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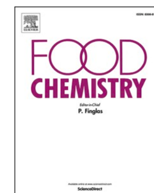

Review

A comprehensive approach to the chemical analysis of fungal biomass – the pitfalls of nutritional standardization

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ABSTRACT

Current protein production methods are reaching their capacity, and therewith their sustainability is at jeopardy. Alternatives like microbial biomass, particularly fungal mycelium, are gaining interest due to their low environmental impact and favorable amino acid profile for human consumption. Current chemical analysis of fungal biomass relies on methodologies standardized for plant biomass or animal protein, often leading to inaccurate and incomplete results. Particularly, overestimation of the protein content is a crucial pitfall, due to the presence of RNA and chitin. Tailored quantification techniques are required, including an adequate nitrogen-to-protein conversion formula, appropriate fiber quantification, and extended solvent selection for lipid quantification. This review provides a novel standardized approach to analyze fungal biomass, enabling accurate quantification of the main constituents such as protein, lipid, RNA, and fiber (e.g. glucan/chitin). Such a comprehensive approach for chemical analysis of fungal biomass is essential for applying fungi in food.

1. Introduction

The current worldwide protein production systems are under pressure due to increased demands related to a larger global population, urbanization, and limitations of conventional protein production systems (FAO, 2011; Van Zanten et al., 2018). As a consequence, not only plant-based protein production, in particular that of soybeans, intensified enormously (FAO, 2021), but also microbial biomass is increasingly explored (Aiking, 2011; Canoy et al., 2024; Maini Rekdal et al., 2024; Todorov et al., 2024). As such, the use of fungi to convert carbohydrate-rich feedstocks into mycelial protein-rich food ingredients is emerging as a sustainable alternative to current protein production systems. Proteins from fungal biomass have an amino acid composition favorable for human consumption, and for their production, significantly less land and water are used compared to those derived from animals and plants (Derbyshire & Delange, 2021; Derbyshire & Finnigan, 2021; Finnigan et al., 2016; Taherzadeh et al., 2022; Wiebe, 2002). Still, also for fungal biomass production, energy is needed to heat fermenters and supplement air, and initial investments to set up such fermentation processes can be high (Chen & Liu, 2021). Moreover, in Europe, most mycelial biomass falls under the Novel Foods regulation, and in the United States of America (USA), either a self-affirmed generally recognized as safe (GRAS) status or a letter of no further questions by the FDA is required for commercial use (Food and Drug Administration (FDA), 1997; The European parliament and council, 2015). As part of the risk assessment and approval procedures in Europe and the USA, compositional data on the novel food is required, often gathered via standardized methods. Such standardized methods, however, are increasingly reported to result in incorrect or incomplete macronutrient composition of fungal biomass. As these methods were originally standardized for plant or animal biomass, they tend to ignore fungal-specific components (Gmoser et al., 2020; Karimi et al., 2021; Svensson et al., 2021). Additionally, an in-depth understanding of the chemical composition of fungal biomass is essential for its efficient utilization and exploitation in

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food products. Moreover, the absence of mycotoxins is crucial for the safe implementation of fungal biomass, but this is not discussed further here. This review advocates a new standardized approach for the compositional analysis of fungal biomass, clearly describing the challenges, pitfalls, and method selection for accurate quantification. More specifically, it targets the contents of protein, RNA, lipid, phospholipid, fiber, e.g. chitin, soluble carbohydrates, vitamins, and ash. Although this review focuses on approaches for compositional analysis of fungal biomass, similar principles can be applied to other micro-organisms or single-cell-based (food) ingredients.

2. Challenges and pitfalls in compositional analysis of fungal biomass

Fungal biomass is generally the insoluble, washed fraction recovered after submerged growth of fungal mycelium in a minimal medium supplemented with soluble carbohydrates (i.e., mainly glucose) and an inorganic nitrogen source (Derbyshire & Finnigan, 2021; Taherzadeh et al., 2022). Generally, fungal hyphae comprise an inner cytosol with its contents, surrounded by a phospholipid bilayer, a chitin/glucan cell wall, and a mannoprotein outer layer, providing rigidity and a protective physical barrier around the cell (Fig. 1; Gow et al., 2017; Vega & Kalkum, 2012).

Current analytical methodologies applied for determination of the composition of fungal biomass, although still accepted in Europe and the US for Novel Food-type dossiers, are recognized to give an incomplete picture or lead to incorrect insights, as further exemplified below. Consequently, a standardized approach tailored for analyzing the macronutrient and chemical composition of fungal biomass is essential when (novel) fungal biomass is to be used responsibly in food products for human consumption.

2.1. The protein content of fungal biomass – a nitrogen balance

Protein in fungal biomass is present in multiple forms, such as enzymes, storage proteins, cell wall proteins, and transporters (Wang et al., 2023). It can be expected that only part of these proteins is water-soluble, while, for example, cell wall-bound or intertwined proteins will remain water-insoluble (Schoffelmeer et al., 1999). Hence, although exact ratios of soluble versus insoluble protein have yet to be published for fungal biomass, it is anticipated that soluble protein quantification methods will result in incomplete data for fungal biomass (Bradford, 1976; Smith et al., 1985). Moreover, as specified above, fungal biomass is considered the insoluble, washed fraction. To circumvent such incomplete protein quantification and not depend on solubility, total nitrogen analysis should be performed, which is the standard method for protein quantification. The nitrogen content is subsequently multiplied by a standardized factor of 6.25, reflecting the average percentage of nitrogen in many food proteins (FAO & WHO, 2022). Nitrogen can be quantified by using the so-called 'Kjeldahl' or 'DUMAS' methods

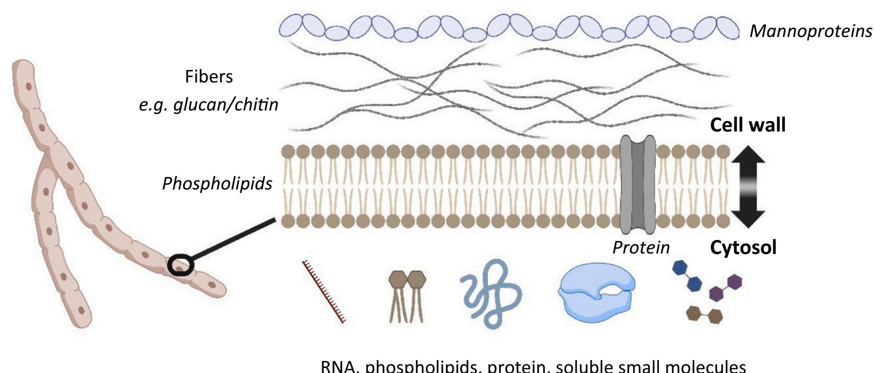

Fig. 1. A simplified overview of the structural organization and composition of fungal biomass. On the left side, the fungal hyphae, on the right side, a magnified representation of the fungal cell wall (top) with various components present inside the cytosol (bottom). Created with BioRender.com.

Table 1
Nutritional composition of fungal biomass (FB) based on dry matter (DM), as specified in GRAS dossiers (3FBIO Ltd, 2020; Marlow Foods Ltd, 2001; The Better Meat Co., 2022). FB #1 is a commercial product from The Better Meat Co., Washington, USA, FB #2 from 3F BIO limited, Glasgow, United Kingdom, and FB #3 is from Marlow Foods, North Yorkshire, United Kingdom.

Component (% w/w DM)[a]	Commercial fungal biomass (FB)		
	FB #1	**FB #2**	**FB #3**
Fat[b]	3.9	6.2	12–14
Crude protein (N% x 6.25)[c]	48.3	54	52–59
Protein (lysine derived)[d]			42–50
Fiber[e]	32.0	28.9	22–28
Carbohydrates[f]	8.5		
Ash[g]	7.3	3.6	3–4
Total	100.0	92.7	89–105

[a] Dry matter (DM) based.
[b] Analyzed according to AOAC 996.06 (Latimer, 2023).
[c] Analyzed according to AOAC 992.15 (Merril & Watt, 1973).
[d] Lysine derived, on acid hydrolysis (23 h at 105 °C, 20 % HCl) followed by colorimetric lysine quantification (2,4,6-trinitrobenzene sulphonic acid), according to Moorhouse et al., 1967.
[e] Analyzed according to AOAC 991.43 (Latimer, 2023).
[f] Based on total DM minus fat, protein, fiber, and ash (Merril & Watt, 1973).
[g] Analyzed according to AOAC 923.03 (Latimer, 2023).

involving either acid hydrolysis followed by NH_4 quantification or combustion of the sample followed by quantification of N_2, respectively (Mæhre et al., 2018). Although this is a valid approach for some plant-based biomass and foodstuffs in which nitrogen is present primarily in protein (Jones, 1941; Mariotti et al., 2008), for fungal biomass, it is not. Here, nitrogen is part of protein, cell wall chitin, and RNA/DNA, of which amounts and ratios vary with fungal species and processing conditions (Derbyshire et al., 2023; Karimi et al., 2021). In Table 1 the crude protein content of three commercial fungal biomass products is shown as an example, alongside the overall chemical compositions as specified in GRAS dossiers. Note that the data in Table 1 might vary depending on, among others, media composition, culture, and processing conditions, and might be strain dependent.

The data in Table 1 Fungal biomass #3 (FB #3; *Fusarium venenatum* ATCC PTA-2684 (Marlow Foods Ltd, 2001) exemplifies that the protein content is likely overestimated when the amount of crude protein is determined (i.e., N% x 6.25; 52–59 %; Table 1), while the lysine-derived protein content for the same FB #3 material results in an already more specific protein content of 42–50 % (Table 1). Still, the colorimetric nature of quantifying protein based on lysine ignores differences in lysine ratios among proteins and fungal biomass. Other GRAS dossiers report only crude protein contents (i.e., based on only nitrogen analysis), for example, in Table 1 for fungal biomass #1 (FB #1; *Neurosporra crassa* NRRL 78076 (The Better Meat Co., 2022) and #2 (FB #2; *Fusarium venenatum* IMI145425 (3FBIO Ltd, 2020)), likely ignoring the overestimation of the protein content by important nitrogen-containing mycelial components other than protein, such as chitin and RNA/DNA.

Overall, although relatively elaborate compared to total nitrogen analysis, quantification of the amino acid content is considered the most specific and accurate method to directly quantify the amount of protein in fungal biomass (Rutherfurd, 2009). Additionally, this method provides insights in the amino acid composition, and therewith the nutritional value. Amino acid quantification is based on severe hydrolysis of the materials aiming at hydrolyzing all peptide (amide) bonds between amino acids. The stability of some of the released amino acids is relatively low and can be prone to oxidation and decomposition, which can lead to a slight underestimation of the total protein content in the presence of oxygen (Rutherfurd & Gilani, 2009). Thus, the absence of oxygen, hydrolysis conditions, and the sample matrix direct the delicate balance between complete hydrolysis of the protein and degradation of already released single amino acids (Rutherfurd & Gilani, 2009). Standard procedures involve three separate methods: acid hydrolysis for most amino acids in the absence of oxygen (AOAC 982.30), oxidation before acid hydrolysis for cysteine and methionine (AOAC 982.30), and alkaline hydrolysis needed for tryptophan due to its instability in acidic conditions (AOAC 988.15). Subsequently, the free amino acids can be quantified via UHPLC-UV or GC-FID after derivatization. The protein content can then be calculated from the sum of the content of each amino acid, considering the addition of one water molecule per amino acid for the loss of the peptide bond.

2.2. Nitrogen-containing compounds interfering with protein quantification

Besides nitrogen related to protein, both RNA/DNA and chitin can contribute significantly to the total amount of nitrogen in fungal biomass (Fig. 2), with generally chitin as largest contributor.

The RNA content in the fungal biomass is generally positively correlated to its growth rate; thus, regulation of growth rates can steer RNA levels (Nielsen, 1997; Waldron & Lacroute, 1975). The amount of DNA in fungal biomass is often negligible since it does not exceed 0.1 % *w*/w of the complete dry biomass (Anderson, 2008). Current legislation allows RNA levels up to a daily intake of 2 g/day from food. However, this is based on limited evidence from around 1970 (Bowering et al., 1970; Edozien et al., 1970; The British Nutrition Foundation, 2016). At the same time, it has been reported that 2 g of RNA per day gives a higher risk of the formation of stones in the urinary tract due to uric acid concentrations (Bowering et al., 1970). As such, in the production process of fungal biomass, an RNA reduction step can be considered, for example, by increasing temperatures to >68 °C, activating naturally present ribonuclease, which degrades RNA into nucleotides, which is consequently solubilized. A main disadvantage of this RNA reduction step is the significant simultaneous fungal biomass loss of 33–38 % (Ward, 1998; Wiebe, 2002). Whether such RNA reduction steps are needed for safe use in food must be reevaluated (The British Nutrition Foundation, 2016). Understanding the safety of RNA and daily doses in food is currently limited because standardization of RNA/DNA quantification methods has yet to be established for nutritional compositional analysis, and available RNA content data in foods are, therefore, scarce. Methods available for RNA/DNA analysis involve the depolymerization of RNA/DNA into nucleotides, for example, via heating at 65 °C in alkaline conditions or via acid hydrolysis, with subsequent quantification by HPLC approaches (Azarani & Hecker, 2000; Li & Breaker, 1999; Zhang et al., 2021). RNA is built out of four types of nucleobases (i.e., adenine (A), uracil (U), cytosine (C), and guanine (G) (Li et al., 2015; Fig. 2), the nucleobases are present in an RNA chain with a backbone of phosphate groups and ribose. Note that varying AUCG ratios are found among different species (Li et al., 2015). To enable a general estimation of nitrogen content in RNA/DNA, an equal nucleobase distribution was assumed. While base composition can vary between fungal strains, this approximation provides a reasonable basis for interspecies comparisons in the absence of relevant sequence data. Considering an even distribution of these nucleobases, RNA comprises 16.1 % nitrogen (calculated, Supplement 1), which is similar to the amount of nitrogen in protein. Hence, based on an even distribution of nucleobases, N_{RNA} will result from the analyzed RNA content divided by 6.2 (=100/16.1 %).

Nitrogenous chitin is an important structural polysaccharide within the cell wall of fungi, built of a linear chain of β-(1→4)-linked 2-amino-2-deoxy-D-glucopyranosyl units. Upon its biosynthesis, the amino group is acetylated (Fig. 2; Roy et al., 2017), this acetyl group can be removed during further growth and cell wall rearrangements of the fungus. In fungal cell walls, chitin can comprise 1–30 %*w*/w of dry biomass (Garcia-Rubio et al., 2020), which would represent a nitrogen content ranging from 0.1 to 2.1 %w/w. When multiplied by the conventional nitrogen-to-protein conversion factor of 6.25, this would lead to a protein overestimation of 0.6 to 13.1 %w/w. Hence, chitin can substantially contribute to the total nitrogen proportion in fungal biomass. N_{chitin} can be calculated, once chitin (glucosamine) is quantified (see methodology in sections 2.3 & 3), from the chitin content divided by 11.5; in which the 11.5 represents the nitrogen percentage of the (anhydrous) glucosamine (i.e., 8.69 %; Fig. 2).

Given the above, and to facilitate total nitrogen content analysis as

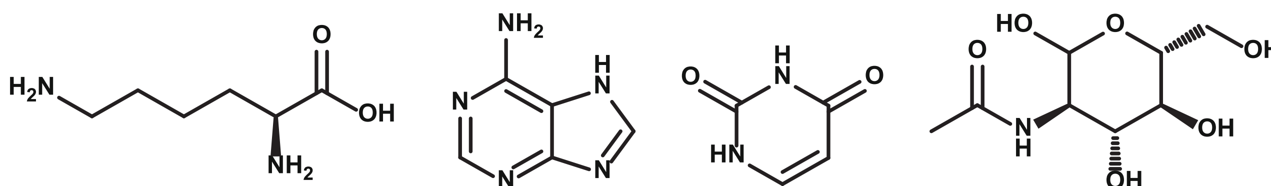

Fig. 2. Nitrogen-containing molecules present in fungal biomass, from left to right of lysine (protein), adenine & uracil (RNA), and acetylated glucosamine (chitin).

an indicator of protein in fungal material, Eq. (1) can be utilized once the amino acid-based protein, the corresponding nitrogen-to-protein conversion factor, chitin, and RNA/DNA contents are determined. Next, under the assumption that, for a certain fungal strain in a specific process, the RNA/DNA and chitin proportions are constant, total nitrogen analysis can be applied to indicate protein contents, in particular relevant to monitor long-term fermentations and for quality control. Other nitrogen-containing compounds, such as head groups of phospholipids and intracellular metabolites, can also be included but are very minor in amount compared to protein, chitin, and RNA/DNA in fungal biomass and are hence considered negligible (Perczyk et al., 2020).

$$\text{Protein (Nitrogen based)} = (N_{Total} - N_{RNA} - N_{Chitin})^{*}F_{NP} \quad (1)$$

In which F_{NP} is the nitrogen-to-protein conversion factor. F_{NP} is calculated using the (anhydro) amino acid profile reported in the GRAS dossiers and is 6.24, 6.10 and 6.09 for FB #1, FB #2, and FB #3 respectively (Supplement 2; 3FBIO Ltd, 2020; Marlow Foods Ltd, 2001; The Better Meat Co., 2022). In the analysis both glutamine and asparagine are converted and analyzed as their acid counterpart. Although, the conversion of glutamine to glutamic acid and asparagine to aspartic acid considerably influences the percentage of nitrogen in fungal protein, in the nitrogen to protein conversion factor, only the analyzed values for aspartic acid and glutamic acid were taken into account. If an accurate nitrogen-to-protein conversion factor is to be established for a specific strain, it is advised to include analysis of asparagine and glutamine.

Coincidentally, the nitrogen-to-protein conversion factors of the three fungal biomasses are very similar, but it must be noted that the nitrogen-to-protein conversion factor can vary considerably among different fungal species.

2.3. Current gravimetric methods underestimate fungal fiber content

Standardized total dietary fiber quantification methods for food and feedstuffs are gravimetrically based (AOAC 991.43 and 2011.25), aiming at an easy-to-operate analysis at affordable costs (Ferjančič et al., 2022). In short, samples are, when relevant, enzymatically treated to degrade and solubilize protein and starch, with subsequent sequential washing of (soluble) fibers in different solvents like acetone, ethanol, and water (AOAC 991.43 and 2011.25). The weights of the dried soluble dietary fiber extract combined with the final dried residue represent the fiber content. Chitin accumulates in the final residue due to its insolubility in the solvents used in the fiber isolation (Roy et al., 2017). The residue is corrected for ash, and for residual protein, which can be present in the form of protease-resistant and insoluble proteins. Hereto, the residue is subjected to ash content analysis and total nitrogen content analysis, applying the conversion factor of 6.25 (Latimer, 2023; Mariotti et al., 2008). As reviewed and discussed in 2.2 and Eq. 1, RNA/DNA and accumulation of chitin in the fiber residue will lead to an overestimation of protein based on total nitrogen and, consequently, an underestimation of fiber content. Moreover, the above gravimetrically based methodology will obviously not distinguish between the variety of fibers present in the fungal cell wall, such as glucan, chitin, chitosan, cellulose, mannan, and polygalactosamine (Bartnicki-Garcia, 1968).

To further distinguish and quantify fibrous (poly)saccharides, a recognized method, usually applied for plant biomass materials, involves an acid hydrolysis to convert all (poly-) saccharides present into their monomeric building blocks. Indeed, fungal cell wall fibers, except chitin-like ones, are also known to be hydrolyzed to monosaccharides rather completely in 1 M sulphuric acid at 100 °C for 2.5 h, sometimes preceded by pre-swelling in 12 M sulphuric acid at 30 °C (Englyst & Cummings, 1984; Kouzounis et al., 2021). The resulting monosaccharides can either be directly separated and individually quantified via HPAEC-PAD analysis (Hounsell et al., 2009), or first derivatized to, for example, so-called alditol acetates to make them volatile for separation and quantification by GC-FID (Englyst & Cummings, 1984). By contrast, this type of hydrolysis does not hydrolyze chitin-like structures, including chitosan, chitin, and polygalactosamine (Roy et al., 2017). Harsher hydrolysis conditions are required to hydrolyze such fibers into their (deacetylated) building blocks, namely 12 M HCl at 100 °C for 1.5 h (Dolgopyatova et al., 2013). Note that under these harsh conditions, (poly-) saccharides other than chitin-like ones are also hydrolyzed, but the formed monosaccharides immediately react further into a range of non-defined reaction products (Dolgopyatova et al., 2013; Ride & Drysdale, 1972). The formed deacetylated glucosamine and galactosamine remain stable for subsequent separation and quantification with HPAEC-PAD.

To determine the degree of acetylation of chitin, another hydrolysis and HPLC-type method are needed to quantify the released acetyl groups (i.e., acetic acid; Zamani et al., 2008). The released acetic acid is unstable in the harsh acidic conditions used for complete depolymerization (12 M HCl). The degree of acetylation can be determined with dilute sulphuric acid (0.25 M) at 120 °C for 1 h (Zamani et al., 2008). If the degree of acetylation is neglected, and assuming a degree of acetylation of 100 %, this can lead to an underestimation of the total chitin content of up to 26 % (calculated based on molecular weight).

Although the above-described hydrolysis procedures can cover the whole range of fibers present in fungal biomass, it will only result in their overall building block composition. Specific structural information, for example, the linkage type and degree of branching, requires other analytical approaches going beyond overall nutritional compositional analyses, which are not further specified here.

Of note, in addition to the analytical approach described here, fungi have been categorized into seven distinct classes based on specific cell wall components and composition (Bartnicki-Garcia, 1968). Although it is helpful to distinguish chitin-rich fungal species from glucan-rich ones, analysis of family-specific fiber present is still foreseen.

2.4. Quantification of the apolar fraction – the challenge of multiple lipid classes

Crude fat quantification is based on its extraction and subsequent gravimetric determination of the dry matter recovered in the extract (Srigley & Mossoba, 2017). Animal and plant-based fats and lipids are known to recover well and rather completely in apolar solvents. Hence, solvents such as hexane, heptane, or petroleum-ether are usually advised in protocols for fat quantification (Srigley & Mossoba, 2017). Depending on the sample matrix, a mild hydrolysis of the samples can be applied to improve fat extraction (Srigley & Mossoba, 2017). Considering fungal biomass, lipids are primarily found in the form of phospholipid bilayers in the cell membrane (Fig. 1; Iwama et al., 2023; Renne & de Kroon, 2018). Phospholipids have a highly polar phosphate head group, which hinders solubilization in aforementioned apolar solvents (Ricciutelli et al., 2006). Instead, more polar solvents, such as chloroform, methanol, a combination thereof, or diethyl ether is needed to extract and quantify the complete phospholipid fraction (Wasserscheid & Stark, 2010).

Underestimation of lipid content as a result of inefficient extraction protocols is exemplified by the published lipid contents of fungal biomass. For example, the lipid content of FB #1 (Table 1) was based on hexane extraction (AOAC 996.06), resulting in a lipid content of 3.9 % *w*/w (based on dry matter), while the lipid content of FB #3, albeit from a different process, is considerably higher (13 %*w*/w based on dry matter). Although the dossier of FB #3 does not specify the solvent used for lipid extraction and quantification, it can be anticipated that this higher value includes phospholipids in the total lipid content. That, and the fact that the dossier of FB #3 mentions explicitly that 30 % of the lipids were found as phospholipids, showcases the relevant proportion of phospholipids being part of the total lipid fraction. Similar observations were made in the analysis of 23 different fungal biomasses, where the lipids were extracted using petroleum ether, and varied between 0.4 and 3.4 %w/w (based on dry matter), neglecting phospholipid content

(Yu et al., 2020). The amount of phospholipids relative to triglycerides in fungal biomass is rarely studied but is likely related to the growth stage (dos Oliveira et al., 2011; Fakas et al., 2009). The discrepancies between the lipid contents of the reported biomasses indicate the importance of adequate solvent selection for lipid quantification in fungal biomass.

2.5. Soluble carbohydrates and small molecules

Fungal biomass is, to a lesser extent, composed of small soluble carbohydrates and small molecules (e.g. polyols) present in the cytosol. An important note for fungal biomass is that, commonly, only the insoluble fraction of submerged fermentations is used, and most soluble metabolites are washed out (Holt et al., 2024). However, carbohydrates and small soluble molecules can still add to the nutritional properties of fungal biomass and cannot be neglected since they are often quickly metabolized by humans. In the USA, the standard procedure dictates calculating such soluble carbohydrates and small molecules by subtracting the amount of quantified protein, fiber, lipid, and ash from the total dry matter (Merril & Watt, 1973; Table 1). Such a procedure, however, will not distinguish between types of free carbohydrates, glycogen, or intracellular molecules such as osmolytes, and does not take into account the incorrect quantification of macronutrients when based on procedures highlighted in this review (Ianutsevich et al., 2023; Von Meyenburg, 1969; Touster & Shaw, 1962). Quantification of said solubles would benefit from using mild (enzymatic) hydrolysis protocols of a collected soluble fraction, after which released specific monosaccharides and/or polyols can be separated and quantified, for example, by using HPLC (e.g., HPAEC-PAD, HPLC-MS) (Hounsell et al., 2009).

3. Proposed novel standardized approach for the analysis of fungal biomass

This review highlights the challenges of the compositional analysis of fungal biomass and the pitfalls of standardized methodologies. The necessity of a novel standardized approach for fungal biomass analysis is evident and is becoming increasingly important, seen the current interest of such microbial biomass in human nutrition. A stepwise approach, with considerations, for the analysis of the chemical composition of fungal biomass is proposed in Fig. 3. The proposed analytical methods are more than standard quality control methods, however, are deemed necessary to properly assess the nutritional value of biomass. Under the assumption that, for a certain fungal strain in a specific process, the composition is constant a selection of the proposed methods would be sufficient to monitor long-term fermentations and for quality control. The analytical methods indicated can be applied to the biomass of any fungal species, and a similar approach can be used for other novel microorganisms or single-cell-based (food) ingredients.

Firstly, the protein content of fungal biomass has to be analyzed via either specific amino acid analysis, or via the here proposed total nitrogen analysis corrected for nitrogen from RNA/DNA and chitin (Eq. 1). We suggest quantifying RNA/DNA contents via acid hydrolysis released nucleotides with subsequent HPLC-UV quantification, as specified by Azarani and Hecker (2000); (see Fig. 3). The next step is fiber (i.e. (poly-)saccharide) quantification with separate specific acid hydrolysis conditions for chitin, its degree of acetylation, and other polysaccharides Fig. 3). The resulting glucosamine and monosaccharides can be quantified using HPAEC-PAD or after derivatization by GC-FID, respectively. The amount of chitin following glucosamine quantification has to be calculated using the degree of acetylation based on HPLC analyses. Lipid quantification of fungal biomass can be well approached via solvent extraction, and subsequent gravimetric quantification, but should consider appropriate solvent types, to include phospholipids (methanol: chloroform; Fig. 3). Methods that remain applicable and accurate for analyzing fungal biomass include AOAC methods for determining moisture content, ash, vitamins, and minerals (Latimer, 2023). Minerals are already incorporated in the ash content but give extra insights into the nutritional benefits of fungal biomass.

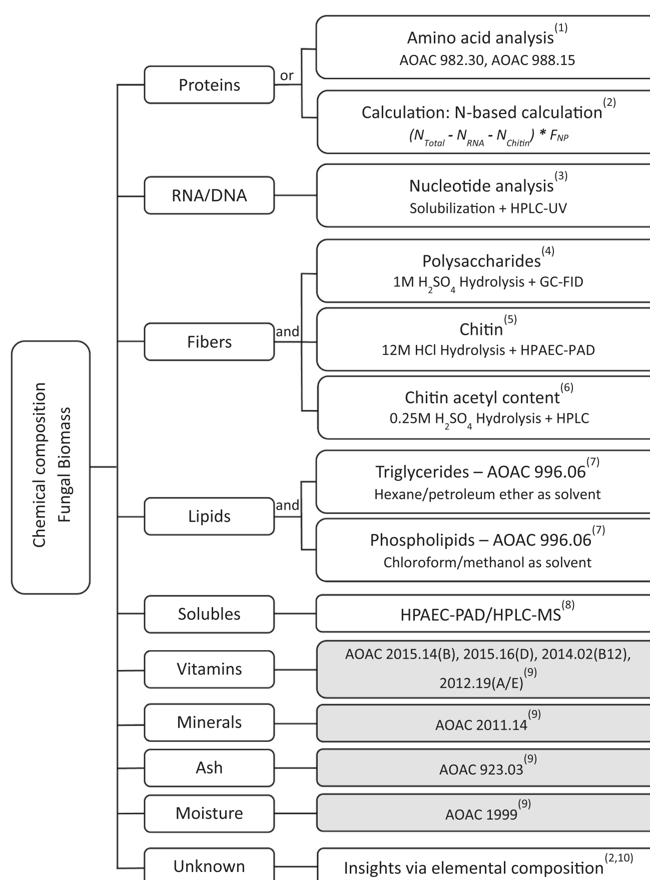

Fig. 3. Schematic flow chart – of a standard approach for the chemical analysis of fungal biomass. Methods in grey boxes are according to standard procedures [(1)]Rutherfurd and Gilani (2009), [(2)]this paper, [(3)]Azarani and Hecker (2000), [(4)]Englyst and Cummings (1984); Kouzounis et al. (2021), [(5)]Dolgopyatova et al. (2013), [(6)]Zamani et al. (2008), [(7)]Srigley and Mossoba (2017), [(8)]Hounsell et al., 2009, [(9)]Latimer (2023), [(10)]Kuveke et al. (2022).

Lastly, elemental compositional analysis can be helpful in validating the mass balance or when the above proposed approach does not yet cover 100 % of the dry matter. The elemental composition of fungal biomass, i.e., CHNSOP, can be determined using combustion-based elemental analyses (Fadeeva et al., 2008). Combining outcomes from the elemental composition with those of all suggested compositional analysis methods will guide the identification of not yet analyzed compounds in the direction of carbon, oxygen, or nitrogen-rich compounds, providing insights into the chemical nature of ‘missing’ components and directions for further analyses (Fig. 3).

4. Conclusion

Fungal biomass has gained increasing interest as an alternative food ingredient over the past decade. Although conventional nutritional composition methods are standardized, these methods do not consider components present in fungal biomass, such as RNA/DNA, chitin, and phospholipids. This review highlights the effect of nitrogen-based protein quantification methods on the incomplete quantification of protein and fibers. A novel approach for the complete chemical analysis of fungal biomass is proposed, considering non-protein-nitrogen, difficulty of fiber separation, the solubility of lipids/phospholipids and provides a strategy for elemental insights into possible gaps. Our proposed strategy results in accurate mapping of the chemical composition of fungal and,

in a broader context, microbial or single-cell biomass. Such compositional understanding is essential to further unlock and implement these novel food ingredients, contributing to a more resilient and sustainable food system.

CRediT authorship contribution statement

Cas H.A. Geerits: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Maloe Kleine Haar:** Writing – review & editing, Methodology, Investigation. **Kirsten C.C. Knobel:** Writing – review & editing, Supervision, Conceptualization. **Jean-Paul Vincken:** Writing – review & editing, Supervision. **Mirjam A. Kabel:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cas HA Geerits reports financial support was provided by The Protein Brewery B.V., Breda, The Netherlands. Cas HA Geerits reports a relationship with The Protein Brewery B.V., Breda, The Netherlands that includes: employment. Kirsten CC Knobel reports a relationship with The Protein Brewery B.V., Breda, The Netherlands that includes: employment. Maloe Kleine Haar reports a relationship with The Protein Brewery B.V., Breda, The Netherlands that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.145443.

Data availability

No data was used for the research described in the article.

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