction buffer (100 mM ammonium bicarbonate, 5 mM DTT, and 4 M urea, pH = 8.2). After incubating for 30 min with shaking device and at room temperature it is centrifuged twice (5 min at 4000 g and 5 min at 7000 g), to separate coarse matrix compounds. To alkylate the cleaved disulfide bonds, 1 mL supernatant is incubated with 30 µL Iodoacetamide solution (IAA) for