DIETARY SUPPLEMENTS

Determination of Glucosamine in Raw Materials and Dietary Supplements Containing Glucosamine Sulfate and/or Glucosamine Hydrochloride by High-Performance Liquid Chromatography with FMOC-Su Derivatization: Collaborative Study

JOSEPH ZIQI ZHOU, TED WASZKUC, and FELICIA MOHAMMED
NOW Natural Foods, Methods Development Laboratory, 395 S. Glen Ellyn Rd, Bloomingdale, IL 60108

Collaborators: M. Blumhorst; R. Buren; R. Das; L. Huang; J. Jabusch; X. Kou; M. Nagarajan; H. Nguyen; K. Orellana; T.S. Peng; B. Podhola; C. Ray; K. Reif; C. Shevchuk; A. Solyom; D. Sullivan; J. Wang; W. Wang; Q.Yang; Q. Zheng

A collaborative study was conducted for determination of glucosamine in raw materials and dietary supplements containing glucosamine sulfate and/or glucosamine hydrochloride by high-performance liquid chromatography (HPLC) with *N*-(9-fluorenyl-methoxycarbonyloxy) succinimide (FMOC-Su) derivatization. Thirteen blind materials, one pair of which were duplicates, were tested by 12 collaborating laboratories. The test samples consisted of various commercial products, including tablets, capsules, drink mix, and liquids as well as raw materials, blanks, and those for spike recovery analyses. The tests with blank products and products spiked with glucosamine showed good specificity of the method. The average recoveries at spike levels of 100 and 150% of the declared amount were 99.0% with a relative standard deviation (RSD) of 2.1%, and 101% with an RSD of 2.3%, respectively. The test results between laboratories on each commercial product were reproducible with RSD values of no more than 4.0%, and the results were repeatable in the same laboratory with an average RSD of 0.7%. HorRat values ranged from 0.5 to 1.7 on both tests of spike recovery and reproducibility between laboratories on commercial products. The average determination coefficient of the calibration curves from the laboratories was 0.9995 with an RSD of 0.03%. All of the 12 collaborating laboratories succeeded in the study and none of their reported test results were outliers, partly indicating the robustness of the method. It is recommended that the method be accepted by AOAC INTERNATIONAL as Official First Action.

Submitted for publication April 2005.
The recommendation was approved by the Methods Committee on Dietary Supplements as First Action. *See* "Official Methods Program Actions," (2005) *Inside Laboratory Management*, May/June issue.
Corresponding author's e-mail: joseph.zhou@nowfoods.com.

The glucosamine high-performance liquid chromatography (HPLC) method with *N*-(9-fluorenyl-methoxycarbonyloxy) succinimide (FMOC-Su) derivatization (1–3) was selected by an AOAC expert review panel (ERP) as the most appropriate method to recommend for further laboratory validation. The results of the subsequent single laboratory validation (SLV) study, when subjected to peer review by the ERP and selected members of the AOAC INTERNATIONAL Methods Committee on Dietary Supplements, indicated the method was suitable for a full collaborative study (4).

The collaborative study was conducted to evaluate the method accuracy and precision based on its intra- and interlaboratory performance (5, 6). In this study, 13 glucosamine test materials, including a pair of blind duplicates, were analyzed by 12 collaborating laboratories. These materials consisted of various commercial products as well as blanks and spike recovery samples.

Glucosamine product is one of the most popular dietary supplements in the United States, and its effectiveness has been clinically proven for the treatment of osteoarthritis (7). Glucosamine hydrochloride (HCl) and glucosamine sulfate are the 2 most important glucosamine salt forms used in these products and claimed on the product labels. The establishment of an official glucosamine method will facilitate the quality control and regulatory compliance of the product.

Collaborative Study

Study Design

The study was conducted on 12 different test materials. One of them was split into 2 identical samples to test repeatability of the analytical results in the same laboratory. The identity or content of these 13 test samples was not

Table 1. Test materials for glucosamine collaborative study[a]

ID No.	Sample name	Form	Ingredient and potency
G1	Spike recovery level I–150%	Powder, laboratory-made	360 ± 10 mg glucosamine HCl reference standard spiked in 70 ± 10 mg blank powder (G12 below)
G2	Spike recovery level II–100%	Powder, laboratory-made	240 ± 10 mg glucosamine HCl reference standard spiked in 190 ± 10 mg blank powder (G12 below)
G3	Glucosamine sulfate raw material from shellfish	Powder, commercial	Minimum 71% glucosamine sulfate and KCl 23–25%. Glucosamine sulfate potassium chloride complex empirical formula: $2GFB{\cdot}H_2SO_4{\cdot}2KCl$
G4	Glucosamine HCl raw material from shellfish	Powder, commercial	Minimum 98.5% glucosamine HCl
G5	Glucosamine HCl raw material from vegetarian source	Powder, commercial	Minimum 98.5% glucosamine HCl
G6	Joint free plus-collagen 10 g, glucosamine 1.5 g, chondroitin 0.5 g, methylsulfonylmethane (MSM) 0.5 g	Drink-mix powder, commercial	Containing per serving size (2.5 tbsp or 12.6 g): glucosamine HCl 1.5 g; hydrolyzed collagen 10 g; chondroitin sulfate 500 mg; MSM 500 mg. Other ingredients: silicon dioxide
G7	Glucosamine complex-joint support	Capsule, commercial	Containing per serving size (2 caps): glucosamine sulfate 200 mg, glucosamine HCl 600 mg, *N*-acetyl glucosamine 200 mg. Other ingredients: gelatin, rice powder, silica, and magnesium stearate
G8	Glucosamine and chondroitin	Tablet, commercial	Containing per serving size (2 tabs): glucosamine sulfate 1.5 g, chondroitin sulfates 1.2 g. other ingredients: potassium chloride, cellulose, stearic acid, silica, and magnesium stearate
G9	Glucosamine lemonade liquid drink mix	Liquid, commercial	Containing per serving size (1 pouch or 59 mL): glucosamine HCl 1500 mg, vitamin C 60 mg. Other ingredients: high fructose corn syrup, water, lemon juice concentrate, natural flavors, citric acid, sodium benzoate, and potassium sorbate
G10	Glucosamine sulfate, 1000 mg	Tablet, commercial	Containing per serving size (1 tab): glucosamine sulfate (from shellfish) 1000 mg. Other ingredients: microcrystalline cellulose, vegetable cellulose, titanium dioxide, silica, vegetable magnesium stearate, and vegetable glycerin
G11	Liquid blank matrix: lemonade juice all natural	Liquid, commercial	Containing no glucosamine, water, high fructose corn syrup, lemon juice concentrate, citric acid, potassium citrate, natural flavor
G12	Combination blank matrix for tablets and capsules	Powder, laboratory-made	Containing no glucosamine, chondroitin sulfates (1%), MSM (1%), vitamin C (3%), SAMe (0.5%), potassium chloride (7%), manganese sulfate in rice flour (18%), microcrystalline cellulose (30%), vegetable grade stearic acid (3%), vegetable grade magnesium stearate (1.3%), silica (0.2%), and calcium carbonate (35%)
G13	The same as G10		

[a] The material names, ingredients, and potencies were not released to collaborators.

released to the collaborators, and random identification numbers were assigned to each of the test materials. A set of these materials and the glucosamine reference standard were supplied to each of the laboratories for the study. Practice samples were also provided to ensure that participants could successfully follow the method and optimize their instruments before proceeding with the actual tests. The Study Director was available for consultation. Table 1 lists the 13 test samples and their ingredients and potencies.

Collaborators

Twelve laboratories participated in the study. Three were industrial manufacturers of dietary supplement finished products, 5 were raw material vendors, 3 were commercial testing laboratories, and one was the university laboratory. Geographically, 10 laboratories were from the United States, one from Canada, and one from Germany.

Study Procedures

Product types.—Basically, 5 types of test materials were analyzed: (*1*) tablets, (*2*) capsules, (*3*) drink mix powder, (*4*) raw materials, and (*5*) liquid products. At least one product of each type was chosen to represent the group. These representative samples also covered different material sources, e.g., glucosamine from shellfish or glucosamine from vegetable source; different salt forms: glucosamine HCl and glucosamine sulfate; and different combination products with

Table 2. Expected values and validation data of the test materials

Sample and identification No.	Expected value[a]	Validation data[b]		
		Results (3 trials), %	Avg., %	RSD[c], %
Spike recovery level I, G1	100% Spike recovery	101.9, 102.0, 102.0	102.0	0.1
Spike recovery level II, G2	100% Spike recovery	101.5, 101.3, 100.9	101.2	0.3
Glucosamine sulfate raw material from shellfish, G3	55.0% GFB[d]	60.2, 61.0, 59.8 GFB	60.3 GFB	1.0
Glucosamine HCl raw material from shellfish, G4	81.8% GFB	84.9, 83.1, 85.3 GFB	84.4 GFB	1.4
Glucosamine HCl raw material from vegetarian source, G5	81.8% GFB	83.9, 84.1, 84.5 GFB	84.2 GFB	0.4
Drink mix powder-joint free plus, G6	9.89% GFB	10.2, 10.2, 10.4 GFB	10.3 GFB	1.1
Glucosamine complex capsule, G7	44.8% GFB	55.0, 54.8, 53.7 GFB	54.5 GFB	1.3
Glucosamine and chondroitin tablet, G8	26.1% GFB	27.7, 28.1, 27.8 GFB	27.9 GFB	0.7
Glucosamine lemonade liquid drink mix, G9[e]	2.11% GFB	2.49, 2.48, 2.48 GFB	2.48 GFB	0.2
Glucosamine sulfate tablet, G10	46.3% GFB	50.3, 50.1, 50.5 GFB	50.3 GFB	0.4
Liquid lemonade blank matrix, G11	No glucosamine contained	No glucosamine found	No glucosamine found	No glucosamine found
Blank matrix for tablets and capsules, G12	No glucosamine contained	No glucosamine found	No glucosamine found	No glucosamine found
Glucosamine sulfate tablet, G13 (identical to G10)	The same as G10	The same as G10	The same as G10	The same as G10
Calibration curve over the ranges per method	Linearity 0.999	0.9997, 0.9998, 0.9999	0.9998	0.01

[a] Calculated based on label claims or input. For commercial products, the true values could be higher due to overages or lower due to poor quality than the expected values.
[b] Obtained by the Study Director's laboratory at NOW Foods (Bloomingdale, IL).
[c] RSD = Relative standard deviation.
[d] GFB = Glucosamine free base.
[e] The density of the liquid product was 1.0 g/mL.

chondroitin, methylsulfonylmethane (MSM), *S*-adenosyl-L-methionine (SAMe), vitamin C, etc.

Sample potency range.—Because of the large variety of test materials covered in this study, the glucosamine concentrations differed greatly in these samples. As shown in Table 1, for example, with the same quantity but different type of samples, 12.6 g commercial drink mix powder (G6) contains only 1.5 g glucosamine HCl. However 12.6 g glucosamine HCl raw material (G4) contains 12.4 g glucosamine HCl. In addition, between different types of glucosamine salt forms, i.e., glucosamine sulfate and glucosamine HCl, 1 g glucosamine sulfate industrial raw material (G3) contains only 0.59 g glucosamine free base (GFB); the material has the complex empirical formula $2GFB{\cdot}H_2SO_4{\cdot}2KCl$, but 1 g glucosamine HCl (G4) contains 0.83 g GFB. These differences make it difficult to use the same test portion size in weight for all the products in the method. Therefore, to ensure the best accuracy of tests, the method has classified different product categories for sampling.

Preparation of test samples.—(*1*) *Commercial products*.—All products were removed from their original commercial packages to conceal the manufacturer's information. Tablets were ground into powder, but capsules were kept unchanged (because contents once emptied from the shells are ready to proceed for analysis). The powders including raw materials and drink mix were well mixed to prevent non-uniformity problems. For shipment, 20–30 g powder or 60 capsules were packed into a film bag. The liquid products were mixed thoroughly and repacked into 60 mL plastic bottles for shipment.

(*2*) *Blank powder*.—A combination blank powder for tablets and capsules was prepared based on the weight percentages shown in Table 1 and treated as a tablet powder for analysis.

(*3*) *Spike recovery samples*.—The 2 products for spike recovery levels I and II were initially made as a liquid using the blank powder and glucosamine reference standard to avoid homogeneity problems, but those products decomposed from microbial action. It was later found that 1% sodium benzoate can be used as a preservative. Because of time limitation and stability concerns on the other products, the collaborators were asked to prepare G1 and G2 (the 2 spike recovery samples) with the glucosamine HCl standard and G12 (the blank powder) provided. But the identity of G1 and G2 and content of G12 (marked as a tablet powder) were kept

unknown to collaborators. G1 and G2 were prepared by accurately mixing 360 ± 10 mg glucosamine HCl standard with 70 ± 10 mg G12 (spike recovery level I 150%), and mixing 240 ± 10 mg glucosamine HCl standard with 190 ± 10 mg G12 (spike recovery level II 100%), respectively. The base for the above spike percentage levels is 240 mg glucosamine HCl. This amount dissolved in the solvent in a 100 mL flask is the expected concentration designed for this method to analyze various glucosamine products (*see Preparation of Test Solutions* in the method).

All of the above test samples were processed under clean and controlled condition to avoid contamination. Glucosamine in solid state is, in general, chemically stable, but under special cases such as hot temperature and chemical reactions as well as microbial action, it may degrade.

Shipment.—Test samples and standard were shipped to collaborators at ambient temperature. Each of the test bags or bottles was labeled for identification (e.g., G1, G2, and product type, e.g., tablet powder) to assist collaborators in differentiating the products and finding the right categories. Collaborators were required to return a receipt acknowledgement form to indicate receipt and condition of the shipped items. They were also directed to store products and standard at room temperature.

Practice samples.—The practice samples were prepared, randomly coded (e.g., P1, P2), shipped, and analyzed as if they were the actual samples. Collaborators were required to analyze the practice samples and report the results to obtain the Study Director's approval before proceeding with the actual study.

Analysis.—Collaborators were required to prepare new calibration solutions and curve each test day. For test material analysis, single preparation and single injection were required.

Data reporting.—Collaborators were asked to report the linearity of each calibration curve, the corresponding concentrations of the calibration solutions, and the percentages of GFB found in each of the 13 test samples on the data reporting sheets. They were also asked to report any observations and deviations to the method.

Expected values and validation data of the test materials.—Table 2 lists the expected values (e.g., the GFB values calculated from the label claims) and validation data of the test materials. The validation data were obtained by the Study Director's laboratory at NOW Foods (Bloomingdale, IL) using the same method and on the same materials as sent to the collaborating laboratoies in this study.

AOAC Official Method 2005.01 Glucosamine in Raw Materials and Dietary Supplements Containing Glucosamine Sulfate and/or Glucosamine Hydrochloride

High-Performance Liquid Chromatography with FMOC-Su Derivatization First Action 2005

(Applicable to the analysis of glucosamine in raw materials and dietary supplements containing glucosamine sulfate and/or glucosamine hydrochloride.)

See Tables **2005.01A** and **B** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Glucosamine sulfate/hydrochloride finished products or raw materials are dissolved in water. The glucosamine free base (GFB) is released by adding triethylamine to the solution and derivatized with *N*-9-fluorenylmethoxycarbonyloxy succinimide (FMOC-Su). The derivative is separated by high-performance liquid chromatography (HPLC) and measured with UV detection. Glucosamine has 2 natural steroisomers (and , and the interconversion of these 2 in aqueous solution is not preventable, resulting in 2 peaks in the chromatogram. The sum of the areas of these 2 peaks is used to quantify the GFB.

Table 2005.01A. Reproducibility data analysis of interlaboratory results on test materials[a]

Sample code	Avg_R %GFB	SD_r	$\%RSD_R$	$\%PRSD_R$	HorRat value, $\%RSD_R/\%PRSD_R$	No. of outlier labs	No. of labs
G3	59.1	1.0	1.7	2.2	0.8	0	12
G4	84.5	2.1	2.5	2.1	1.2	0	12
G5	83.7	1.5	1.8	2.1	0.9	0	12
G6	10.0	0.4	4.0	2.8	1.5	0	12
G7	53.5	2.0	3.7	2.2	1.7	0	12
G8	27.4	1.0	3.6	2.4	1.5	0	12
G9	2.45	0.3	1.2	2.5	0.5	0	12
G10	50.0	1.0	2.0	2.2	0.9	0	12
G13	49.9	1.0	2.0	2.2	0.9	0	12

[a] SD_r = Reproducibility standard deviation; $\%RSD_R$ = reproductibility relative standard deviation; $\%PRSD_R$ = predicted reproducibility relative standard deviation.

Table 2005.01B. Repeatability data analysis of intralaboratory results on 2 identical samples

	Laboratory and intralaboratory results on blind duplicate samples[a]											
G10 and G13	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
Avg_r	49.5	48.2	50.3	49.4	50.3	51.3	49.9	50.3	49.2	49.3	50.1	51.9
SD_r	0.21	0.14	0.35	0.14	0.35	0.71	0.49	0.64	0.07	0.35	0.00	0.64
$\%RSD_r$	0.43	0.29	0.70	0.29	0.70	1.38	0.99	1.27	0.14	0.72	0.00	1.23
$\%PRSD_r$	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
HorRat	0.2	0.1	0.3	0.1	0.3	0.6	0.4	0.6	0.1	0.3	0.0	0.6

Overall repeatability of the method[b]	
Replicates per laboratory	2
Total number of replicates	24
Overall mean of the laboratory values	49.9
Repeatability standard deviation SD_r	0.41
Repeatability relative standard deviation $\%RSD_r$	0.82

[a] The individual results on G10 and G13 are shown in Table 3.

[b] Calculated using AOAC statistical program (2001) version 1.14 for blind replicates (9).

B. Apparatus

Note: Equivalent apparatus may be substituted. All glassware is Class A.

(**a**) *LC system*.—Agilent HPLC 1100 series with pump, degasser, autosampler, thermostatted column compartment, variable wavelength detector, and Chemstation software for system control and data acquisition (Agilent Technologies, Inc., Palo Alto, CA; www.agilent.com). Operate the LC system under the following conditions: mobile phase flow rate, 0.8 mL/min; detection wavelength, 265 nm; column compartment temperature, 30 C; and injection volume, 10 L.

(**b**) *LC column.*—Phenomenex Prodigy (MidBore™) ODS-3 100 Å, m, 150 3.2 mm, Phenomenex Order No. 00F-4097-R0 (Phenomenex, Torrance, CA 90501; www.phenomenex.com).

(**c**) *LC guard column.*—Phenomenex Prodigy SecurityGuard™ Cartridges ODS-3 100 Å, 4 3.0 mm, Phenomenex Order No. AJO-4287 (Phenomenex).

(**d**) *Analytical balance*.—Readability, ±0.0001 g.

(**e**) *Sonicator*.—Branson (Danbury, CT) 8210 ultrasonic cleaner.

(**f**) *Vortex*.—Barnstead (Newton, MA) Type 16700 mixer.

(**g**) *pH meter.*—Beckman (Fullerton, CA).

(**h**) *Grinder.*—One-touch coffee grinder.

(**i**) *Volumetric flasks*.—5 and 100 mL.

(**j**) *Volumetric pipettors.*—10 mL (needed only for analysis of liquids).

(**k**) *LC solvent filters.*—0.45 m nylon membrane.

(**l**) *Syringe filters.*—PTFE, 0.45 m 13 mm, and 0.45 m 25 mm.

(**m**) *Syringe*.—Luer-Lok™, 3 mL.

(**n**) *Eppendorf variable volume pipettors and tips.*—50–200 L (accuracy: ±1.0–0.6%, precision: 0.3–0.2%) and 500–2500 L (accuracy: ±1.5–0.6%, precision: 0.3–0.2%). *Note*: Make sure both pipettors are properly calibrated.

(**o**) *LC injection vials.*—Screw-cap vials with Teflon-coated caps.

C. Reagents

Note: Chemicals from other suppliers meeting the specifications may also be used.

(**a**) *Reference standard.*—D(+)-Glucosamine hydrochloride, minimum 99% pure, available from Sigma (St. Louis, MO; www.sigmaaldrich.com; Product No. G4875).

(**b**) *Derivatization reagent*.—FMOC-Su, 97% pure, available from Lancaster (Windham, NH; www.lancastersynthesis.com; Cat. No. 6908).

(**c**) *Solvents*.—Acetonitrile, LC grade; trifluoroacetic acid (TFA), minimum 99.0%; triethylamine (TEA), minimum 99%; water, LC grade.

(**d**) *FMOC-Su derivatization solution.*—15mM. Dissolve 50 ± 1.0 mg FMOC-Su in 10 mL acetonitrile. Prepare solution fresh for each test.

(**e**) *Mobile phases.—Mobile phase A*.—Water containing 0.05% TFA, pH 2.4: Add 0.5 mL TFA to 1 L volumetric flask containing ca 900 mL water. Dilute to volume with water and mix. Confirm pH of the solution with pH meter. Filter the solution with 0.45 m nylon membrane before use. *Mobile Phase B*.—Pure acetonitrile.

D. Preparation of Test Solutions

Accurately weigh or measure the amount, as indicated in Table **2005.01C**, into separate 100 mL volumetric flasks. For

Table 2005.01C. Test portion size for glucosamine test materials

Product	Amount
Glucosamine·HCl standard	240 mg (stock standard solution)
Test material with label claim	Calculate from label claim amount of test sample to contain 240 mg of the hydrochloride or 340 mg of the sulfate salt
Test material without label claim	Raw material: 300 mg Tablet powder: 430 mg Capsule fill content: 500 mg Drink mix powder: 2000 mg Liquid: 10 mL

tablets, find and record the mean weight of 20 tablets, grind, mix, and weigh. For capsules, empty and record the mean fill weight of 20 capsules, grind the contents, mix, and weigh. For liquid products, shake well before taking the test portion.

Add ca 80 mL water to the test portion, mix on a Vortex mixer for 1 min, and sonicate for 5 min or until all solids dissolve. (*Note*: Some of the products' excipients, e.g., silicon dioxide, may never dissolve.) Pipet 750 L TEA into each of the flasks and dilute to volume with water. Filter ca 1.5 mL of each solution through a 0.45 m 25 mm PTFE filter into an LC injection vial.

E. Derivatization Procedures

Note: Both standards and test solutions must be derivatized simultaneously in 5 mL volumetric flasks.

Pipet the exact amount, as specified below, of the filtered solutions from the LC injection vials into separate 5 mL volumetric flasks: glucosamine standard working solutions (3-point calibration): 50, 125, and 200 L, respectively; and for all other products: 125 L. Add 500 L 15mM FMOC-Su solution to each flask. Cap flasks tightly with Teflon stoppers, mix well with a Vortex mixer, and sonicate all flasks in the sonicator water bath at 50 C for 30 min. Remove the flasks from the bath, let cool to room temperature, and dilute to volume with the mixture of mobile phases A/B (1/1, v/v). Mix well with a Vortex mixer. Filter each solution through 0.45 m 13 mm PTFE filter into an LC vial for injection.

F. Determination

(**a**) *System suitability tests*.—Equilibrate the LC system with the mobile phase for at least 30 min. Make 5 replicate injections of the second (mid-concentration) glucosamine HCl working standard. The typical retention time (t) of glucosamine anomer peak 1 (the earlier eluted peak) should not be <4 min, and the relative retention time (R_r) of glucosamine anomers peaks 2 to 1 should be 1.2 ($R_r = t_2/t_1$). The peak tailing factor (T) should not be >2.0 ($T = W_{0.05}/2f$, where $W_{0.05}$ is width of the peak measured at a point 5% of the peak height from the baseline; and f is horizontal distance from the vertical line at the peak maximum to the point on leading edge of the peak at 5% height. The relative standard deviation (RSD) of the sums of peak area of glucosamine peaks 1 and 2 from the 5 injections should not be >2.0%.

(**b**) *Mobile phase gradient program*.—Elute the analytes with the following gradient mode of mobile phases A and B:

Time, min	Ratio: A:B
0.0–6.0	70:30 isocratic
6.0–11.0	Change to 0:100
11.0–13.0	Change to 70:30
13.0–15.0	70:30 isocratic

(**c**) *Run time*.—15 min.

(**d**) *Injection*.—Make single injection of each standard working solution and unknown solution.

G. Calculations

(**a**) *Concentrations of glucosamine working standard solutions*.—Calculate the concentrations of GFB in working standard solutions, after FMOC derivatization:

$$\text{STD } n, \text{ mg/mL} = 0.83091 \quad d \quad W \quad F$$

where n = 1, 2, 3 for 3 different standard working solutions; 0.83091 is the conversion factor from glucosamine HCl to GFB: 179.17/215.63; d = the dilution factor: v/(100 mL 5 mL), with v = 0.050, 0.125, and 0.200 mL for STD1, STD2, STD3, respectively; W = the amount of glucosamine HCl standard weighed, mg; and F = the purity factor of glucosamine HCl standard used.

(**b**) *Percentage of GFB in all glucosamine contained materials*.—Calculate the % GFB in all glucosamine-contained materials:

$$\%\text{ GFB, mg/mg} = (P - b) \quad 100/(a \quad D \quad W)$$

where P = the sum of peak area of glucosamine peaks 1 and 2 of the unknown test sample; a = slope of the calibration curve; b = intercept of the calibration curve; D is the dilution factor: v/(100 mL 5 mL), with v = 0.125 mL; W = the amount of test portion weighed, mg.

For liquids:

$$\text{GFB, mg/mL} = (P - b)/(a \quad D \quad V)$$

where V = the amount of test portion volume used, mL.

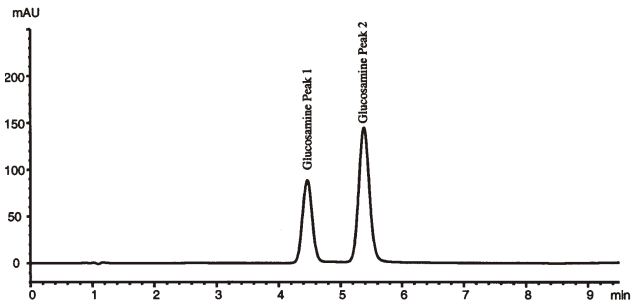

Figure 2005.01. Typical chromatogram of a glucosamine tablet.

(**c**) *Percentage of glucosamine HCl in finished products or raw materials*.—Calculate the % glucosamine HCl in glucosamine HCl finished products or raw materials:

$$\% \text{ G·HCl, mg/mg} = (P - b) \quad 100/(0.83091 \quad a \quad D \quad W)$$

For liquids:

$$\text{G·HCl, mg/mL} = (P - b)/(0.83091 \quad a \quad D \quad V)$$

(**d**) *Percentage of glucosamine sulfate (2GFB·H_2SO_4) in finished products or raw materials*.—Calculate the % glucosamine sulfate in glucosamine sulfate finished products or raw materials:

$$\% \text{ G·sulfate, mg/mg} = (P - b) \quad 100/(0.78511 \quad a \quad D \quad W)$$

where 0.78511 is the conversion factor from glucosamine sulfate (2GFB·H_2SO_4) to GFB: (2 179.17)/456.418.

For liquids:

$$\text{G·Sulfate, mg/mL} = (P - b)/(0.78511 \quad a \quad D \quad V)$$

(**e**) *Amount of glucosamine sulfate (or HCl) per product unit*.—Calculate glucosamine sulfate (or HCl) in mg per tablet (or capsule), or glucosamine sulfate (or HCl) in mg per serving volume (mL):

$$\text{mg G·sulfate (HCl) per tablet (capsule)} = \% \text{ w/w G·Sulfate (HCl)} \quad 100 \quad \text{mg/Tab (Cap)}$$

or

$$\text{mg G·Sulfate (HCl) per serving volume} = \text{mg/mL G·Sulfate (HCl)} \quad \text{mL/Serving}$$

where mg/Tab (Cap) is the average weight of one tablet or the average fill weight of one capsule, in mg; and mL/Serving is the serving volume for liquid products, in mL.

Figure **2005.01** shows a typical chromatogram of a glucosamine tablet.

Reference: *J. AOAC Int.* **88**, 1048(2005).

Results and Discussion

Collaborative Study Results

The analyses were completed by all 12 collaborating laboratories in 2 weeks. The sample identification, which was randomly assigned to the test samples, was decoded after the test results were received, and the names of the participating laboratories were coded from L1 to L12 for their data presentation in this report.

Table 3 shows the complete set of data submitted from the collaborating laboratories in the percentages of GFB for

Table 3. Interlaboratory results of percentages of glucosamine free base found in 8 commercial products and 2 different blank samples

	Glucosamine free base reported from the collaborating laboratories, %											
Sample code	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
G3	58.7	58.1	58.4	57.8	59.1	59.7	61.4	59.0	58.9	58.5	59.6	59.8
G4	84.6	83.5	83.6	84.2	83.5	85.6	89.7	85.8	83.5	81.0	85.3	83.5
G5	84.0	82.5	83.6	83.0	83.3	84.0	86.5	84.4	84.2	80.0	84.8	84.2
G6	10.2	9.66	10.1	9.18	9.70	10.4	10.5	9.50	10.1	10.4	10.6	10.1
G7	53.0	51.9	53.1	54.0	51.1	55.6	54.7	54.3	55.0	49.1	55.0	55.4
G8	26.2	26.5	27.4	27.2	30.1	27.0	27.7	27.6	27.3	26.8	26.6	28.3
G9	2.49	2.41	2.46	2.42	2.46	2.41	2.48	2.48	2.45	2.46	2.42	2.48
G10[b]	49.6	48.1	50.5	49.5	50.0	50.8	49.5	50.7	49.1	49.5	50.1	52.3
G11	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G13[b]	49.3	48.3	50.0	49.3	50.5	51.8	50.2	49.8	49.2	49.0	50.1	51.4

[a] ND = None detected.

[b] G13 and G10 were 2 identical samples. However, the collaborators were not informed they were the blind duplicates.

Table 4. Calibration preparation and linearity reported from collaborating laboratories

	Calibration solution concentration, g/mL			
Lab code	1st Point	2nd Point	3rd Point	Linearity
L1	19.81	49.52	79.23	0.9996
	19.75	49.38	79.00	0.9991
L2	19.85	49.62	79.40	0.9998
	20.65	51.62	82.59	0.9990
L3[a]	19.83	49.56	79.30	1.0000
L4	19.80	49.50	79.20	0.9998
	19.83	49.57	79.31	0.9993
L5	19.60	39.21	78.41	0.9991
	19.93	39.86	79.73	0.9999
L6	19.79	39.58	79.17	0.9985
	19.71	39.42	78.84	0.9989
L7	19.95	49.89	79.82	0.9994
	19.90	49.74	79.59	0.9997
L8[a]	20.15	40.30	80.60	0.9995
L9[a]	20.27	40.55	60.82	0.9998
L10[a]	20.33	40.67	61.00	0.9997
L11	19.95	39.90	79.80	0.9998
	19.97	39.93	79.87	0.9997
L12	19.9	39.8	79.5	0.9997
	19.8	39.6	79.1	0.9992
Avg.				0.9995
Standard deviation				0.0004
RSD, %				0.04

[a] All of the 13 test materials were tested in 1 day. Therefore, only one calibration curve was prepared.

8 commercial products and 2 different blanks. These data indicate the method performance on reproducibility of the analytical results between laboratories. Table 3 also shows the results on 2 identical test samples (G10 and G13) that indicate the method performance on the repeatability of the analytical results in the same laboratory and reproducibility between laboratories. Table 4 shows the concentrations of calibration solutions and linearity of calibration curves reported from each laboratory. Table 5 shows the recovery results of 2 spike levels from all the laboratories. The percentages of spike recovery were calculated by dividing the percent GFB found in each test sample by the percent GFB fortified in the test sample and multiplying by 100. The data indicate both the method accuracy and method precision between laboratories.

The results in Table 3 were used to generate the statistical data presented in Tables **2005.01A** and **B**, respectively, for reproducibility between laboratories and repeatability in the same laboratory.

Average (A), standard deviation (SD), and relative standard deviation (RSD) used for the statistical analysis are defined as follows:

$$A = \quad A_i \quad / N$$

$$SD = \quad A_i \quad A^{\,2} \quad / \; N \; 1 \qquad ^{1/2}$$

$$\%RSD \; = \; 100SD / A$$

where A_i is the individual measurement on the sample; and N is the number of data points needed to achieve $\sum A_i$.

The HorRat value is the ratio of the RSD, expressed as a percent (%RSD) to the predicted RSD, expressed as a percent (%PRSD), i.e., HorRat = %RSD / %PRSD, where %PRSD = $2C^{-0.1505}$ and C = the measured mean concentration of the analyte expressed as a decimal mass fraction (e.g., 1 g/100 g = 0.01). For the spike recovery results presented in Table 5, the true concentrations of GFB (%GFB spiked/100) was used to calculate %PRSD for HorRat values. For the statistical data shown in Tables **2005.01A** and **B**, the experimental concentration values of %GFB/100 were used to calculate %PRSD.

Figure 1 shows sample chromatograms from Laboratory 2 (L2) of glucosamine standard sample 3, commercial glucosamine sulfate tablets (G10), and blank powder combination matrix for tablets and capsules (G12).

Collaborators' Comments

Most collaborators found the method easy to follow and perform. Reasonable modifications of the method (as shown below) without significantly affecting the test results and with the Study Director's permission were allowed to show the method robustness.

Laboratory 2 increased the post-gradient equilibration time from 2 to 7 min before next injection for a total run time of 20 min. The results from the other laboratories were obtained by using the conditions per the method.

Laboratory 5 observed a small peak around a retention time of 4.1 min when the analyst used water as a blank for test. The laboratory also observed this little peak in some glucosamine sample analysis, and suspected it had merged with peak 1 of glucosamine in other tests. The analyst also found in some tests another small peak between peaks 1 and 2 of glucosamine. It is not sure that the peaks were due to some impurities in the particular lot of the reagents. However, because these peaks were very small, the quantitation results from the laboratory were not affected (Tables 3–5 and **2005.01A** and **B**).

Laboratory 6 reported that during a replicate injection of 5, the area of glucosamine peak 1 slightly increased while the area of peak 2 slightly decreased, but the total area of peaks 1 and 2 remained the same (RSD = 0.19 and 0.33%, with 5 injections for each of 2 trials).

Table 5. Interlaboratory results of glucosamine spike recovery

	Laboratory code											
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
Spike level I: 100%												
Amount blank powder[a] used, mg (w_1)	190.4	196.8	190.6	188.1	191.4	198.6	189.5	184.3	191.0	95.0	189.8	190.6
Amount G·HCl std[b] spiked, mg (w_2)	239.8	257.8	241.5	241.6	241.6	242.9	241.3	240.6	244.0	120.0	240.6	240.8
GFB spiked[c], %	46.3	47.1	46.4	46.7	46.4	45.7	46.5	47.1	46.6	46.4	46.4	46.4
GFB found, %	46.3	46.2	45.3	45.3	45.3	47.1	47.4	47.4	45.8	44.7	46.4	45.2
Spike recovery, %	100	98.1	97.6	97.0	97.6	103	102	101	98.2	96.3	100	97.4
$Avg._R$ and RSD_R, %	$Average_{R,\ \%SpikeRecovery}$: 99.0, RSD_R: 2.1											
$PRSD_R$, %	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
HorRat value	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Spike level II: 150%												
Amount blank powder[a] used, mg (w_1)	69.3	72.7	71.7	70.2	72.0	74.8	70.7	63.1	76.0	35.0	70.0	76.2
Amount G·HCl std[b] spiked, mg (w_2)	359.9	380.1	362.1	354.6	361.1	355.8	360.5	355.4	366.0	180.0	360.3	363.1
GFB spiked[c], %	69.7	69.8	69.4	69.4	69.3	68.7	69.5	70.6	68.8	69.6	69.6	68.7
GFB found, %	68.2	69.5	69.2	69.1	68.0	71.7	71.9	71.1	68.1	72.2	72.2	70.1
Spike recovery, %	97.8	99.6	99.7	99.6	98.1	104	103	101	99.0	104	104	102
$Avg._R$ and RSD_R, %	$Average_{R,\ \%SpikeRecovery}$: 101, RSD_R: 2.3											
$PRSD_R$, %	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
HorRat value	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1

[a] Combination blank matrix (powder) for tablets and capsules (G12) was used.

[b] G·HCl std: glucosamine HCl reference standard.

[c] GFB spiked, % = [0.83091 w2 / (w1 + w2)] 100% where 0.83091 is the conversion factor from glucosamine HCl to GFB.

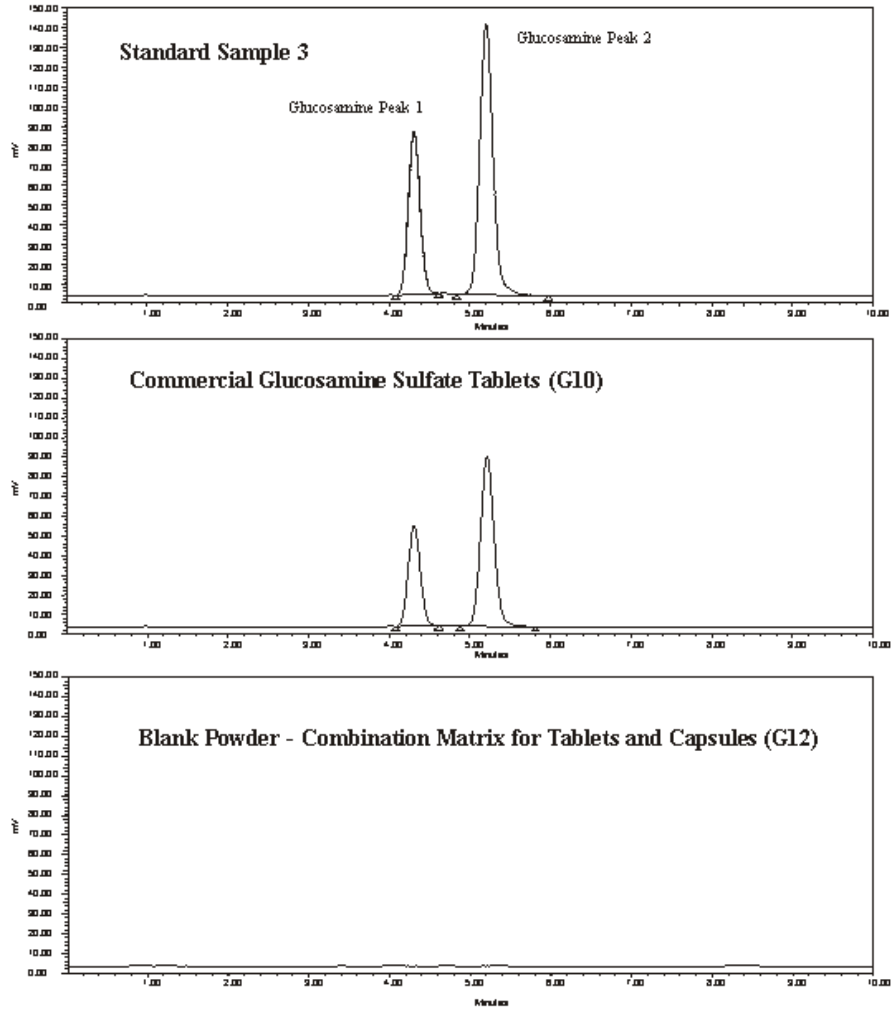

Figure 1. Sample chromatograms of glucosamine standard sample 3, commercial glucosamine sulfate tablets (G10), and blank powder combination matrix for tablets and capsules (G12). Chromatogram source: collaborating Laboratory 2 (Lab Code: L2).

Laboratory 7 analyzed the test samples after they were prepared and stored in LC vials for 4 days at ambient temperature. The results are adequate, indicating good stability of the glucosamine-FMOC-Su derivatives.

Laboratory 10 prepared all the materials by using half of the recommended amount for sampling, and 50 mL instead of the 100 mL volumetric flasks per the method. For derivatization, Laboratory 10 used 10 mL instead of 5 mL volumetric flasks, and all recommended volumes for test solution and reagents were doubled. Some of the differences in test portion weight were shown in Table 4 (amounts of the blank powder and glucosamine HCl standard used). However, all of their test results are acceptable.

Laboratory 11 performed the tests without a guard column.

Laboratory 12 noticed insoluble residues in the sample preparations of G1, G2, G8, G10, G12, and G13.

None of the collaborating laboratories reported any system suitability problems in the study.

Performance Characteristic of the Method

In summaries of the data (Tables 3–5 and **2005.01A** and **B**) the tests with the blank materials and the products with glucosamine spiked showed good specificity of the method. Specificity can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other materials (4, 6). As shown in Table 3, the 2 blank matrixes (G11 and G12) containing SAMe, vitamin A, citric acid, chondroitin sulfates, MSM, lemon juice concentrate, and other ingredients had none detected reported from all laboratories at the retention times corresponding to glucosamine peaks, indicating no interference to glucosamine quantitation. The spike recovery study also bore evidence that the method is accurate. The average spike recoveries presented in Table 5 at the spike levels of 100 and 150% were 99.0% with an RSD of 2.1%, and 101% with an RSD of 2.3%, respectively. The test results between laboratories on each commercial product were reproducible with all RSD values of no more than 4.0%. The results were repeatable in the same

laboratory on 2 identical samples, with an average RSD of 0.68% for all laboratories. The average determination coefficient of the calibration curves from the laboratories was 0.9995 with an RSD of 0.03%.

HorRat values showed 0.9 and 1.1 for the spike recovery analysis at the levels of 100 and 150%, respectively. For the tests of reproducibility between laboratories on each commercial product, HorRat values ranged from 0.5 to 1.7. The tests of repeatability in the same laboratory on 2 blind duplicates showed HorRat values of 0–0.6 for all laboratories. The zero and near zero values for HorRat are the results of some laboratories finding and reporting the exactly same or similar %GFB on 2 identical tablet samples (G10 and G13), which diminished the RSD in the numerator for calculation of the HorRat value. The fact that collaborating laboratories found the same or very similar results on 2 blind duplicates among 13 test materials demonstrated well the reliability of the method.

The method is also rugged and robust (*see* refs 6 and 8 for definitions). The method has been tested by 12 collaborating laboratories on various test materials. Although the same method was followed, the actual use conditions in different laboratories may vary greatly with different equipment brand, quality, and personnel. For example, the water bath may display exactly 50 C, but the real temperature could be 55 C (or more or less) if the temperature sensor was not calibrated, or the regulator on the bath did not work so well (accurately); or, if some laboratory simply accepted 55 C instead of 50 C as required, but did not report the difference to the Study Director, and so on. It is important to note that all 12 collaborating laboratories succeeded in the study and none of their reported test results were outliers, partly indicating the robustness of the method.

Because glucosamine concentration in commercial products changes significantly with product manufacturer, type (tablets, capsules, raw materials, etc.), and glucosamine salt form used (glucosamine hydrochloride and glucosamine sulfate), the tests may be more accurate if the sampling amount is based on its label claims.

Recommendations

Based upon the results of the collaborative study, it is recommended that the method be accepted by AOAC INTERNATIONAL as Official First Action.

Acknowledgments

We appreciate AOAC INTERNATIONAL's Methods Committee on Dietary Supplements and ERP members for their valuable guidance, comments, and suggestions, as well as AOAC INTERNATIONAL staff for its hard work and support for the collaborative study. We thank the following collaborators for their participation:

Chuck Ray and Randy Buren, Cargill, Inc., Eddyville, IA

Wendi Wang and Hao Nguyen, Advanced Botanical Consulting & Testing, Inc., Tustin, CA

Jack Jabusch and Darryl Sullivan, Covance Laboratories, Inc., Madison, WI

Xiaolan Kou and Qiuping Yang, Nature Sunshine Products, Inc., Provo, UT

Aniko Solyom, The University of Arizona, Tucson, AZ

Jonathan Wang, Nature Way Products, Inc., Springville, UT

Tang S. Peng and Quan Zheng, Pure World Botanicals, South Hackensack, NJ

Mike Blumhorst, Archer Daniels Midland Co., Decatur, IL

Mythili Nagarajan and Brandon Podhola, Enzymatic Therapy, Inc., Green Bay, WI

Li Huang and Cathy Shevchuk, JR Laboratories Inc., Burnaby, BC, Canada

Rupa Das and Kevin Orellana, BI Nutraceuticals, Long Beach, CA

Klaus Reif, PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany

This study is the result of a contract between the Center for Food Safety and Applied Nutrition (FDA) and the Office of Dietary Supplements (NIH) with AOAC INTERNATIONAL. The purpose of the contract is to provide the FDA, as well as other government agencies and the dietary supplements industry, with AOAC INTERNATIONAL Official Methods that are applicable to commercially available dietary supplements and their raw materials.

References

(1) Waszkuc, T., Mohammed, F., & Zhou, J. (2005) (in preparation)

(2) Furst, P., Pollack, L., Graser, T.A., Godel, H., & Stehle, P. (1990) *J. Chromatogr.* **499**, 557–569

(3) You, J., Fan, X., Wang, H., Wang, G., Su, J.X., & Zhou, C.L. (1998) *J. Liq. Chromatogr. & Rel. Technol*. **21**, 2103–2115

(4) Zhou, J., Waszkuc, T., & Mohammed, F. (2004) *J. AOAC Int.* **87**, 1083–1092

(5) *Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (2002) AOAC INTERNATIONAL, Gaithersburg, MD

(6) Snyder, L., Kirkland, J., & Glajch, J. (1997) *Practical HPLC Method Development*, 2nd Ed., John Wiley & Sons, Inc., New York, NY

(7) Reginster, J., Deroisy, R., Rovati, L., Lee, R., Lejeune, E., Bruyere, O., Giacovelli, G., Henrotin, Y., Dacre, J., & Gossett, C. (2001) *The Lancet* **357**, 251–256

(8) International Conference on Harmonization (March 1, 1994) *Fed. Regist*. **59**, 9750

(9) Lynch, J.M. (2001) *AOAC Statistical Program for Blind Replicates,* Version 1.14, Ithaca, NY