On the Nitrogen Content and a Robust Nitrogen-to-Protein Conversion Factor of
Black Soldier Fly Larvae (*Hermetia illucens*)

Ruben Smets¹ , Johan Claes¹ , Mik Van Der Borcht¹

¹Faculty of Engineering Technology, Department of Microbial and Molecular Systems, KU
Leuven, Kleinhofstraat 4, 2440 Geel, Belgium.

Ruben Smets <https://orcid.org/0000-0001-7068-4514>

Johan Claes <https://orcid.org/0000-0003-2565-9138>

Mik Van Der Borcht <https://orcid.org/0000-0001-9091-2983>

Correspondence should be addressed to mik.vanderborcht@kuleuven.be

Mik Van Der Borcht, Faculty of Engineering Technology, Department of Microbial and
Molecular Systems, KU Leuven, Kleinhofstraat 4, 2440 Geel, Belgium.

Abstract

Currently, a broad discussion exists in the literature regarding insect protein analysis. At its core, main difficulties and uncertainties are the inconsistent use of analysis methods and nitrogen-to-protein conversion (k_P) factors. While the Kjeldahl and Dumas methods are both used in the literature, their result represents inherently different nitrogen fractions. Thus far, no correlation between them is established for insect matrices, which is a major uncertainty. Despite much effort has been made towards more accurate k_P factors, calculation of these was based on merely one sample while the chemical composition varies depending on rearing conditions. Using a broad variation in black soldier fly (BSF) larvae samples in the present study, a correlation between Kjeldahl and Dumas and a robust k_P factor has been established. Moreover, the nitrogen distribution of BSF samples was also assessed after accurate chitin analyses. A highly significant linear correlation existed between the results of Kjeldahl and Dumas (slope: 1.009; intercept: -0.008 ; R^2 : 0.9997). Consequently, both methods were deemed interchangeable for BSF larvae. Using amino acid data, a practical, more accurate and robust k_P factor of 4.43 was obtained. Concerning the chitin content, the average of all BSF larvae samples was 5.95 ± 0.86 g *N*-acetylglucosamine/100 g dry matter and no correlation with the k_P factor was observed. Regarding the nitrogen distribution of the samples, it was found that the contribution of nitrogenous compounds other than protein and chitin is not only high but also prone to variation (12 – 30 % of the total nitrogen content).

Key words

Hermetia illucens; Kjeldahl; Dumas; Protein conversion factor; Amino acids; Chitin

43 **Graphical abstract** 45 **1. Introduction**

46 As the world population continues to grow, substantial problems concerning the food supply
 47 are expected. Extrapolations of the United Nations predict that the world's population will rise
 48 to 9.7 billion by 2050, which corresponds to an annual increase of 63 million people (1). In
 49 addition to a growing population, consumption of animal proteins is on the rise as well due to
 50 an increased income. As a result, the demand for alternative and more sustainable protein
 51 sources such as insects, microalgae, and plant-based substitutes is increasing (2–4). In this
 52 scope, black soldier fly (BSF, *Hermetia illucens*) larvae are highly promising as they can be
 53 reared efficiently on low-value organic waste streams, converting it into high-value biomass.
 54 Besides being rich in lipids (30 – 40 %), these larvae also contain high amounts of proteins (20
 55 – 50 %) and chitin (4 – 7 %), all expressed on dry matter (5–8). In addition, BSF proteins
 56 contain all essential and limiting amino acids for poultry and pigs (i.e., in order of limitation:
 57 Lys, Arg, Thr, Met, Cys, Trp, Ile, Leu, Val, His, Phe, and Tyr), and salmon (i.e., in order of
 58 limitation: His, Ile, Leu, Lys, Met, Phe, Thr, Val, Arg), among others, in sufficiently high
 59 quantities (9,10).

60 Regardless whether BSF larvae are incorporated directly into human food (3), used as animal
 61 feed (10), or processed into feedstock for industrial applications (5); accurate quantification of

their chemical composition (i.e., lipids, protein, and chitin) is of utmost importance. At this moment, a broad discussion exists in the literature on the subject of insect protein analysis. A nice overview on this discussion was provided by Jonas-Levi and Martinez (11) as they compiled 75+ results of insect protein and chitin analyses and their corresponding analysis procedures. Looking at their data collection, it can be observed that the protein content of insects published in the literature is obtained through different analysis procedures, making comparison difficult. Although this overview is already published four years ago, a close look at more recent literature still shows the same issues (Table 1). In addition, the procedures used in the literature to determine the chitin content of insects are most-often procedures for dietary fibre analysis (i.e., crude fibre, acid detergent fibre, etc.) which do not differentiate between fibres (11). Acidic hydrolysis followed by quantification of the chitin monomers is more performant, as described in our previous work (12). The more accurate quantification of chitin through such approach instead of gravimetric procedures allows to assess the nitrogen distribution in insects with a higher certainty.

The most common approach to determine the protein content is to first determine the nitrogen content of a sample, which is then multiplied with a conversion factor (i.e., k_P factor). Although nitrogen determinations can be performed either by the Kjeldahl or Dumas method, their results represent different nitrogen fractions. The Kjeldahl method consists of an acid digestion step which converts organic nitrogen into NH_4^+ ; followed by a quantification of the latter through titrimetry (31). Compounds with N–N or N–O linkages (e.g., azo- and nitro compounds, hydrazones, etc.) as well as heterocyclic, nitrogenous compounds (e.g., pyridine) are, however, highly resistant to the digestion conditions and are therefore not (completely) included in the nitrogen content determined by the Kjeldahl method ($w_{\text{N, Kjeldahl}}$) (32).

Table 1 Compilation of the recent literature regarding protein analyses of black soldier fly (*Hermetia illucens*), reported after the overview published by Jonas-Levi and Martinez in 2017 (11). This overview shows the developmental stage of *H. illucens* which was investigated in the corresponding study, the used analysis method for the determination of the nitrogen content, and the used nitrogen-to-protein (k_P) conversion factor.

Publication year	Developmental stage	Analysis method	k_P factor	Reference
2017	Larvae	Dumas	4.67 [†]	(13)
2017	Prepupae	Dumas	6.25 [‡]	(7)
2017	Larvae, prepupae, and pupae	Kjeldahl	n.s.	(8)
2018	Larvae	Dumas	6.25	(14)
2018	Larvae	Kjeldahl	4.67	(15)
2018	Larvae	Kjeldahl	6.25	(15,16)
2019	Larvae	Dumas	6.25	(17,18)
2019	Larvae	Kjeldahl	4.76	(19)
2019	Larvae	Kjeldahl	6.25	(20,21)
2019	Prepupae	Kjeldahl	4.76	(22)
2020	Larvae	Dumas	6.25 [†]	(23)
2020	Larvae	Kjeldahl	4.76	(24,25)
2020	Larvae	Kjeldahl	5.75 [†]	(26)
2020	Larvae	Kjeldahl	6.25	(25)
2020	Larvae, prepupae, and pupae	Kjeldahl	4.67	(6)
2021	Larvae	Dumas	4.76	(27)
2021	Larvae	Kjeldahl	6.25	(28)
2021	Prepupae	Dumas	6.25	(29)
2021	Larvae & prepupae	Kjeldahl	4.76 & 6.25	(30)

[†] newly determined; [‡] nitrogen content corrected for chitin; n.s. not stated

Regarding the Dumas method, a combustion furnace is used to convert all nitrogen sources (i.e., organic and inorganic) into NO_x, which are then reduced to N₂ prior to quantification ($w_{N, \text{Dumas}}$) with a thermal conductivity detector (33). Both methods, however, are susceptible to interference of other nitrogenous compounds (e.g., chitin, ammonia, uric acid, etc.) as they increase the nitrogen content of the sample beyond the nitrogen originating from protein. To counter this problem, accurate k_P factors are required which correct for the presence of non-protein nitrogen. Alternatively, amino acid analysis is not prone to such interference and yields more accurate results, albeit more expensive (34). Although the results of the Kjeldahl and

Dumas method represent a different nitrogen fraction, several studies have aimed at comparing them, showing a matrix dependence (31,33,35). While the results of oilseeds and dairy products are shown in the literature to have a ratio ($w_{N, \text{Kjeldahl}}/w_{N, \text{Dumas}}$) of almost 1, samples high in inorganic nitrogen (e.g., fruit and vegetables) show a significantly lower ratio. Consequently, a direct comparison of the nitrogen content of such samples obtained via either Kjeldahl or Dumas is not possible. To the best of the author's knowledge, no such comparison has been made for insect samples thus far, which results in a large uncertainty.

Another difficulty in the comparison is the use of different k_P factors. While the value of 6.25 can be used to calculate the 'crude protein content', this value assumes that all nitrogen in the sample originates from proteins and that their nitrogen content is 16 g/100 g protein (36). This assumption dates back from the beginning of the 20th century and was based on the knowledge that the nitrogen content of proteins analysed at that time (i.e., casein, serum albumin) averaged around this 16 % (37). Since then, it is shown that not all proteins contain 16 g N/100 g besides for a few exceptions; giving rise to various k_P factors depending on the sample matrix (37). As described previously, BSF larvae contain a relatively high amount of chitin (i.e., around 4 – 7 g/100 g dry matter). While the k_P factor of 6.25 is still being used for BSF larvae in the literature (Table 1), significant efforts have been made recently to determined new k_P factors. Values of 4.67 and 5.75 have been determined for BSF larvae whereas values of 5.09 and 5.25 (*Acheta domesticus*), 4.86 (*Alphitobius diaperinus*), 5.00 (*Gryllus bimaculatus*), 5.33 (*Locusta migratoria*), and 4.75 – 5.41 (*Tenebrio molitor*) have been reported in the recent literature as well (2,13,26,38). Although these reported k_P values of BSF larvae are significantly better for estimating the protein content than 6.25, both values were calculated based on only one sample while previous research has already proven that the rearing substrate and larval instar significantly influence the chemical composition of BSF larvae (3,7,8). Ravi and colleagues recently stated in their publication that the obtained k_P values can be case-specific and that,

“More data for the selection of an ideal k_P value or k_P values for BSF larvae, pre-pupae, pupae and adult protein fractions solid or liquid would be necessary to determine the true protein and chitin content” (26).

In order to overcome these difficulties and uncertainties reported in the literature and to clarify what has been bugging researchers working on insect protein analysis, the focus of the present study was threefold. Firstly, the nitrogen content of BSF larvae obtained via the Kjeldahl and Dumas method were compared with each other to assess their ratio, correlation, and interchangeability. Secondly, amino acid analyses were performed on BSF larvae in order to calculate a robust k_P factor. Lastly, the chitin content of BSF larvae was accurately analysed with UPLC-MS after acidic hydrolysis in order to gain insight in the nitrogen distribution of BSF larvae. For this purpose, nine BSF larvae samples reared on different substrates and harvested at different larval instars were used to include as much variation in chemical composition as possible and to gain insight into the variation of the k_P factor for black soldier fly larvae. Using the results of the Kjeldahl method, the amino acid and chitin analysis; the nitrogen distribution in the BSF larvae was assessed as well.

2. Materials and methods

2.1. Chemicals and consumables

Petroleum ether (bp. 40 – 60 °C, technical), H₂SO₄ (95 %, technical), K₂SO₄, CuSO₄.5H₂O, NaOH, and H₃BO₃ were all supplied by VWR Chemicals. Tin pressed capsules (8 mm x 5 mm, standard clean) and all reagents required for the Dumas method were purchased from OEA Laboratories Ltd. Helium was supplied by Nippon Gases. The analytical standard of acetanilide was provided by Thermo Fisher. Chitin (practical grade), HCl (37.0 %), 9-fluorenylmethyl chloroformate (FMOC-Cl), D-glucosamine hydrochloride (analytical standard), and amino acid standards were purchased from Sigma Aldrich. Syringe filters (Chromafil PET 0.20 µm) and Soxhlet thimbles were supplied by Macherey-Nagel. ULC-MS grade acetonitrile and formic

acid were bought from Biosolve. The derivatisation reagent for amino acid analyses (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, AQC) was supplied by Waters Co. Ultrapure water was obtained through the Arium Pro® UV from Sartorius. All reagents were of analytical grade unless specified otherwise.

2.2. *Insect samples*

For the purpose of this study, nine black soldier fly (BSF) larvae samples from different instars and reared on different substrates were used in order to obtain a broad range of nitrogen and lipid contents. Coding of the samples was performed as follows: “BSF X-Y”, in which a different X-value denotes a different rearing substrate and the Y-value denotes time after hatching expressed in days. For the first seven days, all larvae were reared on chicken feed (BSF 1-7), a typically used starter diet for BSF larvae (39). Subsequently, the different rearing substrates or mixtures thereof were added to the rearing crates. Prior to this addition, larvae were sampled as well (i.e., BSF 1-7) to obtain a broad range in rearing substrates and larval instars. The different rearing substrates included: apple waste (BSF 2-Y), brewers’ spent grains (BSF 4-Y), chicken feed (BSF 5-21, BSF 6-21, BSF 7-21), distillers’ dried grains with solubles (DDGS, BSF 2-Y), fermented potato peels (BSF 3-14), fruit and vegetable waste (BSF 4-Y, BSF 5-21), pasta (BSF 4-Y), poultry blood (BSF 6-Y), poultry manure (BSF 7-Y), and wheat flour (BSF 3-14). Sufficient water was added to the rearing substrates as well in order to reach a suitable moisture content (i.e., around 70 %). For example, the larvae of BSF 2-14 and BSF 2-21 were reared on the same substrate (i.e., a mixture of apple waste, DDGS, and water) but harvested 14 and 21 days after hatching, respectively. All larvae samples were washed with running tap water immediately after sampling in order to remove any adhering substrate. After letting the water drain from the larvae in a sieve (1 mm mesh), the BSF larvae samples were inactivated by freezing at -20 °C in thin layers, after which they were ground cryogenically, freeze dried for 48 hours (Büchi, Lyovapor L-200), and homogenised thoroughly.

2.3. Proximate composition

The lipid content of the larvae samples (w_{lipids}) was analysed according to Soxhlet with petroleum ether as described previously (5). After the lipid extraction was complete, the thimbles were emptied on watch glasses and spread out to let excess petroleum ether evaporate overnight. The resulting defatted BSF larvae powder was used for further analyses. The proximate composition of both the full-fat samples and the resulting defatted larvae samples were determined. Dry matter content was determined by drying for 17 hours in a forced-air oven (UFB500, Memmert) at 105 °C (5). The nitrogen content of the samples was determined via the Kjeldahl ($w_{\text{N, Kjeldahl}}$) and the Dumas method ($w_{\text{N, Dumas}}$). For the Kjeldahl method, around 0.7000 g or 0.5000 g of sample was analysed as described in our previous work (6), for the full-fat and defatted samples respectively. The method according to Dumas was adopted from the literature with some modifications (40). To this end, 10 or 7 mg of sample was weighed to the nearest 0.01 mg in tin pressed capsules using an analytical balance (R160P, Sartorius), for the full-fat and defatted samples respectively. The capsules were carefully closed and combusted in the Flash 2000 Organic Elemental Analyzer (Interscience). The combustion tube was maintained at 950 °C and contained platinised alumina pellets and fine copper oxide wires (0.5 mm x 4 mm). The reduction oven containing copper wires (0.5 mm x 4 mm) was kept at 840 °C. A water scrubbing tube, containing magnesium perchlorate flakes, was located between the reduction oven and the thermal conductivity detector. Helium was used as carrier gas at a flowrate of 250 mL/min. Total runtime of the analysis was set at 450 seconds. Calibration curves were established using repeated runs containing different masses of an analytical standard of acetanilide and blank runs.

2.4. Amino acid content

The amino acid content of the larvae samples was determined via UPLC-MS after acidic and basic hydrolysis (41). Briefly, 25.00 mg of defatted sample was weighed into a clean test tube

(20 mL, borosilicate glass) with screw cap. For the acidic hydrolysis, 10.0 mL of 6.0 M HCl (Sigma Aldrich) was added and the tube was flushed with helium gas to minimise amino acid oxidation. In order to determine tryptophan, a basic hydrolysis with 4.0 mL of 4.0 M LiOH was performed as well. After thoroughly mixing the samples, they were hydrolysed at 110 °C for 22 hours. Subsequently, the samples were cooled under running tap water before the pH was adjusted to 7.5 ± 0.2 with 33 % NaOH or 6.0 M HCl. After pH adjustments, the samples were filtered through a 0.45 µm syringe filter prior to derivatisation. A series of calibration solutions were prepared from analytical standards to prepare calibration curves for the quantification of the samples. As asparagine (Asn) and glutamine (Gln) are deamidated during the acidic hydrolysis, they could not be quantified individually. Instead, they were quantified together with glutamic acid (Glu) and aspartic acid (Asp), respectively. The derivatisation procedure with AQC consisted of mixing 10 µL of sample or standard with 70 µL of borate buffer (0.08 M, pH 8.80) and 20 µL of AQC (18.3 mM in acetonitrile), followed by a heating step at 55 °C for 10 minutes. The derivatised amino acids (injection volume of 1 µL) were separated and quantified using a Acquity H-Class System (Waters Co.) equipped with an Acquity QDa Detector (Waters Co.). The separation was achieved on a Cortecs[®] UPLC C18 column (1.6 µm, 2.1 mm x 150 mm, Waters Co.) which was kept at 55.0 ± 0.5 °C. The mobile phase consisted of 0.1 % formic acid in ultrapure water (A) and 0.1 % formic acid in acetonitrile (B) and was delivered at a flowrate of 0.500 mL/min. Gradient elution was as follows: 1 % B at 0-1 min, 1-13 % B at 1-4 min, 13-15 % B at 4-8 min, 15-95 % B at 8-9 min, 95 % B at 9-10 min, 95-1 % B at 10-10.1 min, and then equilibration with 1 % B until 12 minutes. The QDa Detector was operated in electrospray ionisation (ESI) positive mode with the ESI-probe maintained at 600 °C. The capillary and cone voltages of the QDa Detector were kept at 0.8 kV and 10 V, respectively. All data points were acquired in selection ion recording (SIM) mode at the $[M+H]^+$ masses of the derivatised amino acids using acquisition rate of 8 Hz. The Empower 3 software

was used for the calibration and quantification, after which the amino acid content was calculated.

2.5. Chitin content

The chitin content of the BSF samples was determined after acidic hydrolysis into glucosamine as described in our previous work (12). In brief, 20.00 mg of defatted BSF larvae samples were hydrolysed with 10.0 mL of 8.0 M HCl at 100 °C for 2 hours. Following the hydrolysis of chitin into glucosamine, complete derivatisation of the latter was achieved with 9-fluorenylmethyl chloroformate (FMOC-Cl) through applying a sonication step of 30 minutes at 40 °C. The derivatised glucosamine was separated on an Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm x 50 mm, Waters Co.) which was kept at 45.0 ± 0.5 °C throughout the analysis. Calibration was performed by making appropriate dilutions of an analytical standard of D-glucosamine hydrochloride in ultrapure water. The chitin content of the larvae samples was calculated as the mass fraction of *N*-acetylglucosamine (w_{GlcNAc}), assuming a degree of acetylation of 100 %, as described in previous studies (12,42).

2.6. Calculations and statistical analysis

All mass fractions calculated in the present study are expressed on dry matter basis unless explicitly stated otherwise. In order to compare the nitrogen content determined via Kjeldahl ($w_{\text{N, Kjeldahl}}$) to the nitrogen content obtained via Dumas ($w_{\text{N, Dumas}}$), their ratio was calculated for each sample by dividing their mean values. The standard deviation of this quotient was calculated according to Eq. 1 (43).

$$s_{w_{\text{N, Kjeldahl}}/w_{\text{N, Dumas}}} = \frac{\overline{w_{\text{N, Kjeldahl}}}}{\overline{w_{\text{N, Dumas}}}} \times \sqrt{\left(\frac{s_{w_{\text{N, Kjeldahl}}}}{\overline{w_{\text{N, Kjeldahl}}}}\right)^2 + \left(\frac{s_{w_{\text{N, Dumas}}}}{\overline{w_{\text{N, Dumas}}}}\right)^2} \quad (\text{Eq. 1})$$

The true protein content of the larvae samples ($w_{\text{true protein}}$) was calculated as the sum of the anhydrous amino acid residues (Eq. 2) as suggested in the literature (2,44). Amino acid profiles

of the samples were calculated as ratio of each individual anhydrous amino acid residue to the true protein content to allow better comparison between samples. The k_P factors were calculated as the ratio of the total weights of the anhydrous amino acid residues to $w_{N, \text{Kjeldahl}}$ (Eq. 3), identically to previously published studies regarding k_P values (2,36,44). The use of anhydrous residues is preferred for this calculation as the associated error is only minor considering that the amount of end groups is substantially lower than the total number of amino acid residues in proteins (44). On the other hand, not using the anhydrous residues can result in an overestimation of the true protein content. As already noted before, glutamine and asparagine were not quantified individually but together with their acidic counterparts (i.e., Glu and Asp). As already shown in a previous study, this did not influence the calculation of the k_P factor (13).

$$w_{\text{true protein}} = \sum_{i=1}^{18} \left[w_{AA,i} \times \frac{M_{AA,i} - M_{H_2O}}{M_{AA,i}} \right] \quad (\text{Eq. 2})$$

$$k_P = \sum_{i=1}^{18} \left[w_{AA,i} \times \frac{M_{AA,i} - M_{H_2O}}{M_{AA,i}} \right] \times \frac{1}{w_{N, \text{Kjeldahl}}} \quad (\text{Eq. 3})$$

Concerning the calculations of the nitrogen distribution in the BSF samples, the mass fraction of nitrogen originating from chitin ($w_{N, \text{chitin}}$) was calculated from the results of the chitin analysis using the molecular weight of *N*-acetylglucosamine (M_{GlcNAc}) (Eq. 4). This was performed similarly for the mass fraction of nitrogen derived from proteins ($w_{N, \text{protein}}$) by taking the sum of the nitrogen contents originating from each individual amino acid as reported previously (Eq. 5) (13). In this equation, $n_{N,i}$ signifies the amount of nitrogen atoms of the i th amino acid. Since Asn and Gln were deamidated during the acidic hydrolysis, an accurate calculation of $w_{N, \text{protein}}$ was not possible. Instead, three conditions were calculated to gain insight into the possible variation of $w_{N, \text{protein}}$, assuming an amidation degree (AD) of 0 %, 50 %, and 100 %.

50 %, and 100 %. The AD value of 50 % (i.e., equimolar ratios of Asn/Asp and Gln/Glu) was included since recent work on larval proteins of the yellow mealworm (*Tenebrio molitor*) has shown a similar AD (2). The contribution of nitrogen sources other than chitin and proteins was then calculated as $w_{N, \text{ other}}$ by means of Eq. 6. The standard deviation of this subtraction was calculated using Eq. 7 as reported in the literature (43).

$$w_{N, \text{ chitin}} = \frac{w_{\text{GlcNAc}} \times M_N}{M_{\text{GlcNAc}}} \quad (\text{Eq. 4})$$

$$w_{N, \text{ protein}} = \sum_{i=1}^{18} \left[\frac{w_{AA,i} \times n_{N,i} \times M_N}{M_{AA,i}} \right] \quad (\text{Eq. 5})$$

$$w_{N, \text{ other}} = w_{N, \text{ Kjeldahl}} - w_{N, \text{ protein (50\% amidation)}} - w_{N, \text{ chitin}} \quad (\text{Eq. 6})$$

$$s_{N, \text{ other}} = \sqrt{s_{N, \text{ Kjeldahl}}^2 + s_{N, \text{ protein (50\% amidation)}}^2 + s_{N, \text{ chitin}}^2} \quad (\text{Eq. 7})$$

Unless stated otherwise, all measurements were performed in triplicate. The statistical software used for the analysis was JMP Pro 15.1.0 (SAS Institute). All statistical evaluations were carried out at a significance level of 5 %. Prior to the evaluation with ANOVA and Tukey HSD post hoc test, the equality of variances and normal distribution of the data were checked using the Levene's test and Shapiro-Wilk test, respectively. The Welch's ANOVA alongside with the Steel-Dwass All Pairs post hoc test was used in case of unequal variances or a non-normal distribution of the data. The comparison of the nitrogen contents obtained via the Kjeldahl and Dumas method was performed using regression analysis. For this purpose, both 1/X weighted least squares (WLS) and 1/X weighted Deming regression were performed.

3. Results and discussion

3.1. Proximate composition

The proximate composition of the freeze-dried full-fat and defatted BSF samples is shown in Table 2. Concerning the lipid and nitrogen content of the BSF samples, several differences were observed thanks to the broad range of substrates used during the rearing process and larval instars. This range in nitrogen content (5.8 – 8.9 %) made it worthwhile to compare the results from the Kjeldahl analysis with those of the Dumas analysis (see section 3.2) instead of comparing just one sample or samples with similar nitrogen contents. With the exception of BSF 2-14, BSF 2-21, and BSF 6-21; high lipid contents were obtained for older larvae. This increase in lipid content during larval development was also observed in previous research (8). The obtained results were also in good agreement with previous published data listed in a review article (3), except for BSF 1-7. As these larvae were harvested 7 days after hatching, a low lipid content and a high protein content was expected. Instead, a relatively high lipid content of 43 % and a lower nitrogen content than the other samples was obtained. While it has already been proven that the rearing substrate has a significant influence on the protein and lipid content of BSF larvae (3,7), these larvae were reared on chicken feed similarly to the research by Liu and colleagues (8). This unexpected composition could therefore not be attributed to the rearing substrate and more research regarding its cause is therefore in order from a scientific point of view. From the practical point of view of industrial insect rearers, however, only the nutritional composition at the time of harvest is important.

Table 2 Proximate composition of the full-fat and defatted black soldier fly larvae samples (g/100 g dry matter) and their ratios of the nitrogen contents obtained by the Kjeldahl method to those obtained by the Dumas method. Results are shown as the mean of three replicates $\pm$ standard deviation. Different superscripts in the same column indicate a significant difference ($p < 0.05$).

	Full-fat				Defatted		
	W_{lipids}	$W_{\text{N, Kjeldahl}}$	$W_{\text{N, Dumas}}$	$\frac{W_{\text{N, Kjeldahl}}}{W_{\text{N, Dumas}}}$	$W_{\text{N, Kjeldahl}}$	$W_{\text{N, Dumas}}$	$\frac{W_{\text{N, Kjeldahl}}}{W_{\text{N, Dumas}}}$
BSF 1-7	43.32 ± 0.20^a	6.25 ± 0.03^a	6.19 ± 0.09^a	1.01 ± 0.02	10.51 ± 0.06^a	10.34 ± 0.21^a	1.02 ± 0.02
BSF 2-14	28.10 ± 0.13^b	8.26 ± 0.03^b	8.32 ± 0.19^b	0.99 ± 0.02	11.39 ± 0.12^b	11.71 ± 0.20^b	0.97 ± 0.02
BSF 2-21	28.75 ± 0.02^b	7.71 ± 0.01^c	7.83 ± 0.22^b	0.97 ± 0.02	10.49 ± 0.03^a	10.54 ± 0.31^a	1.00 ± 0.03
BSF 3-14	35.22 ± 0.06^c	6.98 ± 0.05^d	6.71 ± 0.09^c	1.04 ± 0.02	10.28 ± 0.06^c	10.48 ± 0.19^a	0.98 ± 0.02
BSF 4-14	42.84 ± 0.12^a	5.77 ± 0.01^e	5.76 ± 0.02^d	1.00 ± 0.04	9.69 ± 0.10^d	10.07 ± 0.11^a	0.96 ± 0.01
BSF 4-21	42.72 ± 0.37^a	6.66 ± 0.03^f	6.76 ± 0.19^{ce}	0.99 ± 0.03	10.77 ± 0.03^e	10.63 ± 0.16^a	1.01 ± 0.02
BSF 5-21	39.65 ± 3.99^{ad}	7.09 ± 0.02^g	7.17 ± 0.16^{ef}	0.99 ± 0.02	10.05 ± 0.01^f	10.15 ± 0.08^a	0.99 ± 0.01
BSF 6-21	21.62 ± 1.07^e	7.17 ± 0.03^h	7.33 ± 0.17^f	0.98 ± 0.01	8.77 ± 0.09^g	8.78 ± 0.33^c	1.00 ± 0.04
BSF 7-21	37.51 ± 0.49^{cd}	7.76 ± 0.02^c	7.95 ± 0.04^b	0.98 ± 0.01	10.50 ± 0.01^a	10.51 ± 0.52^a	1.00 ± 0.05

3.2. Comparison of the nitrogen contents obtained by the Kjeldahl and Dumas method

When comparing the nitrogen content obtained via Kjeldahl ($W_{\text{N, Kjeldahl}}$) to the nitrogen content obtained through Dumas ($W_{\text{N, Dumas}}$), it was observed that they were highly similar for both the full-fat and defatted samples (Table 2). Statistical analysis of the data confirmed this as no significant differences were found between $W_{\text{N, Kjeldahl}}$ and $W_{\text{N, Dumas}}$ for both the full-fat and defatted samples. When inorganic nitrogen would be present in the BSF larvae samples as a result of the rearing substrate (e.g., nitrate), the value of $W_{\text{N, Dumas}}$ would always have been higher than $W_{\text{N, Kjeldahl}}$. Such trend was, however, not observed in the current dataset. Another noteworthy observation was that the standard deviation of $W_{\text{N, Dumas}}$ was always higher in comparison to $W_{\text{N, Kjeldahl}}$. This may have been the result of the much smaller sample size (7 – 10 mg vs. 500 – 700 mg) required for the Dumas method. Despite best efforts of grinding the BSF larvae as fine as possible and homogenising them thoroughly, they might not have been entirely homogenous. In addition, higher standard deviations were also obtained for the defatted samples, which was likely due to the defatted samples being electrostatically charged

which complicated the weighing step. Nevertheless, all standard deviations could still be considered acceptably low as the coefficient of variation was always lower than 5 %.

Looking at the ratios of $w_{N, \text{Kjeldahl}}$ and $w_{N, \text{Dumas}}$ in Table 2, it could be observed that they were almost equal to 1 for both the full-fat and defatted samples, indicating that the Kjeldahl and Dumas method may be interchanged for BSF larvae. In addition, the mean values of $w_{N, \text{Kjeldahl}}$ and $w_{N, \text{Dumas}}$ were also plotted against each other for all larvae samples to visualise their relationship and the scattering of the data points (Fig. 1). Regarding the regression analysis of the resulting dataset, several statistical approaches and assumptions were investigated prior to performing the actual analysis. Since the Levene's test showed the presence of heteroscedasticity in the values of $w_{N, \text{Kjeldahl}}$ and $w_{N, \text{Dumas}}$, it was opted to use 1/X weighted least squares (WLS) over ordinary least squares (OLS) to minimise the impact of this heteroscedasticity (45). In addition, while (weighted) Deming regression analysis might be more fit-for-purpose than OLS or WLS because of the presence of random errors in both the results of the Kjeldahl and Dumas method (31); OLS is found most-often in the literature on comparing the Kjeldahl and Dumas method (33,35,46).

Fig. 1 Linear relationship between the mass fractions of nitrogen (w_N) obtained by the Kjeldahl and Dumas method on the full-fat (blue) and defatted (orange) black soldier fly larvae samples. Data points are shown as the mean of three replicate analyses with their standard deviation depicted as error bars

For the purpose of this study, both weighted Deming regression and WLS were performed. A final assumption that was tested prior to the actual regression analysis was to confirm whether the results of the full-fat and defatted samples (i.e., respectively blue and orange data points in Fig. 1) were indeed independent from each other so they could be combined for the regression analysis. The reasoning for this assumption was based on the fact that different samples had different lipid contents, which varied significantly as shown in Table 2. Advanced regression analyses with JMP, which took into account these paired observations, showed no statistically significant effects; meaning that the full-fat and defatted samples were independent from each other, thus allowing the whole dataset (i.e., 18 combinations of Kjeldahl and Dumas results) to be used for the regression analysis.

Resulting analysis of the data confirmed the presence of a linear relationship between $w_{N, \text{Kjeldahl}}$ and $w_{N, \text{Dumas}}$. Not only was this linear relationship deemed highly significant ($p < 0.0001$), the obtained correlation coefficient ($R^2 = 0.9974$) was very high as well. The slope and intercept of this correlation were found to be 1.009 and -0.008 , respectively; which again confirmed that for the given range (5.8 – 11.4 %), the Kjeldahl and Dumas method can be interchanged for the determination of the nitrogen content of BSF larvae. No differences were observed between the results of WLS and the weighted Deming regression.

In conclusion, the Kjeldahl and Dumas method were found to be equally suitable for determining the nitrogen content of BSF larvae samples, meaning they can be interchanged. Considering that $w_{N, \text{Dumas}}$ was only in few cases higher than $w_{N, \text{Kjeldahl}}$, the influence of inorganic nitrogen was deemed negligible and using the Dumas method instead of the Kjeldahl method will therefore likely not result in an overestimation of the protein content. Considering that the presence of lipids had no influence, it is suggested to perform the Dumas analyses on full-fat samples because of practical considerations and lower standard deviations. Should the Dumas method be applied on inhomogeneous samples, it is also suggested to perform five

replicates for each sample to identify possible outliers as suggested by the Association of Official Agricultural Chemists (AOAC). In addition to being faster, the Dumas method was also preferred over the Kjeldahl method because of safety and environmental concerns. Even though the Dumas method requires a higher initial investment cost because of the equipment, it must also be noted that this method is less labour-intensive and will therefore save on personnel costs.

Although both methods are already used extensively in the literature on black soldier fly larvae (6–8,13), much uncertainty existed whether the obtained nitrogen contents were comparable between procedures. To the authors' knowledge this is the first time that such comparison has been made for insect samples, clarifying this ambiguity. The results of this work therefore not only allow for the comparison of the nitrogen contents of insects already described in the literature and for future analyses; it also allows for research groups working on insects to exchange their labour-intensive Kjeldahl method for the much faster and safer Dumas method.

3.3. Protein conversion factor

Resulting from the amino acid analyses, amino acid profiles were calculated for a better comparison between the BSF samples. These profiles as well as the true protein content of the samples ($w_{\text{true protein}}$) are shown in Table 3. For all nine insect samples, high quantities of Asx (i.e., sum of asparagine and aspartic acid) and Glx (i.e., sum of glutamine and glutamic acid) were found while the sulphur-containing amino acids and tryptophan were detected in lower concentrations. These findings were highly similar to previously published amino acid profiles of BSF larvae (6,13). Although statistically significant differences were observed in the amino acid profiles, the majority of these differences was rather small. The largest differences were observed for histidine, alanine, and tyrosine, between BSF 2-14, BSF 3-14, and BSF 4-14. Considering that these larvae were all harvested 14 days after hatching, these differences probably originated from a different rearing substrate.

393 Although generally only minor differences were observed in the amino acid profiles, much
 394 larger differences were observed in $w_{\text{true protein}}$ of the nine BSF larvae samples with values
 395 ranging from 23.26 to 36.71 g/100 g dry matter. The significantly lower amount in BSF 1-7
 396 was in contrast with a previous study which has demonstrated that a high level of amino acids
 397 are found in the early larval stages and that these levels decrease towards the prepupal stage
 398 while the lipid content increases (8).

399 Table 3 Amino acid profiles (g/100 g true protein) and true protein content ($w_{\text{true protein}}$) (g/100 g dry matter of the
 400 full-fat samples) of the black soldier fly larvae samples, both calculated based on anhydrous amino acid residues.
 401 Results (listed in order of elution) are shown as the mean of three replicates $\pm$ standard deviation. Different
 402 superscripts in the same row indicate a significant difference ($p < 0.05$).

	BSF 1-7	BSF 2-14	BSF 2-21	BSF 3-14	BSF 4-14	BSF 4-21	BSF 5-21	BSF 6-21	BSF 7-21
His	3.62 $\pm$ 0.04 ^c	2.96 $\pm$ 0.11 ^d	4.51 $\pm$ 0.09 ^a	4.39 $\pm$ 0.10 ^{ab}	2.14 $\pm$ 0.23 ^f	2.62 $\pm$ 0.05 ^{de}	3.97 $\pm$ 0.13 ^{bc}	2.34 $\pm$ 0.08 ^{ef}	3.91 $\pm$ 0.08 ^c
Arg	5.90 $\pm$ 0.09 ^{bc}	6.00 $\pm$ 0.04 ^{bc}	6.70 $\pm$ 0.35 ^a	6.91 $\pm$ 0.01 ^a	5.72 $\pm$ 0.25 ^c	5.91 $\pm$ 0.06 ^{bc}	7.04 $\pm$ 0.19 ^a	5.47 $\pm$ 0.09 ^c	6.45 $\pm$ 0.04 ^{ab}
Ser	4.20 $\pm$ 0.00 ^b	3.98 $\pm$ 0.04 ^c	4.47 $\pm$ 0.01 ^a	4.46 $\pm$ 0.06 ^a	4.59 $\pm$ 0.02 ^a	4.53 $\pm$ 0.03 ^a	4.49 $\pm$ 0.08 ^a	4.10 $\pm$ 0.00 ^{bc}	4.53 $\pm$ 0.00 ^a
Gly	4.50 $\pm$ 0.25 ^{bc}	4.48 $\pm$ 0.01 ^{bc}	4.16 $\pm$ 0.11 ^c	4.63 $\pm$ 0.09 ^{ab}	4.90 $\pm$ 0.01 ^{ab}	4.74 $\pm$ 0.02 ^{ab}	4.79 $\pm$ 0.07 ^{ab}	4.94 $\pm$ 0.08 ^{ab}	5.00 $\pm$ 0.16 ^a
Asx	9.45 $\pm$ 0.11 ^{bc}	10.84 $\pm$ 0.32 ^a	9.46 $\pm$ 0.51 ^{bc}	8.90 $\pm$ 0.10 ^c	9.09 $\pm$ 0.06 ^c	9.98 $\pm$ 0.41 ^{abc}	8.90 $\pm$ 0.16 ^c	10.38 $\pm$ 0.30 ^{ab}	8.90 $\pm$ 0.45 ^c
Glx	12.37 $\pm$ 0.15 ^b	11.46 $\pm$ 0.18 ^{cde}	12.34 $\pm$ 0.04 ^b	12.07 $\pm$ 0.08 ^{bc}	14.49 $\pm$ 0.03 ^a	11.95 $\pm$ 0.12 ^{bc}	11.62 $\pm$ 0.02 ^{bcd}	10.82 $\pm$ 0.12 ^e	11.18 $\pm$ 0.49 ^{de}
Thr	4.33 $\pm$ 0.12 ^{ab}	4.34 $\pm$ 0.07 ^{ab}	4.16 $\pm$ 0.00 ^b	4.27 $\pm$ 0.05 ^{a,b}	4.39 $\pm$ 0.09 ^{ab}	4.32 $\pm$ 0.06 ^{ab}	4.52 $\pm$ 0.04 ^a	4.36 $\pm$ 0.11 ^{ab}	4.41 $\pm$ 0.05 ^{ab}
Ala	6.21 $\pm$ 0.23 ^b	5.18 $\pm$ 0.31 ^c	6.22 $\pm$ 0.04 ^b	6.03 $\pm$ 0.14 ^b	7.69 $\pm$ 0.08 ^a	6.17 $\pm$ 0.13 ^b	6.68 $\pm$ 0.23 ^b	6.57 $\pm$ 0.04 ^b	7.39 $\pm$ 0.13 ^a
Pro	7.17 $\pm$ 0.26 ^{ab}	5.91 $\pm$ 0.14 ^d	6.87 $\pm$ 0.26 ^{abc}	7.23 $\pm$ 0.03 ^{ab}	7.66 $\pm$ 0.11 ^a	7.03 $\pm$ 0.22 ^{abc}	6.73 $\pm$ 0.03 ^{bc}	6.40 $\pm$ 0.02 ^{cd}	7.11 $\pm$ 0.35 ^{abc}
Lys	7.45 $\pm$ 0.25 ^{ab}	8.00 $\pm$ 0.08 ^a	7.07 $\pm$ 0.58 ^{ab}	6.77 $\pm$ 0.02 ^b	6.92 $\pm$ 0.29 ^b	7.49 $\pm$ 0.24 ^{ab}	6.57 $\pm$ 0.01 ^b	7.24 $\pm$ 0.03 ^{ab}	6.81 $\pm$ 0.27 ^b
Cys + cyss	0.90 $\pm$ 0.02 ^a	0.57 $\pm$ 0.02 ^{cd}	0.83 $\pm$ 0.02 ^{ab}	0.88 $\pm$ 0.01 ^{ab}	0.64 $\pm$ 0.05 ^c	0.54 $\pm$ 0.04 ^{cd}	0.77 $\pm$ 0.02 ^b	0.47 $\pm$ 0.03 ^d	0.49 $\pm$ 0.04 ^d
Tyr	7.36 $\pm$ 0.24 ^{de}	9.17 $\pm$ 0.04 ^a	8.34 $\pm$ 0.37 ^{abc}	8.09 $\pm$ 0.01 ^{bcd}	6.93 $\pm$ 0.06 ^e	8.34 $\pm$ 0.09 ^{abc}	7.62 $\pm$ 0.31 ^{cde}	8.84 $\pm$ 0.35 ^{ab}	8.04 $\pm$ 0.13 ^{bcd}
Met	1.75 $\pm$ 0.01 ^b	2.13 $\pm$ 0.16 ^a	1.63 $\pm$ 0.07 ^{bc}	1.62 $\pm$ 0.05 ^{bcd}	1.25 $\pm$ 0.05 ^e	1.32 $\pm$ 0.07 ^{d,e}	1.75 $\pm$ 0.09 ^b	1.42 $\pm$ 0.05 ^{cde}	1.35 $\pm$ 0.05 ^{cde}
Val	6.19 $\pm$ 0.09 ^{bcd}	5.98 $\pm$ 0.07 ^d	6.01 $\pm$ 0.03 ^d	6.03 $\pm$ 0.01 ^d	6.16 $\pm$ 0.01 ^{cd}	6.55 $\pm$ 0.12 ^{ab}	6.10 $\pm$ 0.15 ^d	6.68 $\pm$ 0.06 ^a	6.49 $\pm$ 0.15 ^{abc}
Ile	4.74 $\pm$ 0.06 ^{bc}	4.81 $\pm$ 0.10 ^{ab}	4.43 $\pm$ 0.04 ^d	4.57 $\pm$ 0.07 ^{cd}	4.74 $\pm$ 0.04 ^{bc}	4.91 $\pm$ 0.07 ^{ab}	4.67 $\pm$ 0.01 ^{bcd}	5.00 $\pm$ 0.05 ^a	4.74 $\pm$ 0.05 ^{bc}
Leu	7.38 $\pm$ 0.06 ^{bc}	7.51 $\pm$ 0.15 ^{ab}	6.93 $\pm$ 0.00 ^d	6.94 $\pm$ 0.07 ^d	7.25 $\pm$ 0.04 ^{bcd}	7.55 $\pm$ 0.13 ^{ab}	7.19 $\pm$ 0.00 ^{cd}	7.77 $\pm$ 0.09 ^a	7.20 $\pm$ 0.02 ^{cd}
Phe	4.99 $\pm$ 0.22 ^b	5.39 $\pm$ 0.21 ^{ab}	4.96 $\pm$ 0.10 ^b	4.98 $\pm$ 0.10 ^b	4.22 $\pm$ 0.06 ^c	5.03 $\pm$ 0.01 ^b	5.10 $\pm$ 0.07 ^{ab}	5.50 $\pm$ 0.01 ^a	4.96 $\pm$ 0.01 ^b
Trp	1.48 $\pm$ 0.21 ^{a,b}	1.29 $\pm$ 0.27 ^{ab}	0.95 $\pm$ 0.18 ^b	1.23 $\pm$ 0.12 ^{ab}	1.21 $\pm$ 0.08 ^{ab}	1.02 $\pm$ 0.06 ^{ab}	1.49 $\pm$ 0.29 ^{ab}	1.73 $\pm$ 0.16 ^a	1.06 $\pm$ 0.19 ^{ab}
$w_{\text{true protein}}$	23.26 $\pm$ 1.16 ^f	36.68 $\pm$ 1.31 ^a	36.71 $\pm$ 0.58 ^a	30.91 $\pm$ 0.80 ^{cd}	26.68 $\pm$ 0.10 ^e	29.73 $\pm$ 0.12 ^{cd}	28.22 $\pm$ 0.77 ^{d,e}	31.34 $\pm$ 0.36 ^{bc}	33.98 $\pm$ 0.55 ^{ab}
k_p factor	3.72 $\pm$ 0.19 ^c	4.44 $\pm$ 0.18 ^a	4.76 $\pm$ 0.07 ^a	4.43 $\pm$ 0.11 ^a	4.62 $\pm$ 0.02 ^a	4.46 $\pm$ 0.02 ^a	3.98 $\pm$ 0.11 ^{b,c}	4.37 $\pm$ 0.05 ^{ab}	4.38 $\pm$ 0.07 ^{ab}

Asx, sum of asparagine and aspartic acid; Glx, sum of glutamine and glutamic acid;

Although research regarding the k_P factor has already been performed for several insect species (2,13,38), the dependence of larval instar and rearing substrate was not addressed until now. Using the results of the amino acid analyses, k_P factors have been calculated for the nine BSF larvae samples (Table 3). It was clear that these new k_P values were significantly lower than the typically used conversion factor of 6.25. This was in agreement with previous research stating that the protein content in insects would be overestimated by using 6.25 as conversion factor (11). The results in Table 3 did however show a dependence of instar and rearing substrate similarly to the true protein content. The mean of all k_P factors obtained in this study was 4.35 ± 0.32 , which was in line with the previously reported value of 4.67 for BSF larvae (13) though lower than the value of 5.75 reported by Ravi and colleagues (26). Their higher value may only be partially explained through their k_P value not being calculated using anhydrous amino acid residues (AAR). As stated by Boulos and colleagues (2), the k_P factor can be calculated from data in the literature provided that sufficient data are available. More often than not, amino acid profiles reported in the literature are not based on anhydrous amino acid residues. As such, previously published amino acid profiles were first corrected to their anhydrous residues, prior to the calculation of their respective k_P factors. The resulting k_P factors fell in the range reported in the present study with values of 4.02 for larvae (13) and 4.32 – 4.43 for prepupae (7). The k_P factors of our own previous work regarding larvae, prepupae, and pupae were highly similar to each other (i.e. 4.02 – 4.04) and again fell into the range of the present study (6). The k_P factor of Ravi and colleagues (26) after correction was, however, still higher than the reported range of the present study (i.e. 4.88).

Including the significantly lower k_P value of the young larvae of BSF 1-7 (i.e., 3.72 ± 0.19) in the calculation of the mean is not desired from a practical point of view since insect rearers are most often only interested in the larval protein content at the time of harvest (e.g., 14 – 21 days).

With this value excluded, the practical mean k_P factor was calculated as 4.43 ± 0.22 , which was still in line with previously reported values. Although it was demonstrated that the rearing substrate and larval instar indeed influenced the amino acid composition and k_P values of BSF larvae, using a different k_P value for each substrate is not desirable for obvious reasons. As such, it is proposed to use the newly determined protein conversion factor of 4.43 for future calculations of the protein content of BSF larvae. Using this factor still results in some uncertainty on the true protein content, albeit much lower compared to using 6.25. A more accurate yet also more expensive approach is to use amino acid data to calculate the true protein content. Even though the hydrolysis method during amino acid analysis can still be improved, it is the only protein analysis method free of interference due to the presence of other compounds (34).

3.4. Chitin content

Instead of a gravimetric procedure, the chitin content of the nine different BSF larvae samples was determined chromatographically after acidic hydrolysis of the polymer. During the latter, chitin is depolymerised and deacetylated completely as described in our previous work (12). The resulting glucosamine was quantified through ultra-performance liquid chromatography and the results of this analysis are expressed as the mass fraction of *N*-acetylglucosamine (W_{GlcNAc}) of the sample. It is shown in our previous work that this approach is characterised by a high specificity for chitin (12). The results of this analysis (Table 4) showed a rather broad variation between the samples with chitin contents ranging from 4.6 to 7.3 %. The mean value of all samples was found to be 5.95 ± 0.86 %, which was similar to previously published results of BSF larvae despite their chitin analyses being gravimetric procedures (7,47). The relative differences between the current results, however, could not be attributed to a single factor. While the chitin content seemed to increase with larval age; the samples BSF 5-21, BSF 6-21, and BSF 7-21 showed remarkable differences in their chitin contents, even though they were

all harvested 21 days after hatching. It was therefore assumed that the rearing substrate also has a significant impact on the chitin content of BSF larvae samples. Since both the chitin content and k_P factor were shown to be influenced by the rearing substrate and larval instar, regression analysis was performed to assess the relationship between the chitin content and the k_P factor. The results from this regression analysis, however, did not show any correlation with R^2 values of 0.024 (linear), 0.067 (polynomial, degree = 2), and 0.14 (polynomial, degree = 3). Consequently, it was concluded that when either the chitin content or k_P value of a BSF larvae sample is known, it could not be used to estimate the other.

Table 4 Chitin content, expressed as mass fraction *N*-acetylglucosamine (GlcNAc), of the black soldier fly larvae samples (g GlcNAc/100 g dry matter). Results are shown as the mean of three replicates $\pm$ standard deviation. Different superscripts indicate a significant difference ($p < 0.05$).

Sample	w_{GlcNAc}
BSF 1-7	5.88 ± 0.16^{cd}
BSF 2-14	5.90 ± 0.03^{cd}
BSF 2-21	6.18 ± 0.01^{bc}
BSF 3-14	6.99 ± 0.32^{ab}
BSF 4-14	5.03 ± 0.23^e
BSF 4-21	7.33 ± 0.26^a
BSF 5-21	5.31 ± 0.26^{de}
BSF 6-21	4.65 ± 0.28^e
BSF 7-21	6.28 ± 0.08^{bc}

3.5. Nitrogen distribution

While chitin is indeed a source of nitrogen ($w_{\text{N, chitin}}$), the amount of nitrogen only accounts for ± 7 g/100 g of chitin as mentioned previously (12). Yet, the contribution of $w_{\text{N, chitin}}$ to the total nitrogen content, determined either by Kjeldahl or Dumas, is often described in the literature as a large source of overestimation (11,38,42). As can be observed from Fig. 2, this contribution of $w_{\text{N, chitin}}$ in the present study was rather limited as it accounted for only 4 – 7 % of $w_{\text{N, Kjeldahl}}$ (i.e., the ratio of $w_{\text{N, chitin}}$ to $w_{\text{N, Kjeldahl}}$). As mentioned in section 2.6, the nitrogen originating from proteins ($w_{\text{N, protein}}$) was calculated from the amino acid content assuming an amidation

degree (AD) of 0 % and 100 % to gain insight into the variation of $w_{N, \text{protein}}$, and for an AD of 50 % which is similar to *T. molitor* (2). For an AD of 0 % (i.e., only Asp and Glu residues), it was found that $w_{N, \text{protein}}$ ranged from 3.7 to 6.0 g N/100 dry matter for all BSF samples. In this case, $w_{N, \text{protein}}$ was responsible for 60 – 77 % of $w_{N, \text{Kjeldahl}}$. On the other hand, $w_{N, \text{protein}}$ was found to range from 4.3 to 6.9 g N/100 g for an AD of 100 % (i.e., only Asn and Gln residues); which corresponded to a contribution of 69 – 89 % of $w_{N, \text{protein}}$ to $w_{N, \text{Kjeldahl}}$ for all nine BSF larvae samples. The variation in $w_{N, \text{protein}}$ dependent on the AD value, is also visualised in Fig. 2 by means of the red error bars. While the present calculation gave some insight into the variation of $w_{N, \text{protein}}$ for each sample, it is also shown in Fig. 2 that this value differed quite substantially between BSF larvae samples. When comparing the results of the present study with nitrogen distributions of mealworms (*T. molitor*), crickets (*A. domesticus*), and locusts (*L. migratoria*) recently published by Boulos and colleagues (2), a rather large discrepancy was observed. While they found that around 95 % of the total nitrogen could be attributed to proteins, the results of the present study indicated that this contribution was much lower for BSF larvae. Yet, this comparison should be performed with caution considering different insect species were used. While Janssen and colleagues (13) also determined the range in which $w_{N, \text{protein}}$ can vary for BSF larvae (i.e., 74 – 87 % of total nitrogen), they did not elaborate much on this subject in their paper. Nevertheless, their result was much more comparable with those obtained in the present study. Strikingly, Janssen and colleagues determined this range for *T. molitor* as well, which was also considerably lower in comparison to the result of Boulos and colleagues despite it being the same insect species (2,13). Considering the results of the present study showed that a rather large variation on the nitrogen distribution exists for BSF larvae, it was plausible that this is also the cause.

Fig. 2 The distribution of the different mass fractions of nitrogen (w_N) of the nine black soldier fly larvae samples. Blue columns represent the nitrogen content originating from chitin ($w_{N, \text{chitin}}$). Orange columns represent the nitrogen content from proteins with an amidation degree (AD) of 50 % ($w_{N, \text{protein 50\% amidation}}$), corresponding to equimolar ratios of Asn/Asp and Gln/Glu. Grey columns depict the mass fraction of nitrogen, originating from other nitrogenous compounds ($w_{N, \text{other}}$). The sum of the stacked columns indicates the total nitrogen content as determined by Kjeldahl (Table 2). Black error bars represent the standard deviation. Red error bars visualise the uncertainty on $w_{N, \text{protein}}$, showing the range in which $w_{N, \text{protein}}$ can vary for AD values of 0 % to 100 %. Labels display the relative contribution of $w_{N, \text{chitin}}$ (blue), $w_{N, \text{protein with 50\% amidation}}$ (orange), and $w_{N, \text{other}}$ (grey) to the total nitrogen content as determined by Kjeldahl

A very recent study has shown that the AD of larval proteins from *T. molitor* is 50 %, corresponding to equimolar ratios of Asn/Asp and Gln/Glu (2). Using this value as estimate for the AD of BSF larval protein, would mean that the value of $w_{N, \text{protein}}$ varied between 4.0 – 6.4 g N/100 g dry matter for the nine BSF samples. In terms of the relative contribution to $w_{N, \text{Kjeldahl}}$, this would mean that only 64 – 83 % of the total nitrogen content originated from proteins. As such, the remainder (i.e., 12 – 30 %) must have originated from nitrogen sources other than chitin and proteins ($w_{N, \text{other}}$), either organic or inorganic. This rather large variation in $w_{N, \text{other}}$ (Fig. 2) might explain why no correlation was found between k_P and the chitin content. Previously published articles describe ammonia, melanin, nitrates and nitrites, nucleic acids, and uric acid as other possible nitrogen sources in BSF larvae (13,47,48). However, since the present study showed a high similarity between $w_{N, \text{Kjeldahl}}$ and $w_{N, \text{Dumas}}$ (section 3.2), it was presumed that the contribution of (1) inorganic nitrogen sources and (2) heterocyclic nitrogen

sources (e.g., melanin, nucleic acids, and uric acid) to the total nitrogen content was rather limited. As described previously, these sources are not included in $w_{N, \text{Kjeldahl}}$ whereas they are included in $w_{N, \text{Dumas}}$, meaning that much larger differences between these two would have been observed otherwise. In order to gain more insight into the nitrogen distribution of BSF larvae, more research has to be performed regarding these other sources of nitrogen. Moreover, in order to calculate $w_{N, \text{protein}}$ and its contribution to $w_{N, \text{Kjeldahl}}$ more accurately, the amidation degree (AD) of BSF larval protein has to be determined in future work using the mild hydrolysis techniques described in the literature (2,49).

4. Conclusions

While black soldier fly larvae are gaining an increased interest for use in feed or as feedstock for industry, several uncertainties are present regarding their protein analysis. In order to clarify these ambiguities, the Kjeldahl and Dumas method were compared, a new and more robust protein conversion (k_P) factor was established, and the chitin content of BSF larvae was examined in detail to gain insight into the nitrogen distribution of BSF larvae. It was found that no significant differences were found between the results of the Kjeldahl and Dumas method. Moreover, the ratio of the obtained nitrogen contents ($w_{N, \text{Kjeldahl}}/w_{N, \text{Dumas}}$) was almost equal to 1 for all cases and a highly significant linear correlation ($p < 0.0001$, slope: 1.009, intercept: -0.008, R^2 : 0.9974) between these two nitrogen contents was found in the investigated range (5.8 – 11.4 % g/100 g dry matter). All these results showed that the Kjeldahl and Dumas method can be interchanged for determining the nitrogen content of BSF larvae, allowing for a much easier comparison with data in the literature and in future research. The newly determined nitrogen-to-protein conversion (k_P) factor for BSF larvae was found to be 4.43 ± 0.22 . For the first time to the authors' knowledge, it was shown that some degree of variation on this k_P factor exists, depending on the rearing substrate and larval instar. Yet, despite this variation on the k_P factor, using 4.43 will be much more accurate as compared to using 6.25 as conversion factor.

Concerning the chitin content of the BSF larvae samples, values ranging from 5.0 to 7.3 g GlcNAc/100 g dry matter were obtained with a total average of 5.95 ± 0.86 g GlcNAc/100 g dry matter. Despite the fact that the rearing substrate and larval instar had an influence on both the k_p factor and chitin content, no correlation between the latter two was observed. With respect to the nitrogen distribution in the BSF samples, it was shown that chitin was responsible for merely 4 – 7 % of $w_{N, \text{Kjeldahl}}$ (i.e., the ratio of $w_{N, \text{chitin}}$ to $w_{N, \text{Kjeldahl}}$). Considering around 64 – 83 % of $w_{N, \text{Kjeldahl}}$ originated from proteins assuming equimolar ratios of Asn/Asp and Gln/Glu, the contribution of other nitrogenous compounds was not only high but also subjected to large variation (i.e., 12 – 30 % of $w_{N, \text{Kjeldahl}}$). In order to gain more insight into this nitrogen distribution of BSF larvae, the accurate determination of the asparagine and glutamine content of BSF larval proteins and the content of other nitrogenous compounds are both interesting topics worth further research.

Declarations

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The larvae samples used for this research were kindly collected by Dr. Enya Wynants as part of the EDINCO project, funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment (contract RT 15/9 EDINCO). A special thanks goes to Y. Smets and R. Huysmans for their contribution to the experimental work. The authors also want to thank prof. Peter Goos for his assistance regarding the statistical analysis of the data.

References

1. United Nations Department of Economic and Social Affairs Population Division. World Population Prospects 2019: Highlights. 2019.

- 568 2. Boulos S, Tännler A, Nyström L. Nitrogen-to-Protein Conversion Factors for Edible
569 Insects on the Swiss Market: *T. molitor*, *A. domesticus*, and *L. migratoria*. *Front Nutr*.
570 2020 Jul 10;7:89.
- 571 3. Wang Y-S, Shelomi M. Review of Black Soldier Fly (*Hermetia illucens*) as Animal Feed
572 and Human Food. *Foods*. 2017;6(10):91.
- 573 4. FAO. Looking at edible insects from a food safety perspective. Challenges and
574 opportunities for the sector. FAO; 2021.
- 575 5. Smets R, Goos P, Claes J, Van Der Borght M. Optimisation of the lipid extraction of
576 fresh black soldier fly larvae (*Hermetia illucens*) with 2-methyltetrahydrofuran by
577 response surface methodology. *Sep Purif Technol*. 2021;258(2):118040.
- 578 6. Smets R, Verbinnen B, Van De Voorde I, Aerts G, Claes J, Van Der Borght M.
579 Sequential Extraction and Characterisation of Lipids, Proteins, and Chitin from Black
580 Soldier Fly (*Hermetia illucens*) Larvae, Prepupae, and Pupae. *Waste and Biomass*
581 *Valorization*. 2020;11(12):6455–66.
- 582 7. Sprangers T, Ottoboni M, Klootwijk C, Ovyne A, Deboosere S, De Meulenaer B, et al.
583 Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on
584 different organic waste substrates. *J Sci Food Agric*. 2017;97(8):2594–600.
- 585 8. Liu X, Chen X, Wang H, Yang Q, Rehman K, Li W, et al. Dynamic changes of nutrient
586 composition throughout the entire life cycle of black soldier fly. *PLoS One*.
587 2017;12(8):1–21.
- 588 9. D’Mello JPF. Amino acids in animal nutrition. D’Mello JPF, editor. Vol. 2, Amino Acids
589 in Animal Nutrition. Wallingford: CABI; 2003. 125–142 p.
- 590 10. Belghit I, Liland NS, Gjesdal P, Biancarosa I, Menchetti E, Li Y, et al. Black soldier fly
591 larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo*
592 *salar*). *Aquaculture*. 2019 Mar;503:609–19.
- 593 11. Jonas-Levi A, Martinez J-JI. The high level of protein content reported in insects for
594 food and feed is overestimated. *J Food Compos Anal*. 2017 Sep;62(June):184–8.
- 595 12. Smets R, Van Der Borght M. Enhancing the specificity of chitin determinations through
596 glucosamine analysis via ultra-performance LC-MS. *Anal Bioanal Chem*. 2021 May
597 11;413(11):3119–30.
- 598 13. Janssen RH, Vincken J-P, van den Broek LAM, Fogliano V, Lakemond CMM. Nitrogen-
599 to-Protein Conversion Factors for Three Edible Insects: *Tenebrio molitor*, *Alphitobius*
600 *diaperinus*, and *Hermetia illucens*. *J Agric Food Chem*. 2017;65(11):2275–8.
- 601 14. Liu Z, Minor M, Morel PCH, Najar-Rodriguez AJ. Bioconversion of Three Organic
602 Wastes by Black Soldier Fly (Diptera: Stratiomyidae) Larvae. *Environ Entomol*
603 [Internet]. 2018 Dec 7;47(6):1609–17. Available from:
604 <https://academic.oup.com/ee/article/47/6/1609/5145296>

- 605 15. Meneguz M, Schiavone A, Gai F, Dama A, Lussiana C, Renna M, et al. Effect of rearing
606 substrate on growth performance, waste reduction efficiency and chemical composition
607 of black soldier fly (*Hermetia illucens*) larvae. *J Sci Food Agric*. 2018;98(15):5776–84.
- 608 16. Barragan-Fonseca KB, Dicke M, van Loon JJA. Influence of larval density and dietary
609 nutrient concentration on performance, body protein, and fat contents of black soldier fly
610 larvae (*Hermetia illucens*). *Entomol Exp Appl*. 2018;166(9):761–70.
- 611 17. Schmitt E, Belghit I, Johansen J, Leushuis R, Lock E-J, Melsen D, et al. Growth and
612 Safety Assessment of Feed Streams for Black Soldier Fly Larvae: A Case Study with
613 Aquaculture Sludge. *Animals*. 2019 Apr 23;9(4):189.
- 614 18. Wong C-Y, Rosli S-S, Uemura Y, Ho YC, Leejeerajumnean A, Kiatkittipong W, et al.
615 Potential Protein and Biodiesel Sources from Black Soldier Fly Larvae: Insights of
616 Larval Harvesting Instar and Fermented Feeding Medium. *Energies*. 2019 Apr
617 25;12(8):1570.
- 618 19. Matthäus B, Piofczyk T, Katz H, Pudiel F. Renewable Resources from Insects:
619 Exploitation, Properties, and Refining of Fat Obtained by Cold-Pressing from *Hermetia*
620 *illucens* (Black Soldier Fly) Larvae. *Eur J Lipid Sci Technol*. 2019;121(7).
- 621 20. Somroo AA, ur Rehman K, Zheng L, Cai M, Xiao X, Hu S, et al. Influence of
622 *Lactobacillus buchneri* on soybean curd residue co-conversion by black soldier fly larvae
623 (*Hermetia illucens*) for food and feedstock production. *Waste Manag*. 2019;86:114–22.
- 624 21. Ravi HK, Vian MA, Tao Y, Degrou A, Costil J, Trespeuch C, et al. Alternative solvents
625 for lipid extraction and their effect on protein quality in black soldier fly larvae. *J Clean*
626 *Prod*. 2019;238.
- 627 22. Shumo M, Osuga IM, Khamis FM, Tanga CM, Fiaboe KKM, Subramanian S, et al. The
628 nutritive value of black soldier fly larvae reared on common organic waste streams in
629 Kenya. *Sci Rep*. 2019 Dec 12;9(1):10110.
- 630 23. Hasnol S, Lim JW, Wong CY, Lam MK, Ntwampe SKO. Liminal presence of exo-
631 microbes inoculating coconut endosperm waste to enhance black soldier fly larval
632 protein and lipid. *Environ Sci Pollut Res*. 2020;27(19):24574–81.
- 633 24. Ewald N, Vidakovic A, Langeland M, Kiessling A, Sampels S, Lalander C. Fatty acid
634 composition of black soldier fly larvae (*Hermetia illucens*) – Possibilities and limitations
635 for modification through diet. *Waste Manag*. 2020 Feb;102:40–7.
- 636 25. El-Dakar MA, Ramzy RR, Ji H, Plath M. Bioaccumulation of residual omega-3 fatty
637 acids from industrial *Schizochytrium* microalgal waste using black soldier fly (*Hermetia*
638 *illucens*) larvae. *J Clean Prod*. 2020;268:122288.
- 639 26. Ravi HK, Degrou A, Costil J, Trespeuch C, Chemat F, Vian MA. Effect of devitalization
640 techniques on the lipid, protein, antioxidant, and chitin fractions of black soldier fly
641 (*Hermetia illucens*) larvae. *Eur Food Res Technol*. 2020;246(12):2549–68.
- 642 27. Logan LAP, Latty T, Roberts TH. Effective bioconversion of farmed chicken products

- 643 by black soldier fly larvae at commercially relevant growth temperatures. J Appl
644 Entomol. 2021;00:jen.12878.
- 645 28. Fischer H, Romano N. Fruit, vegetable, and starch mixtures on the nutritional quality of
646 black soldier fly (*Hermetia illucens*) larvae and resulting frass. J Insects as Food Feed.
647 2021 Apr 13;7(3):319–27.
- 648 29. Adebayo HA, Kemabonta KA, Ogbogu SS, Elechi MC, Obe MT. Comparative
649 assessment of developmental parameters, proximate analysis and mineral compositions
650 of black soldier fly (*Hermetia illucens*) prepupae reared on organic waste substrates. Int
651 J Trop Insect Sci. 2021 Apr 2;1–7.
- 652 30. Galassi G, Jucker C, Parma P, Lupi D, Crovetto GM, Savoldelli S, et al. Impact of Agro-
653 industrial Byproducts on Bioconversion, Chemical Composition, in vitro Digestibility,
654 and Microbiota of the Black Soldier Fly (Diptera: Stratiomyidae) Larvae. Rumbos C,
655 editor. J Insect Sci. 2021 Jan 1;21(1).
- 656 31. Thompson M, Owen L, Wilkinson K, Wood R, Damant A. A comparison of the Kjeldahl
657 and Dumas methods for the determination of protein in foods, using data from a
658 proficiency testing scheme. Analyst. 2002;127(12):1666–8.
- 659 32. Sáez-Plaza P, Michałowski T, Navas MJ, Asuero AG, Wybraniec S. An Overview of the
660 Kjeldahl Method of Nitrogen Determination. Part I. Early History, Chemistry of the
661 Procedure, and Titrimetric Finish. Crit Rev Anal Chem. 2013;43(4):178–223.
- 662 33. Simonne AH, Simonne EH, Eitenmiller RR, Mills HA, Cresman CP. Could the Dumas
663 Method Replace the Kjeldahl Digestion for Nitrogen and Crude Protein Determinations
664 in Foods? J Sci Food Agric. 1997 Jan;73(1):39–45.
- 665 34. Mæhre HK, Dalheim L, Edvinsen GK, Elvevoll EO, Jensen IJ. Protein determination—
666 method matters. Foods. 2018;7(1).
- 667 35. Watson ME, Galliher TL. Comparison of Dumas and Kjeldahl methods with automatic
668 analyzers on agricultural samples under routine rapid analysis conditions. Commun Soil
669 Sci Plant Anal. 2001;32(13–14):2007–19.
- 670 36. Mariotti F, Tomé D, Mirand PP. Converting nitrogen into protein - Beyond 6.25 and
671 Jones' factors. Crit Rev Food Sci Nutr. 2008;48(2):177–84.
- 672 37. Jones DB. Factors for converting percentages of nitrogen in foods and feeds into
673 percentages of protein. United States Department of Agriculture. 1941.
- 674 38. Ritvanen T, Pastell H, Welling A, Raatikainen M. The nitrogen-to-protein conversion
675 factor of two cricket species - *Acheta domesticus* and *Gryllus bimaculatus*. Agric Food
676 Sci. 2020 Mar 18;29(1):1–5.
- 677 39. Dortmans BMA, Diener S, Verstappen BM, Zurbrugg C. Black Soldier Fly Biowaste
678 Processing - A Step-by-Step Guide. Dübendorf, Switzerland: Eawag: Swiss Federal
679 Institute of Aquatic Science and Technology; 2017.

40. Oftedal OT, Eisert R, Barrell GK. Comparison of analytical and predictive methods for water, protein, fat, sugar, and gross energy in marine mammal milk. *J Dairy Sci.* 2014 Aug;97(8):4713–32.
41. Borremans A, Crauwels S, Vandeweyer D, Smets R, Verreth C, Van Der Borgh M, et al. Comparison of Six Commercial Meat Starter Cultures for the Fermentation of Yellow Mealworm (*Tenebrio molitor*) Paste. *Microorganisms.* 2019;7(11):540.
42. Han X, Heinonen M. Development of ultra-high performance liquid chromatographic and fluorescent method for the analysis of insect chitin. *Food Chem.* 2021 Jan;334:127577.
43. Ku HH. Notes on the Use of Propagation of Error Formulas. *J Res Natl Bur Stand - C, Eng Instrum.* 1966;70C(4):263–74.
44. Tkachuk R. Nitrogen-to-protein conversion factors for cereals and oilseed meals. *Cereal Chem.* 1969;46:419–23.
45. Jain RB. Comparison of three weighting schemes in weighted regression analysis for use in a chemistry laboratory. *Clin Chim Acta.* 2010;411(3–4):270–9.
46. Miller EL, Bimbo AP, Barlow SM, Sheridan B. Repeatability and reproducibility of determination of the nitrogen content of fishmeal by the combustion (Dumas) method and comparison with the Kjeldahl method: interlaboratory study. In: *Journal of AOAC International.* 2007. p. 6–20.
47. Caligiani A, Marseglia A, Leni G, Baldassarre S, Maistrello L, Dossena A, et al. Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids and chitin. *Food Res Int.* 2018;105:812–20.
48. Gold M, Tomberlin JK, Diener S, Zurbrugg C, Mathys A. Decomposition of biowaste macronutrients, microbes, and chemicals in black soldier fly larval treatment: A review. *Waste Manag.* 2018 Dec;82(October):302–18.
49. Mossé J, Huet JC, Baudet J. The amino acid composition of wheat grain as a function of nitrogen content. *J Cereal Sci.* 1985;3(2):115–30.