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Trends in Food Science and Technology

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https://doi.org/10.1016/j.tifs.2021.12.018

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Trends in Food Science & Technology 119 (2022) 371–377

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

Edible insects in the metabolomics era. First steps towards the implementation of entometabolomics in food systems

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ARTICLE INFO

Keywords
Metabolomics
Non-target screening
Edible insects
Food systems

ABSTRACT

Background: Edible insects are a nutritious and sustainable alternative source of animal proteins, and consequently are nowadays promoted as human food and animal feed worldwide. Ensuring the quality, safety, and traceability of insect foods is thus critical to maintain a safe and economically sustainable supply chain. Providing a simultaneous analysis of a wide range of chemical compounds, metabolomics and non-target screening (NTS) have become important mass spectrometry-based tools in the field of food sciences. At this moment, however, the application of these analytical techniques to edible insect-related topics has been largely overlooked.

Scope and approach: In this Commentary, we explore the potential of "entometabolomics" for food science and industry. We address the hurdles associated with safety, quality, and traceability of insects as food and feed and current issues related with insect industrial applications.

Key findings and conclusions: Current applications of metabolomics and NTS in traditional food systems are presented. We propose ways to translate and implement such techniques for the edible insect sector. Even if the broad applicability of metabolomics and NTS approaches is still hampered by technical challenges, we show the high potential of entometabolomics when implemented into the insect food and feed industry and research.

1. Introduction

The world faces the challenge of meeting an increasing demand for protein sources from meat and fish products due to the exponential growth and rising prosperity of its population. The livestock industry can hardly satisfy this demand, considering that it occupies already 70% of all agricultural land and is regarded as a leading cause of anthropogenic-driven climate change. Therefore, worldwide there is a search for alternative protein sources. Compared to the farming of conventional livestock, insects are considered as one of the most sustainable alternatives (B. F. Kim et al., 2020), as they emit less greenhouse gases and ammonia (Oonincx & de Boer, 2012), have a high food and feed conversion efficiency (van Huis, 2013), require less land and water (Oonincx & de Boer, 2012), and can transform low-value organic by-products into high-quality food or feed (Surendra et al., 2020). Therefore, insects are promoted worldwide as human food and animal feed (henceforth referred to as edible insects). In this scenario, the market will require massive and guaranteed amounts of high-quality insect products. Ensuring the quality, safety, and traceability of insect-based products is thus critical for their marketing and for the credibility of the insect food and feed sector.

In the last decade, omics technologies and big data approaches, such as metabolomics and non-target screening (NTS), have become important methodological tools in the field of food science (Castro-Puyana, Pérez-Míguez, Montero, & Herrero, 2017; García-Cañas, Simó, Herrero, Ibáñez, & Cifuentes, 2012; S.; Kim, Kim, Yun, & Kim, 2016; Rubert, Zachariasova, & Hajslova, 2015). Metabolomics is the study of global changes in the entire metabolite set (i.e. the "metabolome", consisting of small molecules <1500 Da) of biological samples, and applies to endogenous compounds which can be accumulated, depleted, synthesized, or bioconverted by a given organism (S. Kim et al., 2016; Wishart, 2008). In NTS studies, chemical structures of xenobiotic compounds,

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https://doi.org/10.1016/j.tifs.2021.12.018
Received 25 March 2021; Received in revised form 15 October 2021; Accepted 15 December 2021

taken up by an organism from exogenous sources, are detected in a sample using no prior information (Schymanski et al., 2015).

Both techniques strongly rely on analytical platforms, such as gas chromatography (GC) or liquid chromatography (LC) hyphenated to high-resolution mass spectrometry (HRMS). They are often applied to obtain patterns of molecules without necessarily identifying or quantifying specific compounds. These patterns can be used to build classification models (fingerprinting, e.g. for food authentication and confirmation of origin) or to discover new entities (exposure assessment, e.g. biomarker elucidation or drug discovery) (Castro-Puyana et al., 2017; López-Ruiz, Romero-González, & Garrido Frenich, 2019). Since the acquired datasets generated by these techniques are of great complexity, data mining procedures relying on bioinformatics tools have been developed (e.g. MS-DIAL, XCMS and MZmine) to extract the valuable information from the raw data. Chemometric tools, including multivariate analysis (like principal component analysis, random forest classification, and support vector machines), are applied to statistically assess the relevance of the high number of variables across the relatively limited number of samples (Castro-Puyana et al., 2017; S.; Kim et al., 2016). Algorithms are then applied to match the features against spectral databases to identify them as either endogenous compounds (genuine metabolomics study) or as xenobiotics of interest (NTS). The certainty of identification relies on the matching score with those libraries and important annotations are often confirmed with chemical standards (Schymanski et al., 2014). The annotation of features can be used to convert the observed effects into a biological interpretation (López-Ruiz et al., 2019) (Fig. 1).

The application of metabolomics and NTS approaches in food systems is commonly referred to as ‘‘food metabolomics’’ and can be used for both small scale optimization processes and for whole food systems, including food and feed component analysis, food consumption monitoring, and physiological monitoring in diet and nutrition studies (Bayram & Göklrmakll, 2018; S.; Kim et al., 2016; Li et al., 2020; Wishart, 2008). Providing a simultaneous identification of a wide range of endogenous and exogenous compounds, metabolomics and NTS have already shown high potential in food optimization processes (Beleggia et al., 2011), food quality and safety studies (Cajka, Danhelova, Zachariasova, Riddellova, & Hajslova, 2013; Castro-Puyana et al., 2017; Kendall et al., 2019; Li et al., 2020), and authentication branding (Consonni & Cagliani, 2019; Cubero-Leon, De Rudder, & Maquet, 2018). At this moment, however, the use of these holistic techniques to investigate topics regarding edible insects has been largely unexploited.

In 2015, Snart et al. proposed the term “entometabolomics” to describe the application of metabolomics techniques to a variety of aspects of insect biology to help understand their physiology and behavior (Snart, Hardy, & Barrett, 2015). In this Commentary, we further explore the potential of entometabolomics, this time investigating the application of metabolomics and NTS approaches to both the “farm-to-fork” continuum and bioengineering aspects of the insect industry. By doing this, we intend to broaden the spectrum of applicability of metabolomics techniques to insect study systems and to provide the scientific community with food (and feed) for thought.

1.1. Entometabolomics for the safety of edible insects

To maintain an appropriate degree of security for human and animal consumers, the monitoring of the food and feed safety is continuously required from primary production to final consumption (Castro-Puyana et al., 2017). When developing food or feed from edible insects and insect-based ingredients, potential safety issues regarding consumer and animal health should be identified and mitigated to minimize repercussions (van Huis, 2013, 2015). Among these risks, the detection of potential food pathogens, allergens, toxins (Schlüter et al., 2016) and environmental pollutants (Poma et al., 2017, 2019) can raise significant concerns. In addition, compliance with existing legal requirements for food and feed should be ensured. The assessment of safety of edible insect-based food and feed should be performed not only during rearing (through the accumulation of hazardous natural and xenobiotic compounds), but also during their processing (potential contamination occurring during industrial handling, such as cross-contamination during manufacturing of insect products and migration from food packaging materials) and consumption.

The main advantage of NTS approaches to investigate food and feed safety issues is the potential to holistically screen for multiple compounds in a single analysis, and to detect unknown and unexpected compounds (Castro-Puyana et al., 2017; Li et al., 2020) (Fig. 2). Such approaches are currently used in an increasing number of food-related investigations. NTS has been successfully employed to detect mycotoxin contamination (e.g. aflatoxins) (Bhatnagar et al., 2008; Giacometti, Tomljanović, & Josić, 2013), toxic chemicals in food and feed (like veterinary drug and pesticide residues) (De Paepe et al., 2019), foodborne pathogens (e.g. *Salmonella* spp.), and microbial contaminants leading to spoilage (e.g. *Pseudomonas* spp.) (Li et al., 2020).

In addition to NTS, food safety can also benefit from metabolomics approaches, since they can be successfully employed to investigate livestock safety in non-invasive monitoring processes to detect subtle

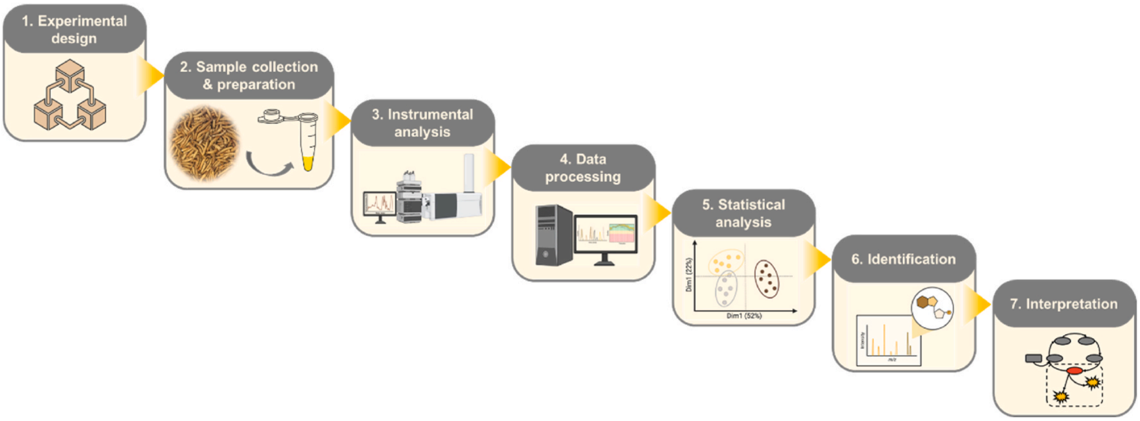

Fig. 1. General metabolomics-based screening workflow. (1) Experimental design, including sufficient quality assurance and quality control (QA/QC) measures to assure high-quality data; (2) Sample collection and preparation procedure to ensure a reliable acquisition of data representing the organism; (3) High-end analytical instruments efficiently acquire a representative characterization of the extracts; (4) Data processing either based on vendor software or open-source platforms converts thousands of signals into variables called (mass) features; (5) Statistical interpretation based on univariate and/or multivariate analysis to select significant biomarkers; (6) Annotation and identification of a feature is based on the acquisition metadata (Schymanski et al., 2014); (7) Interpretation of an exposure inventory or of a pathway analysis to integrate the identified features into biologically relevant mechanisms.

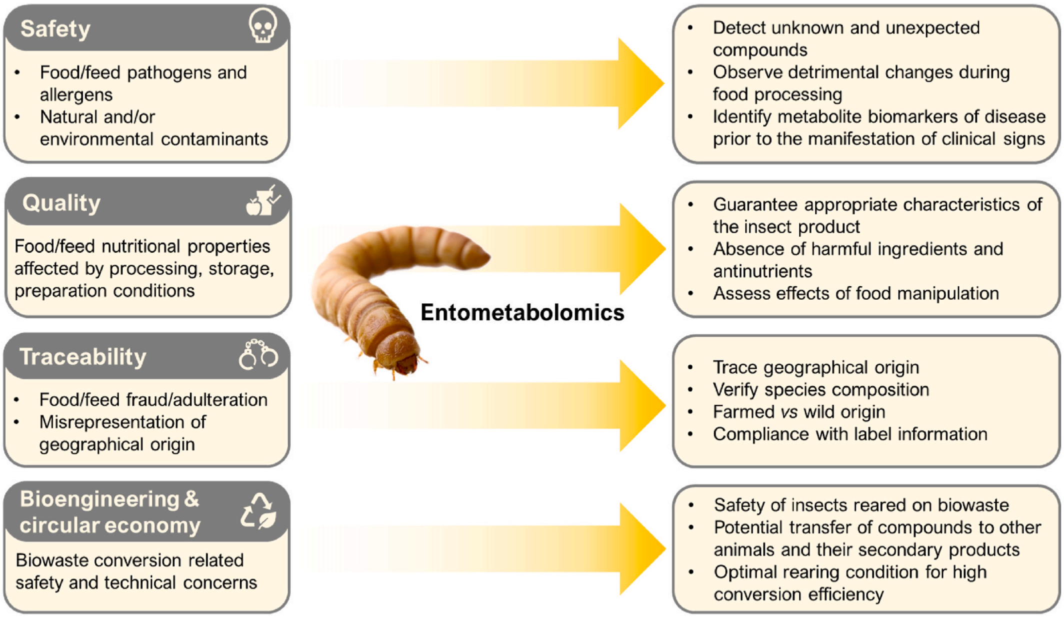

Fig. 2. Application of metabolomics and NTS to edible insects (entometabolomics). General overview of how metabolomics and non-target screening techniques can help overcome the most common hurdles and concerns associated with safety, quality and traceability of edible insects intended as food and feed and current issues related with insect industrial applications.

phenotypic changes, innate phenotypic propensities, and dietary responses in livestock research and monitoring (Goldansaz et al., 2017). A particularly relevant application of livestock metabolomics lies in its potential to identify and monitor biomarkers of animal disease (such as metabolic disorders, diabetes, and heart disease), not only by facilitating their diagnosis, but also by allowing to predict disease prior to the manifestation of clinical signs. This allows farmers and breeders to change the cascade of biological events leading to that disease (Fig. 2) and make early informative decisions, positively impacting animal care (Goldansaz et al., 2017). While in the past edible insects were harvested in the wild, modern insect farming techniques have now been developed and are promoted on an industrial scale (Kok, 2021; Nischalke et al., 2020). Evidently, macro-livestock (cows, sheep, goats, and pigs) farming techniques could in turn be adapted for mini-livestock, such as edible insects (DeFoliart, 1995), providing essential information to the rearing companies regarding their management and leading to significant financial and sustainable outcomes.

1.2. Entometabolomics for the quality of edible insects

Food quality represents the sum of all properties and assessable attributes of a food item and comprises several different aspects, including economic value, nutritional and health value, and even psychological (due to consumers' opinions and expectations) and ecological value (due to direct or indirect impact of food on the environment) (Leitzmann, 1993). Over the past few decades, both producers and consumers acknowledge a significant increase in interest in the nutritional quality of food, the origin of raw materials, and the environmental impacts of food production (i.e. land and water use footprint of food, greenhouse and ammonia emissions, food miles, food waste, etc.).

The nutritional value of edible insects is highly variable and depends on the insect species, metamorphic stage, abiotic conditions, and diet (Finke & Oonincx, 2014). Many edible insects provide satisfactory amounts of energy and proteins, are rich in mono- and polyunsaturated fatty acids, and in essential micronutrients (Gowda, Sasaki, Hasegawa, Chiba, & Hui, 2021; Rumpold & Schlüter, 2013). Edible insects can be consumed whole, processed into products such as flour and granular or paste forms, or they can be reduced into their basic components (such as chitin, proteins, and fats) (Dossey, Tatum, & McGill, 2016) and incorporated into other food products to improve nutritional qualities (Duda, Adamczak, Chelminska, Juszkiewicz, & Kowalczewski, 2019; Parker et al., 2020). Like most food, however, processing, storage, and preparation methods (including boiling or frying) applied before consumption can significantly alter or influence insect nutritional composition (Baek et al., 2019; S. Kim et al., 2016). The objective of food quality is thus to guarantee the appropriate characteristics of the products, the absence of anti-nutritive components and harmful ingredients, and to assess the effects of food manipulation and processing (Li et al., 2020). Because the food metabolome reflects the nutritional value, flavour, and colour of a food product, metabolomics approaches can evaluate changes during processing (Rubert et al., 2015) and provide valuable information on the composition and characteristics of food products (Fig. 2). This way, it allows the food industry to design, modify, and refine processes along the production chain according to the desired quality and nutritional properties of the final food products (Beleggia et al., 2011; Diez-Simon, Mumm, & Hall, 2019; Herrero, Simõ, García-Cañas, Ibáñez, & Cifuentes, 2012).

While metabolomics studies on insects as food for human consumption are still scarce, a few recent studies have explored opportunities for insect exploitation in the feed sector. Leni, Caligiani, and Sforza (2019) investigated if metabolic changes are observed during and following different killing methods of black soldier fly larvae (*Hermetia illucens*) that could affect their protein nutritional quality. They demonstrated that the type of killing method affects the extractability, processability, and final nutritional profile of insect proteins, e.g. killing by freezing stimulates the activation of several enzymatic pathways, including melanisation (Leni et al., 2019). Roques et al., 2020 used a metabolomics approach to assess the effects of insect-protein extract on fish metabolism, concluding that the tested insect-based feed is a valuable substitute of plant-ingredients for the improvement of fish performance (Roques et al., 2020). A multi-omics approach was applied by Meyer et al. (2020) to investigate if the replacement of a conventional protein source by insect-based feed could impact the metabolism of growing pigs. They showed that insect meal could be used without causing adverse effects on pig metabolism (Meyer et al., 2020). Although these examples illustrate the possibility to successfully incorporate new ideas in already existing farming processes, these omics and big data techniques can also support research towards the use of insects as end-product for human consumption.

1.3. Entometabolomics for the traceability and authenticity of edible insects

Food traceability, often referred to as "from-farm-to-fork", aims to provide continuous monitoring of a food item along all steps of the supply chain. Such steps include farming, processing, logistical handling, distribution, retailing, and consumption (Castro-Puyana et al., 2017). Along the way, a wide range of food ingredients may be adulterated by adding banned chemicals or inferior quality products, resulting in a health risk for consumers (Li et al., 2020). Episodes of food frauds, including the deliberate misrepresentation of geographical origin and the adulteration of food ingredients or packaging, may have a negative impact on consumer confidence. In response, the food industry and regulatory mechanisms are in a continuous search to develop tools to guarantee food authenticity (Kendall et al., 2019). Traceability is of utmost importance when dealing with edible insects, whose global market is rapidly rising due to their significant potential to contribute to meeting future global food demands, while reducing environmental impact. While insects are commonly consumed as a food source in many regions of the world, Western consumers are still strongly biased against eating insects, which are often considered with disgust (Looy, Dunkel, & Wood, 2014). This attitude is however gradually changing, likely due to the emphasis on their nutritional properties and environmental sustainability (van Huis, 2016). The recent opening and positive interest of Western countries towards entomophagy is however still precarious and needs to be nourished, encouraged, and preserved.

Most methods applied for the monitoring of food frauds are based on targeted chemical analysis, focusing on the detection and quantification of one or more pre-defined classes of compounds (Cavanna, Righetti, Elliott, & Suman, 2018). The main advantage of using metabolomics and NTS approaches in food authentication lies in the untargeted nature of data acquisition, since unexpected changes in the food composition and metabolic profile (e.g. by the addition of adulterants) can be detected without the need of an *a priori* hypothesis (Cubero-Leon et al., 2018). This has already been performed on tomato paste, saffron, honey, coffee, balsamic vinegar (Consonni & Cagliani, 2019), beer (Cajka, Riddellova, Tomaniova, & Hajslova, 2011), meat (Cajka et al., 2013), virgin olive oil (Lioupi, Nenadis, & Theodoridis, 2020), wine (Pinu, 2018), and oranges (Díaz, Pozo, Sancho, & Hernández, 2014).

Applied along the insect "farm-to-fork" continuum, metabolomics-based screening approaches are thus valuable tools to *inter alia* i) guarantee the geographical origin of insect foods and the compliance with label information, ii) verify the species composition of insect-based products, and iii) ensure that the insects have been reared rather than collected from the wild, preserving thus the sustainability of natural field populations. To the best of our knowledge, metabolomics studies have not yet been performed on edible insects, while a proteomics-based approach has already been applied to efficiently detect edible insect species in insect-flour (Francis et al., 2020). For such authentication purposes, similar strategies using metabolomics can have additional value, as it focuses on other types of molecules than proteomics and is closer to the functional metabolic phenotype of an organism (Snart et al., 2015).

Furthermore, fingerprinting metabolomics approaches combined with chemometrics and data mining can be used to differentiate between wild and farmed species, as previously performed in phytobiological research (Loskutov et al., 2017). Multivariate classification models can be developed to test the origin of an unknown sample based on its LC-HRMS feature profile (Cubero-Leon, Peñalver, & Maquet, 2014). Robust classification models should then be engineered through various experimental studies that consider multiple sources of variation (geographical location or climate, intra- and inter-individual variability, metamorphic stage, instrumental variability, etc.).

1.4. Entometabolomics for circular economy and bioengineering

A number of insect species naturally feed on organic side streams and play an important role in biowaste reduction and renewable organic material production (van Huis & Oonincx, 2017; Čičková, Newton, Lacy, & Kozánek, 2015). Processing of biowaste with larvae of the black soldier fly (BSFL, *Hermetia illucens*) is an emerging technology for the conversion of biowaste into potentially more sustainable and marketable high-value products, such as protein-rich animal feed ingredients, biofuel (Gold, Tomberlin, Diener, Zurbrügg, & Mathys, 2018), cosmetics (Verheyen et al., 2018), and surfactants (Verheyen et al., 2020), supporting circular economy principles.

The main obstacles which currently need to be overcome in order to up-scale the technology and introduce BSFL as a viable biowaste converter include safety concerns, techno-functional properties, nutritional aspects, consumer attitude, and product applications (Bessa, Pieterse, Marais, & Hoffman, 2020). Currently restricted substrates (IPIFF, 2020), like biowaste deriving from processed animal proteins (PAPs), may include a high number of (bio)chemicals and xenobiotics, which can potentially be transferred and accumulated in insects, such as BSFL. By screening for multiple chemicals in a single analysis, NTS and metabolomics approaches are a useful tool to assess if and how insects are affected by the presence of chemicals in the rearing substrate, and to monitor the eventual transfer of compounds to animals they are fed to and to their eventual secondary products (like milk and eggs) (Fig. 2). In addition, entometabolomics can elucidate and elaborate on alterations caused by chemical uptake by comparing exposed and unexposed individuals in a controlled setting. Subsequent pathway analysis, elucidation of important nutrients and screening for potentially toxic compounds can be used to clarify the mechanism of action and the importance of alterations.

Moreover, high conversion efficiency of biowaste requires optimal conditions of the insect environment and diet in terms of moisture content, pH, macronutrients, proteins, carbohydrates, fibre, and lipids (Gold et al., 2018). Biowaste induced upregulation of metabolic pathways can be elucidated using untargeted metabolomics. When the level of upregulation shows a good correlation with the conversion efficiency of biowaste, upregulated metabolites can be targeted during optimization of insect rearing environment and diet to yield an optimal conversion. In addition, these metabolites can be used to select the best insect (sub)species for biowaste conversion. Elucidation of affected metabolic pathways can guide in the design of optimization experiments by pinpointing lack or excess of certain metabolites, which might be related with the conversion rate. This knowledge can be applied during the rearing process to enhance their conversion performances and/or direct the conversion efficiency towards desired end-products. In a study of Melis et al., 2019, the metabolome of yellow mealworm larvae (*Tenebrio molitor*) revealed changes related to growth and body composition upon feeding on wheat bran or by-products of agri-food industry.

By means of such metabolomics approaches, the optimization of processing parameters will ensure the production of high-quality outcome, which is a major aspect determining public acceptance.

1.5. Critical considerations

In this Commentary, the potential of entometabolomics in assessing insect-based food and feed safety, quality, and traceability was explored. The broad applicability of metabolomics and NTS approaches is still hampered by technical challenges, such as analytical sensitivity, complex data analysis workflows, compound coverage, and often lengthy procedures. In addition, sample preparation required for detection of compounds at low concentrations is an often-overlooked challenge, as insects are complex matrices consisting of a wide range of different components, such as fatty acids, chitin, and pigments, which potentially interfere with the instrumental analysis based on LC-HRMS. Depending on the biological question, an untargeted, semi-targeted or targeted approach can be suitable. While for untargeted metabolomics, the sample preparation method needs to be non-selective to ensure an unbiased broad metabolic coverage, sample preparation can be optimized specifically for certain metabolites in a targeted approach. Therefore, suitable sample preparation for metabolomics (like solvent extraction and solid-phase extraction) or NTS (like QuEChERS) and detection techniques, including QA/QC workflows, should be optimized to efficiently monitor the (bio)chemical entities. Although multivariate statistical approaches are sensitive tools to differentiate between groups, e.g. rearing techniques, they should be applied carefully. To avoid pitfalls, including bias, overfitting and improper test selection, a reliable statistical toolbox should be designed, preferably in collaboration with biostatisticians. Besides analytical workflow considerations, challenges concerning the biological perspective should be envisaged, like the sample size or whether to analyse only certain parts of the insect, the whole organism, or composite samples. Since the inter-individual variability of insects is unknown, a suitable sample size must be established during the design of experiment. This is done by ideally selecting organisms belonging to the same species and population, originating from the same source, and reared under the same controlled conditions. Also, the decision of the “resolution” of the analysis can have major implications. While the metabolome of given tissues provides detailed information representing affected biochemical pathways, it requires a labour-intensive sampling procedure and is often limited by low available biomass. On the contrary, the analysis of whole/multiple organisms in a composite pool is straightforward and helps investigating the average effect on the reared population. However, in this case, detrimental effects would be detected less quickly, as metabolic changes need to be more pronounced to be distinguished from physiological variation. There are also potential differences in the metabolic profile of insects compared to other food commodities. This hurdle could be overcome by applying untargeted metabolomics for profiling purposes, which would enable subsequent studies using semi-targeted/targeted approaches. While reliable annotations for untargeted metabolomics would be a major challenge, researchers could still rely on advanced tools such as molecular networks to discover possibly new molecules (e.g., GNPS web-tool) that were not found in commercial (e.g., NIST, WILEY) or publicly available libraries (e.g., MassBank Europe, MoNA, METLIN) (Misra, 2021; Wang & et.al., 2016). In addition, previously unmatched features can be elucidated by generation of possible formulae and structures followed by in silico generation of fragmentation spectra (e.g., CFM-ID) (Djoumbou-Feunang et al., 2019). Furthermore, the composition of edible insects could be incorporated to FooDB (www.foodb.ca), while discoveries of contaminants could be added to the Global Environment Monitoring System (GEMS)/Food Contamination database (https://extranet.who.int/gemsfood). An example of such species-specific initiative is the metabolomic atlas created for the model organism *Drosophila melanogaster* (FlyMet) (Chintapalli, Al Bratty, Korzekwa, Watson, & Dow, 2013), a web-tool that contains metabolites identified in this insect species with links to spectral libraries, their distribution in different tissues and extraction protocols (http://flymet.org/met_explore/).

Finally, to be a valuable tool from an agro-engineering perspective, the turn-around-time from sampling to the final biochemical interpretation of metabolomics approaches should be as short as possible. This time constraint might be the largest challenge to overcome, as most applications are still performed in a research setting, rather than in a high-throughput infrastructure. Therefore, as metabolomics and NTS are consolidated research approaches, their implementation into food systems and translation to industrial settings could further improve the feasibility of insect rearing strategies.

By defining R&D priorities, developing workable frameworks, and executing proof-of-concept projects, the combination of omics techniques and novel food matrices into the food chain of edible insects can be explored by both industry and research. In the rapidly expanding sector of insects as food and feed, these new and efficient techniques may contribute to producing them in a safe and sustainable manner.

Credit authorship contribution statement

Giulia Poma: Conceptualization, Writing – Original Draft preparation, Visualization. Matthias Cuykx: Conceptualization, Writing - Review & Editing. Katyeny Manuela Da Silva: Writing - Review & Editing. Elias Iturrospe: Writing - Review & Editing. Alexander L.N. van Nuijs: Conceptualization, Writing - Review & Editing. Arnold van Huis: Writing - Review & Editing. Adrian Covaci: Conceptualization, Resources, Writing - Review & Editing, Supervision, Project Administration, Funding acquisition.

Declaration of competing interest

The authors do not have conflicting financial interests to declare.

Acknowledgements

This research was supported by the Exposome Centre of Excellence of the University of Antwerp (BOF grant, Antigoon database number 41222). EI is funded by the Research Scientific Foundation Flanders (FWO) - project number 1161620N. KMdS acknowledges a doctoral fellowship BOF DOCPRO 4 from the University of Antwerp. Graphical icons in Fig. 1 were provided by BioRender, licence n. 2641–5211 (Adrian Covaci). We thank T. McGrath for language revision.

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