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Chitin quantitation (as glucosamine) in food raw materials by spectrophotometry

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A R T I C L E I N F O

Method name:
Chitin quantitation (as glucosamine) in food raw materials by spectrophotometry

Keywords:
Cricket
Shrimp
Lobster
Crab
Crustaceans
Insects
Mealworm
Mushroom
Fungi
Tenebrio molitor
Chitosan
Colorimetry

A B S T R A C T

Chitin is a water insoluble nitrogen-containing polysaccharide made from N-acetyl-D-glucosamine containing β-(1→4)-linkages. In food, chitin is considered as a source of fiber with prebiotic properties to gut microflora. Chitin content varies widely in nature from 1% (yeasts) up to 64% (butterfly cuticles) and is mostly found in filamentous or mushroom forming fungi, insects and crustaceans. This spectrophotometric method is suitable for chitin quantitation (reported as glucosamine) in food raw materials like insects (mealworm larvae, crickets), shrimps, mushrooms and fungi in a research (non-routine) laboratory. To remove interferences, the sample is defatted (Soxhlet) prior to acid hydrolysis in 6 M HCl. The color complex is developed after the addition of Katano's reagent (a mix of 0.05 mol/L sodium metasilicate, 0.6 mol/L sodium molybdate, 30% dimethyl sulfoxide and 1.42 mol/L acetic acid) at 70 °C for 30 min and measured at 750 nm against blank. A five-point linear calibration (5–100 µg/mL) is used. Limit of detection is 3 µg GLCN/mL. The correlation (R^2) with an HPLC method for chitin analysis is at least 0.93.

- a reliable alternative to an HPLC method
- does not require expensive equipment
- deproteination by alkali is not necessary for most matrices - saves about 30% of time

Specifications table

Subject area:	Food Science
More specific subject area:	Analytical chemistry, food chemistry
Name of your method:	Chitin quantitation (as glucosamine) in food raw materials by spectrophotometry
Name and reference of original method:	H. Katano, M. Takakuwa, H. Hayakawa, H. Kimoto, Determination of chitin based on the colorimetric assay of glucosamine in acidic hydrolysate, Anal Sci 32 (2016) 701–703 [1]
Resource availability:	n/a

Method details

Chemicals

1. Compressed nitrogen – cylinder, 20 L, 200 bar, ≥99.99% (e.g. Linde)
2. Petroleum ether – 40–65 °C > 90%, ACS grade, CAS 64,742–49–0 (e.g. Lachner 20045-CT0-M1000–1)

Abbreviations: DMSO, dimethyl sulfoxide; GLCN, glucosamine; LOD, limit of detection; LOQ, limit of quantitation; MQ H_2O, HPLC grade water, prepared in Milli-Q system, filtered through 0.2 µm membrane.

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3. Hydrochloric acid – 35%, ACS grade, CAS 7647–01–0 (e.g. Lachner 10033-A35-M1000–1)
4. Sodium hydroxide – pellets, ACS grade, CAS 1310–73–2 (e.g. Lachner 10006-AP2-G1000–1)
5. Acetic acid – glacial, 100%, ACS grade, CAS 64–19–7 (e.g. Merck 1.00063 by Supelco)
6. Dimethyl sulfoxide - ACS grade, CAS 67–68–5 (e.g. Merck 1.02952 by Supelco)
7. D-(+)-glucosamine hydrochloride - >99%, analytical standard, (e.g. Merck 346299 by Millipore)
8. Sodium molybdate$^{(VI)}$ dihydrate ($Na_2MoO_4 \cdot 2H_2O$) - >99.5%, CAS 10102–40–6 (e.g. Merck M1003 by Sigma Aldrich)
9. Sodium metasilicate$^{(IV)}$ nonahydrate ($Na_2SiO_3 \cdot 9H_2O$) - >98%, CAS 13517–24–3 (e.g. Merck S4392 by Sigma Aldrich)
10. Chitin from shrimp shells – practical grade, powder, used as in-house reference material (e.g. Merck, Sigma-Aldrich, C7170)
11. Water – HPLC grade, >18.2 MΩ, prepared on site by Milli-Q water purification system, filtered through 0.2 μm membrane

Materials

1. Analytical balance – readability 0.1 mg and 0.01 mg
2. N_2 evaporator – for 1.5 mL HPLC vials, dry (stainless steel block), 60 ˚C, with timer, hooked up to N_2 cylinder
3. Ultrasonic bath – 5 L volume, 60 kHz
4. Volumetric flasks – borosilicate glass, grade A, 25, 100, 1000 mL
5. Glass pipettes – 5 mL
6. Soxhlet extractors – glass, with condenser (hooked up to cool tap water or cooling fluid), with round bottom flasks (500 mL)
7. Soxhlet extractor heater – for up to 100 ˚C
8. Thimbles for Soxhlet extraction – able to fit Soxhlet extractor above, cellulose, 30×80 mm (or bigger), e.g. Ahlstrom Munksjo
9. Flasks for mobile phase – 1000 mL with GL45 screw (e.g. Schott Duran)
10. Pipettors – variable volume up to 100, 1000, 5000 and 10000 μL, regularly calibrated
11. Pipette tips – for pipettors listed above
12. Vortex – up to 3000 vibrations/min, e.g. IKA
13. Volumetric flasks – glass, 100 and 1000 mL
14. Volumetric cylinder – borosilicate glass, 50, 250, 500 and 1000 mL
15. Glass beads (3–4 mm) or boiling stones
16. Blender
17. Round or folded filter paper – cellulose, fast filtering, 15 cm in diameter, suitable to accommodate 50 mL of liquid when folded
18. Filtration funnel – glass or plastic, with stem
19. Beakers – glass, 500-mL
20. Petri dishes – glass, about 15 cm in diameter
21. Sieve – screen 1 mm and 3 mm, approx. 20 cm in diameter
22. Ceramic mortar with pestle – 10 cm in diameter
23. (Micro-)centrifuge tubes – conical, 1.5 or 2 mL, polypropylene, with attached cap
24. 15-mL glass Hungate type anaerobic culture tubes (125×16 mm) with butyl rubber stopper and screw cap
25. Centrifuge tubes – conical, 50 mL, polypropylene with a screw cap
26. Centrifuge – with rotor for 50-mL conical centrifuge tubes (above) and able to achieve 10000 rpm
27. Laboratory oven – with variable temperature setup up to 110 ˚C
28. pH indicator strips – 7–12 pH scale
29. HPLC vials – screw top, 1.5 ml, clear, with graduation and caps with rubber membrane
30. Water purification system – Milli-Q water purification system for HPLC grade water, >18.2 MΩ, filtered through 0.2 μm membrane
31. Cuvettes – semi-micro (=1.5 mL volume), 12.5 × 12.5 × 45 mm, suitable for VIS light, single use polystyrene, 1 cm (e.g. Brand #7590 15)
32. Spectrophotometer – able to accommodate 1 cm cuvettes, VIS detection at 750 nm

Solutions

Adjust the amounts of all solutions below according to the number of samples analyzed.

1. **Acetic acid (10 mol/L)**

Note: For each sample 135 μL of this solution is needed.

Into a 100-mL volumetric flask put 57 mL glacial acetic acid and fill up with MQ H_2O up to the mark and mix. The solution is stable for three months at laboratory temperature.

2. **Mo+Si solution (0.1 mol/L sodium metasilicate and 1.2 mol/L sodium molybdate)**

Note I: For each sample 450 μL of this solution is needed.

Note II: Each milliliter of this solution consists of 28.42 mg sodium metasilicate nonahydrate and 290.35 mg sodium molybdate dihydrate. These salts dissolve very well in water.

Into a 50-mL volumetric flask weigh 1.421 g sodium metasilicate nonahydrate and 14.518 g sodium molybdate dihydrate. Fill the volumetric flask to $\frac{3}{4}$ of its volume with MQ H_2O, dissolve the salts by swirling and fill up with MQ H_2O up to the mark and mix. A clear solution must be obtained. Prepare fresh solution each day of analysis.

3. **Katano's reagent**

Note I: For each sample 900 μL of Katano's reagent are needed.

Note II: Each milliliter of Katano's reagent consists of 0.5 mL Mo+Si solution, 0.3 mL DMSO and 0.15 mL 10 mol/L acetic acid (final concentrations in Katano's reagent are: 0.05 mol/L sodium metasilicate, 0.6 mol/L sodium molybdate, 30% DMSO and 1.42 mol/L acetic acid).

Into a 25-mL volumetric flask pipette 12.5 mL Mo+Si solution (see above) and 7.5 mL DMSO, swirl and let stand for 5 min. The solution becomes white and opaque due to a full saturation of the solution. Then, dropwise add 3.75 mL 10 mol/L acetic acid while swirling the volumetric flask. The solution will turn yellow and its temperature will slightly increase. Let cool down to room temperature and fill up with MQ H_2O up to the mark and mix. Let stand on a lab bench for 45 min mixing occasionally. After 45 min, transfer the (yellow) Katano's reagent into 50-mL propylene centrifuge tube and centrifuge for 10 min at 10000 rpm (10730 rcf). After centrifugation the solution should be clear (note: unclear solution will interfere with spectrophotometric detection). For further analysis, use only clear Katano's reagent. Prepare fresh solution each day of analysis.

4. **Hydrochloric acid solution (6 mol/L)**

Note: For each sample 10 mL of this solution is needed.

Work in the fume hood. Into 1000 mL volumetric flask transfer 530 mL HCl (35%) with a volumetric cylinder, add slowly about 200 mL MQ H_2O, mix and cool the flask under running tap water, add another 200 mL MQ H_2O, mix and keep cooling under running tap water. Let the flask equilibrate to room temperature and fill up to the mark with MQ H_2O, stopper and mix well. There is no need to check the concentration by titration since this solution is used for sample hydrolysis and not for analytical purposes.

5. **Glusosamine standard stock solution (1 mg/mL)**

Note: For each sample set 100 mL of this solution is needed.

Into a 100-mL volumetric flask, weigh 120.3 mg glucosamine hydrochloride, dissolve in 50 mL MQ H_2O using ultrasonic bath for 2 min, then make up to the mark with MQ H_2O, stopper, mix. Prepare fresh solution each day of analysis. Calculate the exact concentration of glucosamine using the purity as given on the certificate of analysis supplied by the vendor and keep in mind that a hydrochloride salt has been used for making this solution (do not forget to correct for that).

6. **Glucosamine calibration solutions (5, 35, 70 and 100 μg GLCN/mL)**

Into four 1.5-mL clear HPLC screw top vials, pipette 5, 35, 70 and 100 μL glucosamine standard stock solution (1 mg/mL). Place the HPLC vials into the N_2 evaporator under a gentle stream of nitrogen, into the block heated to 60 ˚C for 15 min and evaporate the solution to dryness. After the evaporation, visually check that the vials are completely dry. Then, calibration solutions undergo the steps as described in section D (in Sample preparation). Prepare fresh calibration solutions each day of analysis.

Sample preparation

The sample (at least 30 g) undergoes subsequently defatting and hydrolysis. Make sure the sample is homogeneous and the sample has been taken (sampled) properly. If needed, grind the sample to create particles 1–3 mm in size (with particles less than 1 mm in size it might be difficult to work with during sample preparation procedure). Do not forget to prepare a blank sample and an in-house reference sample (chitin from shrimp shells, e.g. Merck C7170) for method monitoring purposes. Samples are measured in triplicates.

(A) Defatting

This part takes about 24 h.

1. With a pencil, label an empty cellulose Soxhlet extraction thimble. Using an analytical balance, record the weight of the empty labelled thimble ($m_{thimble}$) and a cotton plug (m_{plug}) with 0.1 mg precision.
2. Into the labeled empty thimble (from step 1), put 10–15 g of sample (pack loosely to allow the petroleum ether to penetrate the sample entirely), record the net sample weight (m_{sample}) with 0.1 mg precision and use the pre-weighted cotton plug (step 1) to gently seal the top of the thimble. Do not overfill the thimble (between the cotton plug and the thimble top rim should be at least 1.5 cm free space). Place the thimble with the sample into the Soxhlet extractor.
3. Work in a fume hood. Into a 500-mL round bottom extraction flask which fits the Soxhlet extractor, place a few boiling stones (or glass beads) and 300 mL of petroleum ether.
4. Assemble the whole Soxhlet apparatus placing the round bottom flask onto the Soxhlet heater, hook it up to the pre-cooled condenser (8–15 ˚C) and heat the flask containing petroleum ether to 70 ˚C for 4 h. The extraction takes place in the fume hood.
5. After 4 h, turn off the apparatus and let it cool down. Safely dispose the petroleum ether with the fats extracted (they are not needed for the chitin analysis). Then, take out the thimble containing the defatted sample and let it dry standing upright (in a stand) overnight (for at least 16 h) in a fume hood. Keep the fume hood turned on during the drying phase.

6. Next day, using an analytical balance, weigh the thimble containing the defatted sample and cotton plug ($m_{afterextr}$) with 0.1 mg precision.
7. Gently remove the cotton plug, make sure the cotton fibers have not contaminated the sample and place the sample into a suitable properly labeled container (polypropylene or glass). Visually and/or by touch check that the sample is not wet and is powdery and loose. Store at room temperature until further analysis (no longer than two weeks).
8. Calculate the content of the fat-free residue in percent rounding the result to one decimal place:

$$\text{fat} - \text{free residue\% (w/w)} = \frac{m_{afterextr} - m_{thimble} - m_{plug}}{m_{sample}} \times 100$$

For mass descriptions see the text in section A. Use masses in grams recorded with 0.1 mg precision. $m_{afterextr}$ – sum of the weight of the dry sample residue after the fat has been removed + thimble + cotton plug [g]; $m_{thimble}$ – weight of the cellulose (Soxhlet) thimble [g]; m_{plug} – weight of the cotton plug [g]; m_{sample} – net weight of the sample before fat extraction [g].

(B) **Hydrolysis**

In this part of sample preparation, do not forget to include a blank sample and an in-house reference sample (chitin from shrimp shells). This part also demineralizes (decalcifies) the sample. This part takes about 28 h. If the matrix contains more than 7% proteins a deproteination may be necessary (for sample deproteination procedure see reference [2]).

9. Preheat laboratory oven to 110 ˚C. In the oven, preheat one or two 1 L glass bottles with a cap, depending on the number of samples to be analyzed. One bottle fits about 15 samples. See note in step 15 below.
10. Work in a fume hood. Into an appropriately sized volumetric cylinder put sufficient amount of 6 M HCl needed for all samples and bubble with stream of N_2 gas for at least 30 min.

Note: The hydrolysis is in done in oxygen-free environment to prevent possible decompositions.

11. Pulverize/grind the defatted sample (as prepared in section A) in a ceramic mortar with pestle or by any other grinding technique suitable for very small sample sizes. The particles created should be less than 1 mm to ensure good hydrolysis. If in doubt, use 1 mm screen sieve if necessary and make sure not to leave any residue on the screen.
12. Into a 15-mL glass Hungate anaerobic culture tube weigh (this time with 0.01 mg precision!) 10 mg of defatted sample (m_{defat}). To monitor method performance and hydrolysis process, include 3 other tubes into the sample set. Use nothing for the blank (tube I), 10 mg chitin from shrimp shells (supplied by Merck) as an in-house reference sample (tube II) and 10 mg of glucosamine standard (tube III).
13. Add 10 mL of nitrogen flushed 6 M HCl (with a glass pipette, step 10), flush the headspace above the liquid with nitrogen (about 3 s) and close tightly.

Note: Work quickly (yet safely) after flushing the headspace and allow no air into the Hungate tube. Shake by hand to disperse the sample. Check visually that there are no lumps.

14. Place the Hungate tubes into a rack and put them into the ultrasonic bath for 15 min, mixing every 3 min by hand to avoid ultrasonic clumping.
15. Place the Hungate tubes into the preheated 1-L glass bottles with a cap, close tightly and put into the oven for 24 h at 110 ˚C. Contents are hot, use heat resistant gloves!

Note: Placing the Hungate tubes into the 1-L glass bottles with a cap serves as oven corrosion protection in case of tube breakage or hydrochloric acid vapor leakage.

16. Very carefully mix the insides of the Hungate tubes every 60 min by very carefully inverting the 1-L bottles ten times. Skip the mixing process during the night. Check the tubes for damages or breakage. Contents are hot, use heat resistant gloves!

Note: Hydrolysis solution will turn yellow or brown during hydrolysis.

17. After 24 h, take the Hungate tubes out of the oven and let cool down to room temperature. Visually check that the liquid level is the same in all tubes (If not, then the tube was not closed properly and some hydrolysis solution has evaporated. In this case, do not further process the sample).

Note: Open the 1-L bottles containing the Hungate tubes in the fume hood in case some of the 6 M HCl has escaped during the hydrolysis or in case of a broken tube.

18. Mix the Hungate tubes using vortex for 10 s and let stand for 30 min to sediment any unhydrolyzed residue (mostly mineral ashes).
19. Into a labelled 1.5-mL HPLC vial (clear one) pipette 100 μL of the respective sample hydrolyzate.
20. Work in a fume hood. Evaporate the hydrolyzate in the 1.5-mL HPLC vial under stream of nitrogen at 60 ˚C for 15 min. After evaporation, visually check each vial that it is completely dry.

Note: Since 6 M HCl is being evaporated, use preferably plastic needles for N_2 evaporation. If this is not possible, wipe the N_2 evaporator after use well with water to prevent corrosion on metallic parts of the equipment.

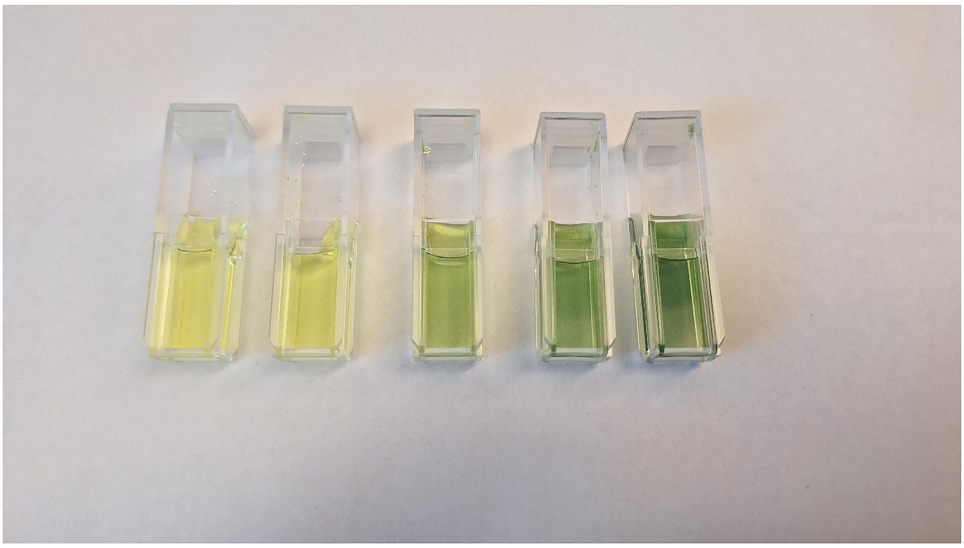

Fig. 1. Color of calibration solutions. From left to right: blank, 5, 35, 70 and 100 µg GLCN/mL.

(C) **Joint sample preparation for calibration standards, samples, blanks and in-house reference sample**

Do this part immediately after completing section B. This part takes about 3 h.

21. Preheat the laboratory oven to 70 ˚C.
22. To the evaporated calibration standards (described in section Solutions), samples (as prepared in sections A-B), blank and an in-house reference sample (as prepared in section B) in the 1.5-mL HPLC vials add 100 µL MQ H_2O and vortex for 5 s.

Note: Choose such vortex speed that the liquid washes the sides of the vial at least up to 1/2 of its total volume.

23. Let stand on the lab bench for 10 min (to re-dissolve the dry residue properly).
24. Again vortex the HPLC vials for another 5 s.
25. Add 900 µL Katano's reagent (see section Solutions), cap the HPLC vials tightly and vortex for 5 s.
26. Put all the vials with calibration standards, samples, blank and an in-house reference sample into the oven for 30 min at 70 ˚C. After heating, the solution will change color from yellow to yellow-green or green depending on the concentration of glucosamine in the solution (Fig. 1).

Note: All the vials (the entire sample set) must go into the oven at once! Use the same solutions for all vials (check beforehand if you have sufficient volumes). Do not prepare the sample set in batches!

27. After 30 min, take the samples out of the oven and refrigerate them at 4 ˚C for 30 min.
28. Vortex the cooled HPLC vials for 5 s.
29. Uncap the HPLC vials transfer their content into single-use polystyrene cuvettes (1 cm) and measure absorbance in a spectrophotometer at 750 nm against blank.

Calibration

Use the spectrophotometric software or Microsoft excel spreadsheet to make calibration. First, calculate the actual concentration of GLCN calibration standards used in µg/mL. Do not forget to correct for standard purity and the fact that hydrochloride salt has been used. Use linear calibration based on absorbance at 750 nm. R^2 should be greater than 0.99. An example of a calibration line is given in Fig. 2. The calibration is linear up to 1000 µg GLCN/mL.

Results

Use current calibration curve equation (see section Calibration) and calculate sample results based on their absorbance at 750 nm in µg GLCN/mL in Microsoft excel spreadsheet. Then, recalculate the results in Microsoft excel sheet using the fat-free mass percent (see formula in section A). Round the final result of CLCN content (mg/g) to the whole number (do not use decimal places).

$$\text{GLCN content } [\text{mg/g}] = \frac{\text{GLCN spectrophoto result} \times \text{fatfree residue}}{m_{defat} \times 100}$$

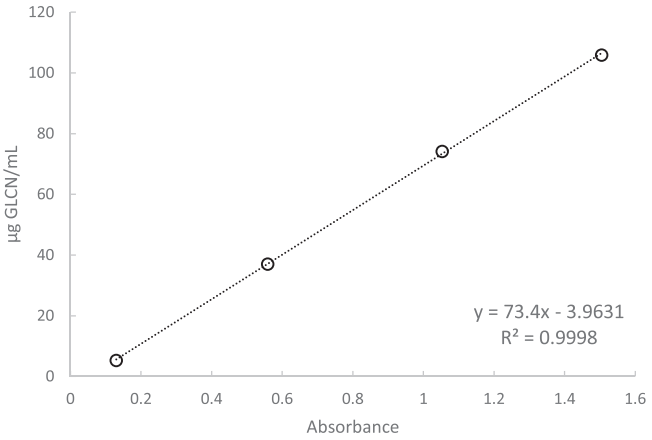

Fig. 2. An example of spectrophotometric calibration curve.

Table 1
Validation performance data. [a] used as an in-house reference sample (purchased from Merck, article C7170).

	Glucosamine	Chitin from shrimp shells[a]	Forest mushrooms	mealworm larvae
Chitin content [mg GLCN/g]	n/a	768.2	55.1	110.8
Repeatability CV(r) [%]	1.9	5.0	6.4	8.1
Repeatability r [%]	4.3	7.8	10.8	15.3
intermediate reproducibility CV(iR) [%]	6.9	10.6	18.1	17.9
Intermediate reproducibility iR [%]	9.2	16.9	28.3	23.2

GLCN spectrophoto result – content of GLCN [µg/mL] as read from the calibration curve; fatfree residue – result as given by the formula in section A [in%, rounded to one decimal place]; m_{defat} – sample weight used for hydrolysis [mg, recorded with 0.01 mg precision]

Method validation

To obtain method performance data has been a rather challenging task for several reasons. To my knowledge, no certified reference material for chitin is available to date and second, chitin is also not available as an analytical standard. The only chitin available is technical grade, coming from shrimp shells and is lacking purity assessment. Chitin is a polymer substance that is insoluble in water and unpolar solvents. This property is utilized at the beginning of sample preparation (defatting and possible deproteination) to obtain chitin-containing residue that is then hydrolyzed into glucosamine. To validate the performance of an analytical method without a certified reference material is possible if spiking experiments are performed. However, without the ability to use chitin as a standard substance with a known purity, the method validation becomes very difficult. Spiking experiments with glucosamine would not reflect the polymer nature of chitin and if deproteination step would be necessry it would get lost since it is soluble in water. Alternatively, the use of chitosan is questionable because it is a deacetylated form of chitin meaning it is a different substance. Also, spiking any food matrix other than shrimp shells with technical grade shrimp chitin is raising questions too. For these reasons, the validation parameters given in Table 1 have to be considered as estimates.

Validation data have been gathered in a single-laboratory validation settings using two analysts on 6 different dates during three-month period in 2023 and two 300 g matrices. Linearity of the calibration curve was being proven up to 1000 µg GLCN/mL. Due to the lack of blank matrices, it has not been possible to reliably determine the limit of detection (LOD) by spiking experiments, yet it is estimated to be 3 µg GLCN/mL. The limit of quantitation (LOQ) has been defined as the lowest concentration of the linear working range (5 µg GLCN/mL). Due to the lack of certified reference material the trueness and recovery of the method cannot be accurately reported. If chitin from shrimp shells could be considered as pure (=100%), then the mean recovery would be 83.8%. In case of GLCN (omitting defatting and deproteination) the mean recovery would be 84.1%. The correlation (R^2) with an HPLC method for chitin analysis is at least 0.93 (Fig. 3).

Ethics statements

Not applicable, this is a chemical method.

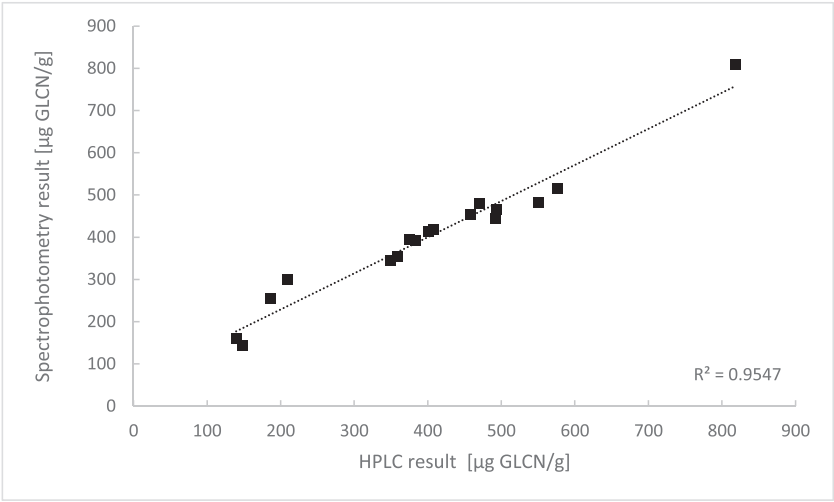

Fig. 3. An example of correlation between spectrophotometry and HPLC results on matrices comprising shrimp, mushrooms and mealworm larvae.

Additional information

Glucosamine is able to reduce the Mo^{VI} species to form a blue molybdosilicate anion with maximum absorbance at 750 nm. Neither N-acetylglucosamine nor chitin react with this ion species thus do not interfere with the method as well as calcium ions, amino acids, proteins and di-, oligo- and polysaccharides [1,3]. A recent detailed review on chitin analysis by multiple approaches could be found here [4]:

Important notes to the method

- no certified reference material with chitin reference value is commercially available
- samples with <7% fat do not have to be defatted as described in step A
- the use of calibrated pipettors is mandatory
- this method is suitable for food chemistry and microbiology application

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Miloslav Šulc: Methodology, Validation, Investigation, Writing – original draft.

Data availability

Data will be made available on request.

Acknowledgments

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