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Departementet har i bestillingen fremsendt d. 15. august 2019 bedt DCA – Nationalt Center for Fødevarer og Jordbrug – om en litteratur gennemgang af tilgængelige analysemetoder for grønne proteinkilder, og deres anvendelighed i hhv. foder og fødevarer.

Nedenfor følger besvarelsen med titlen: Authentication of future green proteins – possible methods of analysis. Rapporten er udarbejdet af Lektor Jette Feveile Young, Tenure Track Ulrik Sundekilde, Akademisk medarbejder Marianne Danielsen and Lektor Nina Poulsen fra Institut for Fødevarer. Rapporten er fagfællebedømt af Seniorforsker Søren Krogh Jensen fra Institut for Husdyrvidenskab, og rapporten er revideret i lyset af hans kommentarer.

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Venlig hilsen

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Authentication of future green proteins – possible methods of analysis

DCA – National Centre for Food and Agriculture

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1 Background

The National Bio-economy Panel has with the publication "Proteins for the Future" (Børsting et al. 2018) recommended a study to elucidate whether existing traceability systems can adequately handle new protein products for food and feed. As part of the implementation of the recommendations of the National Bio-economy Panel, the Danish Ministry of the Environment and Food has commissioned this report. The aim of the combined recommendations in "Proteins for the Future" is that alternative Danish protein products with better environmental and climate footprint will be able to match existing protein products at both price and quality in key market areas in feed and food.

Whether existing traceability systems are sufficient to identify the origin of new protein sources and thus their presence in feed and food is explored by a literature review of available assay methods and their applicability in feed and food.

Traceability / identification of these new green protein sources in feed and food is in this context concentrated on the biological identity i.e. which plant the protein is extracted from.

2 Introduction

New alternative protein sources are currently rapidly expanding in feed and foodstuff and the contribution of these alternative sources are expected to increase in the future. If primary producers and the food industry follow the recommendations set out by "The National Bio-economy Panel", then within five years Danish alternative protein products can compete with traditional protein products economically and quality wise with a better environmental and carbon footprint (Børsting et al., 2018). Currently, there is a lack of research-based evidence on the ability of current technology to identify and differentiate feed and food produced from these new raw material sources. Identification and differentiation of raw materials in products are key steps in authentication. Authentication is a process to avoid food fraud by confirming the identity of a product i.e. ensuring that the product is identical to the labelling claim. This assurance may be pursued in one of two ways 1) tracking and tracing technologies (forgery-proof) or 2) reliable analytical strategies (targeted or non-targeted) (Creydt et al., 2018).

At present, several techniques are being investigated for their suitability of tracking and tracing. Barcodes and 2D quick response (QR) codes used and radio frequency identification (RFID) using microchip are under development for tracking labelled products. Furthermore, addition of coded sugar or cellulose tablets into dry foods or DNA-sequenced nanoparticles into liquids are investigated for tracking non-packaged foods and ingredients (reviewed by Creydt and Fischer, 2018). Moreover, traceability based on novel applications of block-chain mechanisms are developing, but limitations in the current state of development exists (Galvez et al., 2018) .

Reliable analytical strategies for authentication of food is very commodity dependent and authentication of plant protein products from alternative protein sources is in its infancy. This report focus on possible methods for authentication of plant proteins, which may be applicable for future authentication of new protein products from alternative sources (proteins originating from plant materials). In a Danish context, such products could derive from industrial side streams and the feed industry has mainly used rapeseed and sunflower protein rich press cake from oil. Concerning plant side streams for food the best example is

the production of potato flour, where potato protein has become a small but high-value side stream product to the Danish starch industry. Another source could be isolated protein from green biomass, i.e. crops such as lucerne (alfalfa), red/white clover and ray grass, which so far exclusively have been used for ruminant feed. The authentication could also include more established plant products like pea and faba bean flour, the former used increasingly in (vegetarian) food products as a plant protein substitute to meat and the latter merely as feed protein.

The most common authentication application so far is species identification and differentiation, which also will serve as a first step in the authentication of new green biomass derived protein products and hence be the primary focus of this report but also quantification, traceability of geographical origin and the possible consequences of processing and bio-refining will be included.

Plant species identification, quantification, geographical origin and processing can be pursued at different molecular levels typically genes, proteins or metabolites either by very specific targeted analysis or by explorative omics analysis (Böhme et al., 2019) at gene (Zhao et al., 2019), protein (Böhme et al., 2019), and metabolite (Medina et al., 2019) levels.

This report includes description and examples of analytical methods for authentication of plant protein at DNA, protein and metabolite levels, which may be applicable for future authentication of new protein sources.

3 Plant species identification techniques

3.1 DNA-based methods

Traditionally, polymerase chain reaction (PCR) has been used for specific amplification of unique genetic markers, which can be analysed by electrophoresis and used to identify species of origin or even allergens and genetically modified organisms (GMO) (Mafra et al., 2008). The superiority of DNA based methods is the reliability and accuracy towards targeting different tissues, mixed samples and even some processed food items as DNA is

more stable than e.g. proteins and RNA. Furthermore, PCR is a very simple analytical method that has a short processing time and is very cost effective. It has a great sensibility and specificity for the targeted species based on species-specific primers, which are used to target and amplify the DNA fragment of choice, which subsequently can be confirmed by agarose gel electrophoresis. Combined with e.g. restriction endonucleases or sequencing, single or multiple fragments can be identified per species. Especially, microsatellites (single sequence repeats) or mitochondrial DNA fragments are popular choices for use in relation to food adulteration. For quantification, the development of real-time PCR has been important. PCR based methods have been used with great success in animal products for detection of adulteration including animal products from different species or even breeds (reviewed by Mafra et al., 2008). For foods with plant origin, PCR has also been used to target different legumes, spices and cereals as well as for validation of olive oils and detection of allergens from e.g. nuts or gluten from a number of cereals (Mafra et al., 2008). The targeted DNA fragments and genes include microsatellites and specific genes like chloroplast *trnL,* ω-secalin or ω-gliadin.

Recent advances in sequencing together with reduced costs paved the way for development of DNA barcoding, which is now the molecular method of choice for identification of raw materials and processed foods in relation to food fraud and traceability (Galimberti et al., 2013, 2014). According to Hollingsworth et al. important principle for selection of DNA barcodes are standardisation, minimalism, and scalability (Hollingsworth et al., 2011). Thus DNA barcodes relies on universal informative DNA regions (preferable one or a few loci), which are selected based on their low intra-species variation but high inter-species variation and which easily can be routinely amplified by PCR and sequenced in a reliable manner using a universal primer set (Galimberti et al., 2013; Mishra et al., 2016).

Figure 1. DNA based barcodes can be used for identification of plant proteins from different species.

While no universal barcode has been identified, reliable barcodes for different taxonomic groups have been identified including the mitochondrial gene cytochrome oxidase 1 (*CO1*) in animals (Hebert et al., 2003). Comparison of these loci within the taxonomic group of interest must display a high discriminatory power, which enables different species to be distinguished. As mitochondrial DNA is haploid, uniparentially-inhereted and holds a low recombination rate and lack of introns, mitochondrial genes have received a lot of attention in relation to barcoding. However, due to slow substitution rates in plants, the discriminatory power of mitochondrial DNA is too low. In the search for reliable plant based barcodes the Consortium for the Barcode of Life (CBOL) plant working group was established in 2004 (CBOL Plant Working Group, 2009). They proposed a core-barcode for all land plants consisting of two plastid coding regions, namely the coding genes comprising ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, RuBisCO (*rbcL*) and maturase K (*matK*), supplemented with other plastid or nuclear DNA regions depending on the target species (CBOL Plant Working Group, 2009). Like mitochondria, plastids contain their own DNA, but have higher substitution rates in plants and the proposed barcodes, *matk + rbcL*, are both chloroplast genes. However, their phylogenetic resolution is sometimes limited and several other barcoding genes and combination of these for plants have been proposed (Hollingsworth et al., 2011; Mishra et al., 2016). The genomic revolution emerged from next generation sequencing has enabled vast amounts of sequence data at a low cost and instead of focusing on specific barcoding genes, whole nuclear or chloroplast genomes can

be sequenced and used (Hollingsworth et al., 2011; Mishra et al., 2016). This is probably where the future will go, but so far next generation sequencing is still a more expensive solution and may demand higher bioinformatics competences for quality control of data.

3.2 Protein-based methods

A whole range of protein-based methods has been applied to authenticate food. Table 1 lists examples of these methodologies, including electrophoretic, immunological and mass spectrometric methods. As indicated in the table, the methods differ greatly in terms of specificity, sensitivity and quantification capabilities. The trend in the scientific literature for species authentication move towards molecular techniques like genomics and proteomics (Danezis et al., 2016). Mass spectrometry (MS) methods, particularly multiple-reaction monitoring (MRM) (also termed single reaction monitoring (SRM)) (Lange et al., 2008) and SWATH (sequential windowed acquisition of all theoretical fragment ion mass spectra) mass spectrometry (Ludwig et al., 2018) are frontline technologies for analyzing proteins. These MS-based methods rapidly replace other protein-based authentication methods due to unsurpassed sensitivity, selectivity, throughput and multi-analyte capacity (Lange et al., 2008; Danezis et al., 2016; Ludwig et al., 2018).

Species authentication using MRM (and partly using SWATH-MS) rely on identification of proteotypic peptides, which are unique peptides not only to a specific protein, but also to the species in mind. Peptides (including proteotypic) are derived from digestion of proteins with an enzyme, often trypsin (Mann and Wilm, 1994).

Table 1. Examples of protein-based methods for food authentication. Number of stars indicate level of confidence in method specificity, sensitivity and quantification.

Methods	Specificity	Sensitivity	Quantification	Purpose of analysis	Reference
Electrophoretic methods					
SDS-PAGE	*	*	*	Identification of raw and cooked bivalves	(Etienne et al., 2000)
Isoelectric focusing (IEF)	*	*	*	Species origin of raw meat	(Różycki et al., 2018)
2D-gel electrophoresis	**	**	**	Distinguish fresh and frozen octopus	(Guglielmetti et al., 2018)
Capillary electrophoresis	*	*	*	Authentication of sweet cherry varieties	(Serradilla et al., 2008)
Immunological methods					
ELISA	**	***	***	Meat adulteration in food products	(Renčová and Tremlová, 2009)
Western blot	**	***	*	Adulteration of bovine milk with cheese whey	(Chávez et al., 2008)
Mass spectrometric methods					
MALDI-TOF MS Protein fingerprinting	**	**	*	Authentication of fish	(Stahl and Schröder, 2017)
Peptide profiling, untargeted MS/MS	**	***	**	Authentication of processed fish products	(Wulff et al., 2013)
MRM	***	***	***	Authentication of wheat, rye and spelt in bread	(Bönick et al., 2017)
SWATH-MS	***	**	**	Geographical origin of dried sea cucumber	(Zhang et al., 2019)
SWATH-MS + MRM	***	***	***	Authentication of leguminous-based products	(Huschek et al., 2018)

For quantification, MRM rely on synthetically produced heavy isotope labelled counterpart of these proteotypic peptides as internal standards, which allow unsurpassed quantification (Lange et al., 2008). SWATH-MS supports quantification of peptides covering 1000's of proteins without the need for internal standards, but currently SWATH-MS is three- to 10-fold less sensitive than MRM approaches (Ludwig et al., 2018). However, the ability of analyzing a large range of peptides opens up for analysis with a specificity that reaches beyond

species identification (Zhang et al., 2019). A powerful approach, which is likely to become more widely used in the future, is the combination of SWATH for biomarker identification followed by development of a MRM method for high sensitivity and good quantification (Hu et al., 2018; Huschek et al., 2018).

Table 2. Examples of protein-based methods for detecting plant protein isolates in food matrixes

Plant protein isolates	Food matrix	Analytical method	Purpose of analysis	Reference
soybean, pea	raw milk	LC-MS/MS (untargeted) + chemometrics	milk adulteration	(Lu et al., 2017)
soybean, pea	skimmed milk powder	LC-MS/MS (untargeted)	milk adulteration	(Cordewener et al., 2009)
soybean, pea	skimmed milk powder	LC-MS/MS (untargeted)	milk adulteration	(Luykx et al., 2007)
soybean, pea, wheat	milk powder	immunoassay	milk adulteration	(Haasnoot and Du Pré, 2007)
soybean, pea, hydrolyzed wheat, hydrolyzed rice	pasteurized milk	LC-MS/MS (untargeted) + principal component analysis	milk adulteration	(Yang et al., 2018)
soybean, pea, wheat	milk products	2D-gels + MS	milk adulteration	(Yang et al., 2018)
soybean, pea, wheat	raw milk	FIMS + chemometrics	milk adulteration	(Du et al., 2018)
soybean, peanut, pea	meat products, heat stable	MRM (targeted)	meat adulteration	(Li et al., 2018)
soybean	various meat-containing food products	ELISA	meat adulteration	(Renčová and Tremlová, 2009)
lupine, pea, soybean	meat products	LC-MS/MS (untargeted) + MRM (targeted)	meat adulteration	(Hoffmann et al., 2017)
wheat, rye, spelt	bread	MRM (targeted)	Authentication of types of flour	(Bönick et al., 2017)
pea	durum flour and pies	Combination of SWATH-MS and MRM (targeted)	authentication of vegetarian foods	(Huschek et al., 2018)

The MS methods mentioned above are known as "targeted" approaches, as they use pre-defined lists of analytes. In contrast, "un-targeted" methods are data-dependent, meaning that the most abundant analytes in a sample are analyzed. One drawback of targeted methods (and in most cases also of untargeted methods) is that they, just like DNA-based

methods, often require that the entire genome, or at least specific proteins or genes, are sequenced from the species of interest. MS targets are built from genome sequence information and acquired untargeted data is searched against genome sequence databases. This can be problematic as not all organisms have their genome sequenced. At the same time, the link to genome sequence information is the very reason, that these methods can be highly selective, even to the level of distinguishing breeds or cultivars (Hu et al., 2018). The untargeted approach is superior for detecting intended or unintended food adulteration, as this approach detects changes in protein profiles without any pre-defined knowledge. Furthermore, the literature also contain examples of untargeted MS methods combined with multivariate analysis, which allow authentication independent of genome sequence information (Wulff et al., 2013).

Future new protein products are likely to be used in the form of protein isolates (powders). Mass spectrometry-based methods for authentication of plant protein powders from plant sources like pea, soybean and wheat, within food matrices are already available. Also, the literature contains examples of authentication of alternative protein sources like cricket powder (Montowska et al., 2019). A particular focus has been the detection of economically motivated adulteration (EMA) of milk and meat products by addition of plant protein, whereas verification of vegetarian foods is a new focus area (Huschek et al., 2018). Table 2 lists examples available in the literature for detecting plant protein products in food matrices.

These methods may very well be directly applicable to authentication of new protein products for food and feed. However, a few points regarding these new alternative protein sources need to be taken into consideration.

Genome sequenced-based methods (such as MS-methods) are challenged by the fact that far from all sources of alternative protein products are genome sequenced. Another issue is that, unlike animal or other plant protein products, the processing of green plant material is complicated by the fact that in many types of green biomass, the activity of polyphenol oxidases (PPO's) or other redox enzymes is high. When these enzymes encounter their substrates (e.g. polyphenols) during harvest and plant processing, extensive, random protein cross-binding reactions may occur (Bittner, 2006). These challenges regarding crosslinking

is being reviewed in current ministry reports.[1] To which extent redox enzyme activity will influence the sensitivity and specificity of protein-based analytical methods for authentication of green biomass plant protein isolates, will be highly species dependent and remain so far unclear and needs to be further investigated.

Protein-based methods have been applied to authenticate a range of food claims besides species authentication. A great advantage of protein-based methods is that proteins do not only carry information on species origin, but also information on what happened to the living organism before harvest/slaughter, and to the food product during processing. Examples in the literature include determination of; geographical origin (Zhang et al., 2019), production methods, for example farmed versus wild fish (Martinez et al., 2007), quantification of banned processed animal proteins for livestock feed (Steinhilber et al., 2019) and technological food processing. The latter covers several aspects such as distinguish fresh and frozen food (Guglielmetti et al., 2018), determination of product processing methods e.g. smoked, steamed and fried products (Wulff et al., 2013) or determination of a high degree of food processing (Von Bargen et al., 2014). The latter suggests that this method may be suitable for identifying highly processed green proteins.

As such, protein-based analysis can provide valuable evidence for various food-labeling claims, which cannot be identified using other methodologies. Most likely, with the increasing focus on food fraud, these types of food authentications will gain more focus in the future.

3.3 Metabolomics-based methods

Metabolomics is the study of low molecular weight chemical compounds, also termed metabolites, in biological materials. As such, metabolites exists in food items and concentration may be related to cultivar, origin, soil-type and more. Specific regions may

[1] DCA – National Center for Food and Agriculture. Science based policy advice on "Green plant based protein sources for food and feed with regard to feed efficiency, nutrition, food functional properties and their potential as novel food products" and "Undersøgelse af efterspørgsel af grøn protein hos forbruger, fødevare- og fodersektoren", both in preparation.

contain specific mineral composition with a distinct stabile isotope composition that can be traced in processed food items and thus used in authentication procedures.

Metabolomics-based studies of plant-based raw material composition and profiling is widespread and has shown a wealth of metabolites present in raw material as well as processed foods (Ernst et al., 2014). Plant material contains many metabolites that are varying due to the many external factors the plant are subjected to. Cultivar and year-to-year variation is one of the major factors that result in diverse metabolite fingerprints. At Department of Food Science, Aarhus University, we have studied seven cultivars of white-flowered rapeseed in a field experiment over two consecutive years to also obtain seasonal differences. The resulting metabolite profiles revealed distinct metabolite fingerprints of each cultivar but also significant year-to-year variations (Groenbaek et al., 2019). One study of metabolite composition of a *Lonicera* flower buds revealed 82 tentatively assigned chemical compounds, where only six compounds were unambiguously determined (Gao et al., 2012). Still, the chemical fingerprints showed some discriminative power in differentiating between the seven different species (Gao et al., 2012). Nevertheless, the findings by Gao et al. is not translatable into other cultivars as extraction and compound detection is optimized for these specific samples and new samples cannot be analysed without an extensive library.

Many metabolomics-studies utilize advanced laboratory-size analytical equipment. A recent study utilize a miniaturized mass spectrometer using ambient ionization-technique that can be used real-time on site (Gerbig et al., 2017). Using this equipment Gerbig et al. report classification of various foodstuffs including two coffee varieties. It was possible with 96.4% accuracy to classify coffee beans into Robusta or Arabica varieties (Gerbig et al., 2017).

Another external factor that influence plant composition is soil and climate. Traceability of geographic origin has also been investigated by metabolomics approaches. In a study of rice metabolome, Chae and Kim report that geographic region but not cultivar could be identified by an NMR-based metabolomics approach (Chae and Kim, 2016). Another study used UPLC-TOF-MS for metabolite identification and quantification in extracted samples of white asparagus (Creydt et al., 2018). The study showed that only if sufficiently large

geographical distance is tested then the method could differentiate between different regions and only within the same year. The metabolite fingerprint could not be extrapolated across years, indicating there is a larger year-to-year variation than variation originating from cultivation, which the authors also state is widely similar (Creydt et al., 2018). Furthermore, the study by Creydt et al. also report on the usefulness of isotope ratios in determining geographic region of production. Isotopic ratios were determined by isotope-ratio mass spectrometry (IRMS). The IRMS based technique offered comparable results to the metabolomics-based approach (Creydt et al., 2018). Other authors have reported that isotope ratios, specifically on $^{87}Sr/^{86}Sr$ ratios, can determine if asparagus is originating from different geographical regions, again only if the distance is sufficiently large (Zannella et al., 2017).

Literature on isotope ratios during processing is scarce. Plant raw material may undergo various processing steps before consumption, which can affect the metabolome. Recently, studies on wheat and tomato processing have revealed that certain isotopes remain unchanged by the processing (Bontempo et al., 2011, 2016; Wadood et al., 2019).

A case where an extensive metabolite library exists is the proprietary WineScanner utilizing nuclear magnetic resonance spectroscopic technology developed by Bruker. Using this technology it is possible to trace geographic origin of the processed food item, in this case wine produced from grapes, through metabolite levels (Godelmann et al., 2013). One of the requirements of this approach is a large database with known metabolites and their occurrence in food items produced in specific regions. The approach is thus limited to already known variations in metabolite levels and needs constant updates in order to retain its usefulness.

Other external factors that can alter the metabolite fingerprint is stage of development, adulteration, and processing (reviewed by Sobolev et al., 2017). Table 3 list examples of how metabolic fingerprints has been used in different applications.

Thus far, metabolomics-based applications of identification of botanical variety, geography and processing is immature. Often the existing studies published in journals are pilot studies or studies with little translatability. For metabolomics, researchers also have a large number of analytical techniques, inadequate metabolite identification, and a general lack of

standard operating procedures for extraction and analysis. All these points renders comparison between and meta-analysis of multiple studies difficult.

Table 3: Plant molecular fingerprints related to different applications. Adapted from (Sobolev et al., 2017)

Foodstuff	Application	*Metabolites*	References
Apple	Variety	Acetaldehyde, sucrose, leucine, isoleucine, valine, alanine, malic acid, and phenolic compounds	(Vermathen et al., 2011)
Kiwi-fruit	Variety	*Neochlorogenic acid, 3-O-α-l-rhamnopyranosyl quercetin* Amino acids, sugars, organic acids, ATP	(Capitani et al., 2013a)
	Stage of development	Sugars (glucose, fructose, sucrose, galactose), amino acids, O3-β-d-glucopyranosyl-trans-caffeic acid, epicatechin	
Peach	Variety	Glucose, xylose, sucrose, fucose, myo-inositol, choline, isoleucine, valine, alanine, fumaric acid, quinic acid, chlorogenic, neochlorogenic acid	(Capitani et al., 2013b)
Tomato	Variety	Fructose, citric acid and malic acid	(Sánchez Pérez et al., 2011)
	Geographical origin	Fructose, glucose, fatty acids, alanine, methanol, acetylglutamic acid, GABA, glutamine, glutamic, aspartic acids. trigonelline, tryptophan, tyrosine	(Mallamace et al., 2014)
	Stage of development	GABA, glutamate, glucose, fructose, organic acids	(Sánchez Pérez et al., 2011)
Sweet pepper	Variety	Sucrose, glucose, polyunsaturated fatty acids, arginine, GABA, acetate, fatty acids	(Ritota et al., 2010)
	Geographical origin	Sugars (glucose and fructose), organic acids, that is, malate and ascorbate, amino acids, cinnamic acids, phenylalanine, *cis*-olefins, unsaturated fatty acids, acetate, GABA	
Garlic	Variety	Formiate, citrate	(Ritota et al., 2012)
	Geographical origin	Organic acids, fatty acids, amino acids, organosulphur compounds, allicin, sugars, ethanol, methanol	
Saffron	Geographical origin	Picrocrocin, glycosyl esters of crocetin	(Sobolev et al., 2014)
	Adulteration	Curcuminoids, fatty acids, picrocrocin, glycosyl esters of crocetin	(Yilmaz et al., 2010; Cagliani et al., 2015; Petrakis et al., 2015)
Pistachio	Geographical origin	Gallic acid, allantoin, sucrose, glycine-betaine, malic acid, raffinose, proline, uridine, ethyllactate, succinic acid, valine	(Sciubba et al., 2014a)

Foodstuff	Application	*Metabolites*	References
	Stage of development	Alanine, sucrose, quinic acid, shikimic acid, glucose, tyrosine, esters of gallic acid	(Sciubba et al., 2017)
Hazelnut	Variety	Sugars, amino acids, organic acids	(Sciubba et al., 2014b)
Rice	Variety	Acetic acid, glucose, sucrose, and fructose	(Monakhova et al., 2014)
	Geographical origin	Gluconic acid, ethanolamine, valine, lysine, aspartic acid, leucine, threonine, glutamine, sucrose, maltose, and betaine, GABA	(Chae and Kim, 2016)

4 Conclusion

Current state-of-the-art in authentication of feed and food rely on numerous years of research and development, with significant development since the next-generation sequencing era that made genome and proteome sequencing available. Translating this state-of-the-art requires a similar focus on sequencing the new alternative protein sources to generate the necessary databases. DNA is relatively stable and **gene-based methods** are reliable and accurate but rely on known sequences. In general, it is superior for species/varieties identification and differentiation, but not for revealing processing or geographical origin. The consequences of extensive bio-refining of green biomass on gene-based methods need to be elucidated. Identification of raw materials, mixes and processing can be done by the traditional **PCR** method, which is simple, quick and cost effective. Quantification require **real-time PCR** while the more advanced method of **next generation sequencing** may be the choice in the future with the high discriminatory power but too expensive for routine analysis at present. However, gene-based methods may be challenged for analysing harshly processed protein samples due to difficulties in extracting DNA in a standardized manner.

A whole range of **protein-based methods** have been applied to (plant) species authentication. Hereof the most sensitive, selective and reliable is MS-methods, specifically **MRM** and **SWATHMS** where a combination of the two is regarded as the future approach of

choice. For quantification, these methods rely on known proteotypic peptids and MRM on isotope labelled standards.

However, the need for extensive processing and biorefining of green biomass may in some species induce extensive enzyme and oxidation induced protein cross-linking, which could pose a challenge to protein-based methodologies and require optimization
Protein-based methods are often suitable for species authentication and quantification, but not for determination of geographical origin. There is a potential for authenticating other claims, such as processing and storage conditions, but still the consequences of extensive bio-refining of green biomass on protein-based methods needs to be elucidated.

Metabolite-based methods include **MS**-methods and **NMR** for metabolic profiling and **IRMS** for determining geographical origin. The metabolic profiling analysis rely on extensive metabolite libraries from known sources. Processing will impact on metabolite fingerprints while isotope ratios are likely unaffected by the processing enabling tracability of geographical origin. Of the methods covered in this report, metabolite-based methods are superior for revealing geographical origin, may be suitable for species/varieties differentiation and processing but not suitable for identification.

Thus, methods for authentication of known species, varieties and geographical origin is available for entire plants. Some analysis can reveal specific processing steps but analysis of extracted or isolated new green protein products is not readily available in the literature. So far, the scientific literature does not contain publications that directly address species authentication of protein isolates from green biomass (like red and white clover, lucerne and ryegrass) using either DNA, protein or metabolite-based analytical methods, thus existing authenticity methods of analysis are insufficient to identify the origin of new protein sources and thus their presence in feed and food

5 Perspectives

Many of the DNA, protein and metabolite-based analytical tools used for authentication of foods including plant-based foods will be relevant for further investigation and optimization in order to meet the challenge of authentication of new green proteins. The processing conditions taken into account the omics techniques and even a combination of these may be necessary for improving the explanatory power leading to reliable identification. However, single or multiple biomarker compounds may be identified from experimental data, based on these non-targeted omics-approaches, and used to develop targeted and more robust cost effective methods.

For this work **reference sequences** of the demanded species is a prerequisite, i.e. **authentic reference material** from "clean" monocultures has to be cultured and **methods of analysis optimized** and possibly combined for reliable authentication. Another important task will be to monitor DNA and protein quality after various processing methods and in different species in order to validate the impact of processing on reliable species identification.

As the existing methods of analysis for authenticity (traceability and identification) are insufficient to identify the origin of new protein sources after processing and hence the protein as ingredient in feed and food, **it is recommended to initiate work on relevant species specific reference sequences as well as isolation and analysis procedures.**

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