2017 AOAC OFFICIAL METHODS BOARD AWARDS

2014 - 2016 RESEARCH INSTITUTE FEEDS & FERTILIZER METHODS TO BE REVIEWED FOR

2017 METHOD OF THE YEAR

OFFICIAL METHODS OF ANALYSIS OF AOAC INTERNATIONAL

METHOD OF THE YEAR
OMB may select more than one method in this category each year.

Selection Criteria
The minimum criteria for selection are:

a. The method must have been approved for first or final action within the last three years.
b. Generally, some unique or particularly noteworthy aspect of the method is highlighted as making it worthy of the award, such as innovative technology or application, breadth of applicability, critical need, difficult analysis, and/or range of collaborators.
c. The method demonstrates significant merit in scope or is an innovative approach to an analytical problem.

Selection Process:

a. AOAC staff lists all eligible methods for consideration and forwards that list with supporting documentation (e.g. ERP chair recommendation(s)) to the Chair of the Official Methods Board (OMB).
b. The Chair forwards the list along with any supporting information to the members of the OMB.
c. The OMB selects the Method of the Year. The winner is selected by 2/3 vote. If necessary, the OMB chair may cast tie-breaking vote.

Award
An appropriate letter of appreciation and thanks will be sent to the author(s) of the winning method. The corresponding author will be announced at the appropriate session of the AOAC INTERNATIONAL annual meeting, with presentation of an award. All authors will be acknowledged at the annual meeting, will receive an award and a letter of appreciation. The name of the winner(s), with supporting story, will be carried in the announcement in the *ILM*.

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AGRICULTURAL MATERIALS

Determination of Phosphorus and Potassium in Commercial Inorganic Fertilizers by Inductively Coupled Plasma–Optical Emission Spectrometry: Single-Laboratory Validation, First Action 2015.18

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A previously validated method for the determination of both citrate–EDTA-soluble P and K and acid-soluble P and K in commercial inorganic fertilizers by inductively coupled plasma–optical emission spectrometry was submitted to the expert review panel (ERP) for fertilizers for consideration of First Action *Official Method*℠ status. The ERP evaluated the single-laboratory validation results and recommended the method for First Action *Official Method* status and provided recommendations for achieving Final Action. Validation materials ranging from 4.4 to 52.4% P_2O_5 (1.7–22.7% P) and 3–62% K_2O (2.5–51.1% K) were used for the validation. Recoveries from validation materials for citrate-soluble P and K ranged from 99.3 to 124.9% P and from 98.4 to 100.7% K. Recoveries from validation materials for acid-soluble "total" P and K ranged from 95.53 to 99.40% P and from 98.36 to 107.28% K. Values of r for citrate-soluble P and K, expressed as RSD, ranged from 0.28 to 1.30% for P and from 0.41 to 1.52% for K. Values of r for total P and K, expressed as RSD, ranged from 0.71 to 1.13% for P and from 0.39 to 1.18% for K. Based on the validation data, the ERP recommended the method (with alternatives for the citrate-soluble and the acid-soluble extractions) for First Action *Official Method* status and provided recommendations for achieving Final Action status.

Several AOAC Methods (**935.02**, **949.01**, **955.06**, **957.02**, **958.01**, **958.02**, **960.02**, **960.03**, **962.02**, **969.02**, **969.04**, **971.01**, **978.01**, and **983.02**) exist for the determination of P and K in fertilizer materials. Although the methods have worked well, most use labor-intensive methodologies (e.g., gravimetric, titrimetric, photometric, and colorimetric techniques) and several use chemical reagents that have safety concerns (1). Inductively coupled plasma (ICP) optical emission spectrometry (OES) instruments can provide simultaneous determination of P and K, whereas most existing methodologies require the separate determination of each. The primary waste generated by this method is excess sample extract solution, which can be disposed of safely in the laboratory environment, and requires only basic personal protective equipment (1).

Because most laboratories engaged in fertilizer testing have ICP-OES instrumentation, an AOAC INTERNATIONAL–approved method for P and K determination by ICP-OES for both citrate-soluble and acid-soluble P and K was established as a priority need by the Fertilizer Methods Forum. The Fertilizer Methods Forum is a meeting for stakeholders to suggest and prioritize method needs, communicate and discuss method validation results, organize and coordinate collaborative studies, and support volunteers involved in the method development and validation. The Forum also provides a venue for the evaluation of validation data (2). Bartos et al. proposed to the Fertilizer Forum a method offering alternative extractions for citrate–EDTA-soluble and acid-soluble P and K. The citrate–EDTA-soluble extraction alternative yields "direct available phosphate" and "soluble potash," whereas the acid-soluble extraction yields "total" P and K.

James Bartos, Office of the Indiana State Chemist, along with colleagues Barton Boggs, J. Harold Falls, and Sanford Siegel, completed the single-laboratory validation (SLV) and published the results (1). Validation materials ranging from 4.4 to 52.4% P_2O_5 (1.7–22.7% P) and 3–62% K_2O (2.5–51.1% K) were used for the validation.

Spike recoveries for citrate-soluble P and K ranged from 100.30 to 101.26% P and from 99.67 to 101.03% K; standard addition recoveries for citrate-soluble P and K ranged from 101.86 to 102.44% P and from 98.96 to 99.90% K; and recoveries from validation materials for citrate-soluble P and K ranged from 99.3 to 124.9% P and from 98.4 to 100.7% K. Values of r for citrate-soluble P and K, expressed as RSD, ranged from 0.28 to 1.30% for P and from 0.41 to 1.52% for K (1).

Spike recoveries for acid-soluble total P and K ranged from 98.82 to 99.63% P and from 99.97 to 100.12% K; standard addition recoveries for acid-soluble total P and K ranged from 99.23 to 100.80% P and from 101.08 to 101.65% K; and recoveries from validation materials for acid-soluble total P and K ranged from 95.53 to 99.40% P and from 98.36 to 107.28% K. Values of r for total P and K, expressed as RSD, ranged from 0.71 to 1.13% for P and from 0.39 to 1.18% for K (1).

Submitted for publication February 18, 2016.
The method was approved by the Expert Review Panel on Fertilizers as First Action.

The Expert Review Panel on Fertilizers invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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After the SLV demonstrated satisfactory accuracy, precision, ruggedness, and selectivity for both extraction alternatives in inorganic fertilizers, Bartos et al. (1) proposed the method for consideration to the AOAC ERP for fertilizers. The method protocol follows.

AOAC Official Method 2015.18
Determination of Phosphorus and Potassium in Commercial Inorganic Fertilizers
Inductively Coupled Plasma–Optical Emission Spectrometry
First Action 2015

A. Scope

This method is applicable for the determination of both citrate–EDTA-soluble P and K (Alternative A) and acid-soluble P and K (Alternative B) in commercial inorganic fertilizers by ICP-OES. Citrate–EDTA-soluble P and K (Alternative A) is directly synonymous with "available phosphate" and "soluble potash," respectively. Acid-soluble is sometimes referred to as total P and K; however, Alternative B may underestimate the total P and K content when acid-insoluble compounds are present. Values of r for citrate-soluble P and K, expressed as RSD, range from 0.28 to 1.30% for P and from 0.41 to 1.52% for K. Values of r for acid-soluble P and K, expressed as RSD, range from 0.71 to 1.13% for P and from 0.39 to 1.18% for K. *Note*: For liquid fertilizers containing phosphite and for organic fertilizers, an alternative AOAC Method such as **960.03** or **993.31** should be used because the ICP-OES will recover P that is not considered readily plant available in these materials.

Alternative A: Neutral Ammonium Citrate–Disodium EDTA–Soluble P and K using ICP-OES

B. Apparatus

(a) *Analytical balance*.—Readability to 0.1 mg, AT 200 (Mettler Toledo, Columbus, OH), or equivalent.

(b) *pH Meter*.—Readability to pH 7.00, Model 8005 (VWR Scientific, Radnor, PA), or equivalent.

(c) *pH Combination electrode*.—Orion 9102BNWP (Thermo Fisher Scientific, Waltham, MA), or equivalent.

(d) *Constant-temperature water bath*.—Capable of maintaining bath temperature of 65 ± 2°C, BK53 (Yamato Scientific, Santa Clara, CA), or equivalent.

(e) *Heated shaking water bath*.—Capable of maintaining bath temperature of 65 ± 2°C, and set to approximately 200 reciprocations/min.

(f) *ICP-OES instrument*.—Vista-PRO axial view (Agilent Technologies, Santa Clara, CA), or equivalent.

(g) *Gated riffle splitter*.—SP-177 Jones Standard Aluminum Splitter (Gilson Co., Inc., Lewis Center, OH), or splitter with equivalent or improved splitting performance (such as a rotary splitter).

(h) *Grinding mill*.—Model ZM200 rotor mill (Retsch, Haan, Germany) equipped with a 0.5 mm screen, or equivalent. Grinding to a fineness of 0.420 mm corresponding to U.S. standard sieve size No. 40 or Tyler No. 35 mesh is preferred.

C. Reagents

(a) *Ammonium citrate, dibasic*.—$(NH_4)_2HC_6H_5O_7$, formula weight (FW) 226.19, American Chemical Society (ACS) grade, purity >98% (EMD Chemicals, Darmstadt, Germany).

(b) *EDTA, disodium salt, dihydrate*.—$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, FW 372.24, purity >99% (J.T. Baker Chemicals, Center Valley, PA).

(c) *Ammonium hydroxide*.—NH_4OH, FW 35.05, 28.0–30.0% as NH_3 (Mallinckrodt Chemicals, Center Valley, PA).

(d) *Nitric acid*.—HNO_3, 67–70%, OmniTrace grade (EMD Chemicals).

(e) *Potassium dihydrogen phosphate*.—KH_2PO_4, certified at 22.73% P and 28.73% K, National Institute of Standards and Technology (NIST) 200a (Gaithersburg, MD), http://www.nist.gov/srm.

(f) *Potassium chloride*.—KCl, FW 74.55, ACS grade, purity >99% (Mallinckrodt Chemicals).

(g) *Potassium nitrate*.—KNO_3, certified at 38.66% K, NIST 193.

(h) *Triton X-100*.—Octylphenol ethoxylate (J.T. Baker Chemicals).

(i) *10 000 µg/mL beryllium (Be) standard*.—In 4% HNO_3, Cat. No. 10M5-1 (High-Purity Standards, Charleston, SC).

(j) *10 000 µg/mL Scandium (Sc) standard*.—In 4% HNO_3, Cat. No. 10M48-1 (High-Purity Standards).

(k) *Cesium chloride*.—CsCl, FW 168.36, purity >99.999% (Sigma-Aldrich, St. Louis, MO).

(l) *Lithium nitrate*.—$LiNO_3$, FW 68.95, purity >99%, (EM Science, Gibbstown, NJ).

(m) *Citrate–EDTA extraction solution (0.11 M ammonium citrate and 0.033 M disodium EDTA)*.—Weigh and completely transfer 25 g disodium EDTA (*see* **b** above) and 50 g dibasic ammonium citrate (*see* **a** above) to a 2 L volumetric flask containing approximately 1500 mL deionized (or equivalent) water. Adjust the pH to near neutral by adding 30 mL of a solution of ammonium hydroxide–water (1 + 1, v/v; *see* **c** above) in a fume hood. Adjust the final pH to 7.00 (±0.02) using a pH electrode [*see Alternative A: Neutral Ammonium Citrate–Disodium EDTA–Soluble P and K using ICP-OES*, section **B(b)**] and meter [*see Alternative A*, section **B(c)**] while adding the ammonium hydroxide–water (1 + 1, v/v) solution drop-by-drop and stirring. After obtaining a stable pH of 7.00 (±0.02), dilute to volume with deionized water and mix. Larger volumes of this solution can be prepared; however, it is susceptible to microbial degradation, resulting in a maximum shelf life of 2 weeks when stored in a dark location.

(n) *0.5% Triton-X*.—Add 1 mL Triton X-100 (*see* section **h** above) to a 200 mL volumetric flask and dilute to volume with deionized water.

(o) *Internal standard/ionization buffer (10 µg/mL Sc in 0.018 M CsCl and 4% nitric acid)*.—Add 1 mL 10 000 µg/mL Sc stock standard (*see* **j** above), 3 g CSCl (*see* **k** above), 20 mL nitric acid (*see* **d** above), and 1 mL 0.5% Triton-X (*see* **n** above) to a 1 L volumetric flask containing approximately 500 mL deionized (or equivalent) water. Dilute to volume with deionized (or equivalent) water and mix. If Be is used as an internal standard, add 4 mL of 10 000 µg/mL Be (*see* **i** above) stock standard to obtain a concentration of 40 µg/mL Be.

(p) *2000 µg/mL P as orthophosphate (PO_4)*.—Commercial custom standard prepared in a water matrix preserved with

a biocide (Inorganic Ventures, Christiansburg, VA). *Note*: a commercial stock standard preserved in acid is not acceptable because the acid will change the matrix of the pH-neutral ammonium citrate–EDTA and produce erroneous results.

(q) *3000 µg/mL K from potassium chloride*.—Commercial custom standard prepared in a water matrix preserved with a biocide (Inorganic Ventures). *Note*: a commercial stock standard preserved in acid is not acceptable because the acid will change the matrix of the pH-neutral ammonium citrate–EDTA and produce erroneous results.

D. Calibration

(a) *Standard solution*.—Prepare calibration standards from potassium dihydrogen phosphate, potassium chloride, and potassium nitrate [*see Alternative A*, sections **C(e)**, **C(f)**, and **C(g)**, respectively] as recommended in Table **2015.18A**. Several calibration standards are required because (*1*) multiple ICP-OES wavelengths are used, (*2*) some wavelengths are split into multiple calibration segments, and (*3*) a minimum of five points per curve is recommended. Table **2015.18A** provides the P and K concentrations, expressed in micrograms per milliliter, and the percentage of oxide forms.

(b) *Stock standards*.—A 2000 µg/L custom blend commercial P standard and a 3000 µg/mL custom blend commercial K standard [*see Alternative A*, sections **C(p)** and **C(q)**, respectively] can also be used, but commercial stock standards preserved in acid should not be used because the acid changes the pH and matrix of the calibration standards and can produce erroneous results. Table **2015.18B** provides the details for preparing standards from custom purchased standards.

(c) *ICP-OES calibration*.—Emission intensity for each of the calibration standards is plotted against concentration. A minimum of five calibration standards is recommended for each wavelength. Use an internal standard [*see Alternative A*, section **C(o)**] to adjust the concentration of the calibration standards and the test solutions. The recommended wavelengths, standards, concentration ranges, curve fit, and neighboring wavelengths that may produce spectral interference are listed in Table **2015.18C**. Linear regression is preferred, whenever possible. Quadratic curve fit may be necessary because of the dynamic range in fertilizer K concentration, but ensure that the curvature is not excessive as established by the manufacturer's criteria. Many ICP software programs have algorithms to detect excessive curvature of second-order or quadratic calibration curves. Alternatively, linear calibration can be achieved by removal of the high-concentration K standards; however, secondary dilution of high-concentration test solutions will be required. Dilutions must maintain the solvent matrix, which is prepared by diluting 400 mL citrate–EDTA extract solution [*see Alternative A*, section **C(m)**] to 1 L.

(d) *Empirical calibration (optional)*.—The combination of an organic solvent, high salts, and high P in the test portion can result in suppression of signal intensity. This method is designed to address these issues by matrix and aliquot dilution using the recommended pump tube configuration, plus the use of robust plasma conditions and an internal standard. However, if this recommended configuration still produces low P recoveries for the fertilizer concentrates (i.e., 40–52% P_2O_5), then empirical calibration may be necessary. Fertilizer concentrates with certified or accepted consensus values can be obtained from Laboratory Quality Services International (LQSI; http://www.sgs.com/en/mining/Analytical-Services/Proficiency-Testing-Programs-LQSi.aspx) and the Magruder (http://www.magruderchecksample.org) and Association of Fertilizer and Phosphate Chemists (AFPC; http://www.afpc.net) check sample programs. Note that calibration solutions obtained from these certified or consensus reference materials are prepared by following the recommended extraction procedure (*see Alternative A*, section **F**) and that these standards can be used

Table 2015.18A. ICP calibration standards from stock reagent salts for citrate–EDTA-soluble P and K

Standard ID	Volume, mL	Citrate, mL	Stock 1, mL[a]	Stock 2, mL[b]	P concn, µg/mL	P_2O_5, µg/mL	P_2O_5 solution, %	P_2O_5 fertilizer, %	K concn, µg/mL	K_2O, µg/mL	K_2O solution, %	K_2O fertilizer, %
Blank	1000	400	0	0	0	0	0	0	0	0	0	0
1	250	100	10 of Std 7[c]	NA	12	27.5	0.00275	1.4	15.15	18.25	0.00182	0.9
2	250	100	20 of Std 7[c]	NA	24	55	0.00550	2.7	30.3	36.5	0.00365	1.8
3	250	100	5	NA	50	115	0.01146	5.7	63.1	76	0.00760	3.8
4	250	100	10	NA	100	229	0.02291	11.5	126	152	0.01521	7.6
5	250	100	15	NA	150	344	0.03437	17.2	189	228	0.02281	11.4
6	250	100	22[d]	NA	220	504	0.05041	25.2	278	335	0.03345	16.7
7	250	100	30	NA	300	687	0.06874	34.4	379	456	0.04562	22.8
8	250	100	40	NA	400	917	0.09165	45.8	505	608	0.06083	30.4
9	250	100	50	NA	500	1146	0.11457	57.3	631	760	0.07603	38
10	250	100	NA[e]	25	NA	NA	NA	NA	747	900	0.08998	45
11	250	100	NA	30	NA	NA	NA	NA	897	1081	0.10805	54
12	250	100	NA	35	NA	NA	NA	NA	1046	1260	0.12600	63

[a] Stock 1 = 2500 µg/mL P stock standard: 2.7461 g potassium dihydrogen phosphate (KH_2PO_4)/250 mL prepared in deionized water.

[b] Stock 2 = 7472 µg/mL K stock standard: 3.5615 g potassium chloride or 4.8299 g potassium nitrate/250 mL in deionized water.

[c] Serial dilution from another standard (e.g., 10 of Std 7 = add 10 mL from Standard 7).

[d] A volume of 22 mL can be achieved by using a 15 mL and a 7 mL class A pipet, or equivalent combination.

[e] NA = Not applicable.

Table 2015.18B. ICP calibration standards from commercial custom blend stock standard solutions

Standard ID	Volume, mL	Citrate, mL	Stock 1, mL[a]	Stock 2, mL[b]	P concn, µg/mL	P_2O_5, µg/mL	P_2O_5 solution, %	P_2O_5 fertilizer, %	K concn, µg/mL	K_2O, µg/mL	K_2O solution, %	K_2O fertilizer, %
Blank	100	40	0	0	0	0	0	0	0	0	0	0
1	100	38	5 of Std 6[c]	12	12	27.5	0.00275	1.4	360	434	0.04336	21.7
2	100	36	10 of Std 6[c]	10	24	55	0.00550	2.7	300	361	0.03614	18.1
3	100	36	10 of Std 9[c]	8	50	115	0.01146	5.7	240	289	0.02891	14.5
4	100	40	5	6	100	229	0.02291	11.5	180	217	0.02168	10.8
5	100	40	8	4	160	367	0.03666	18.3	120	144	0.01441	7.2
6	100	38	12	5 of Std 6[c]	240	550	0.05499	27.5	63	76	0.00759	3.8
7	100	40	15	NA[d]	300	687	0.06874	34.4	NA	NA	NA	NA
8	100	38	20	5 of Std 6[c]	400	917	0.09165	45.8	36	43	0.00434	2.2
9	100	38	25	5 of Std 6[c]	500	1146	0.11457	57.3	18	22	0.00217	1.1
10	100	40	NA	15	NA	NA	NA	NA	450	542	0.05421	27.1
11	100	40	NA	20	NA	NA	NA	NA	600	723	0.07227	36.1
12	100	40	NA	25	NA	NA	NA	NA	750	903	0.09034	45.2
13	100	40	NA	30	NA	NA	NA	NA	900	1084	0.10841	54.2
14	100	40	NA	35	NA	NA	NA	NA	1050	1265	0.12648	63.2

[a] Stock 1 = 2000 µg/mL P as PO_4 custom stock standard [*see Alternative* A, section **C(p)**].

[b] Stock 2 = 3000 µg/mL K from KCl custom stock standard [*see Alternative A*, section **C(q)**].

[c] Serial dilution from another standard (e.g., 5 of Std 6 = add 5 mL from Standard 6).

[d] NA = Not applicable.

only for calibration within the batch of test solutions with which they were extracted. These standard extract solutions have the same shelf life (i.e., approximately 16 h) as the other fertilizer extracts and must be prepared fresh with each run. Calculations for converting the percentage P_2O_5 in these materials to milligrams per liter P are provided in the *Calculations* section (*see Alternative A*, section **H**). Fertilizer materials below 40% P_2O_5 (approximately 350 µg/mL P) typically do not experience this suppression issue, so standards below this concentration can be obtained using those listed in Tables **2015.18A** and **2015.18B**. Empirical calibration is not the preferred option and should be used as a last resort.

The test solution and internal standard/ionic buffer solution [*see Alternative A*, section **C(o)**] are blended using a Y-connector (Part No. 30703-90; Cole-Parmer, Bunker, CT) or T-connector (Part No. 116-0522-01; Bran+Luebbe, Mequon, WI) just before the nebulizer, using the conditions described in Table **2015.18D**.

E. Sample Preparation

Collect a primary field sample using one of the recommended AOAC sampling procedures (i.e., Method **929.01**, **969.01**, or **992.33**) or other recognized protocol. Prepare solid fertilizer materials by riffling [*see Alternative A*, section **B(g)**] the entire laboratory sample to select an approximate 100 g subsample. Grind the entire 100 g subsample [*see Alternative A*, section **B(h)**] to pass through a 0.50 mm mesh screen. Place the ground analytical sample into a one-quart (0.946 L) glass jar

Table 2015.18C. Calibration criteria for direct-available P and soluble K by ICP-OES

Element ID	Wavelength, nm[a]	Calibration range, µg/mL	Standards used[b]	Curve fit	Spectral deconvolution
P	177.434 (*1*)	0–100	Blank, 1, 2, 3, 4	Linear	None
P	177.434 (*2*)	100–500	4, 5, 6, 7, 8, 9	Linear	None
P	178.222 (*1*)	0–100	Blank, 1, 2, 3, 4	Linear	None
P	178.222 (*2*)	100–500	4, 5, 6, 7, 8, 9	Linear	None
P	213.618 (*1*)	0–100	Blank, 1, 2, 3, 4	Linear	Cu 213.598
P	213.618 (*2*)	100–500	4, 5, 6, 7, 8, 9	Linear	Cu 213.598
P	214.914 (*1*)	0–100	Blank, 1, 2, 3, 4	Linear	Cu 214.898
P	214.914 (*2*)	100–500	4, 5, 6, 7, 8, 9	Linear	Cu 214.898
K	766.485 (*1*)	0–126	Blank, 1, 2, 3, 4	Quadratic	None
K	766.485 (*2*)	50–379	3, 4, 5, 6, 7	Quadratic	None
K	769.897	126–505	4, 5, 6, 7, 8	Quadratic	Possible $LiNO_3$
K	404.721	505–1046	8, 9, 10, 11, 12	Quadratic	None

[a] The designators (*1*) and (*2*) are used to distinguish between the same wavelength selected twice to cover two separate concentration ranges.

[b] The standards correspond to those listed in Table **2015.18A**.

Table 2015.18D. Final ICP-OES conditions used for citrate–EDTA-soluble P and K validation

Factor	Setting
Power, kW	1.45
Plasma flow, L/min	19.5
Auxiliary flow, L/min	2.25
Nebulizer pressure, L/min	0.7
Nebulizer type	Seaspray
Spray chamber	Cyclonic
Sample pump tube	Black/black[a]
Buffer/internal standard pump tube	Gray/gray[a]
CsCl ionic buffer concn, M	0.018
Internal standard and concn, µg/mL	10
Buffer matrix	4% nitric acid
Exposure length, s	10
No. of exposures	3
Rinse time, s	35
Total analysis time, min	2

[a] An orange/white sample pump tube and a red/red buffer/internal standard pump tube provide approximately the same dilution factor, but use less volume of solution. Ensure that a sufficiently large waste pump tube is used to prevent flooding of the spray chamber.

and mix by careful rotation and inversion. For liquid materials, shake the laboratory sample vigorously to thoroughly mix. Invert and rotate the container again (for solid materials) or shake (for liquids) immediately before selecting a test portion. Other validated sample preparation techniques that result in a representative test portion are also acceptable. When the analytical sample is split or the mass is reduced for any reason, the splitting process should be validated to not introduce unintended sampling error.

F. Extraction

Weigh a ~0.5 g test portion to the nearest 0.01 g (*see Alternative A*, section **E**) and completely transfer to a 250 mL wide-mouth class A volumetric flask. Dispense 100 mL 65 ± 2°C preheated citrate–EDTA extraction solution [*see Alternative A*, section **C(m)**] into each flask and insert a rubber stopper. Shake test solutions in a 65 ± 2°C preheated water bath set to approximately 200 reciprocations/min for 60 ± 1 min, remove from the water bath, allow to cool to room temperature (20–25°C), dilute to volume with deionized (or equivalent) water, stopper, and mix. Filter any test solution containing suspended debris using P- and K-free filters. Due to a very limited shelf life, analyze test solutions within 16 h of extraction. After repeated heating and cooling cycles of the 250 mL volumetric flasks, check the calibration of the flasks by adding 250 g deionized (or equivalent) water and verify that the volume is at the meniscus. When a flask loses calibration, either use the corrected volume established by water weight, or discard it.

G. ICP-OES Conditions

The optimal instrument conditions identified during method validation of citrate–EDTA-soluble P and K are listed in Table **2015.18D**. Monitor the rinse time and buffer concentration closely, because they are sensitive to change (1).

ICP-OES instruments differ in their design and options, so minor adjustment to the conditions listed in Table **2015.18D** may be necessary; however, any adjustments to these conditions must be performance based and validated. Special attention should be paid to the recovery of P in fertilizer concentrates or fertilizers containing ≥40% P_2O_5, because these materials pose the greatest need for optimal instrument performance.

H. Calculations

Several variables exist in the instrument software for data reporting, including units, test portion weight, test solution volume, and dilution factor. The calibration standards are prepared as micrograms per milliliter P and K, and the final fertilizer results are reported as percentage P_2O_5 and K_2O, which requires the following two calculations, respectively:

$$P_2O_5, \% = [P \times (250/W) \times 142/(31.0 \times 2)]/10\,000$$

where P is the ICP-OES P reading in micrograms per milliliter, 250 is the final volume in milliliters, W is the test portion weight in grams, 142 is the FW of P_2O_5, 31.0 is the FW of P, 2 is the mole ratio of P_2O_5 to P, and 10 000 is the conversion of percentage to micrograms per milliliter; and

$$K_2O, \% = [K \times (250/W) \times 94.2/(39.1 \times 2)]/10\,000$$

where K is the ICP-OES K reading in micrograms per milliliter, 250 is the final volume in milliliters, W is the test portion weight in grams, 94.2 is the FW of K_2O, 39.1 is the FW of K, 2 is the mole ratio of K_2O to K, and 10 000 is the conversion of percentage to micrograms per milliliter.

Alternatively, the standards can be entered as equivalent theoretical percentages of P_2O_5 and K_2O in solution values, listed in Tables **2015.18A** and **2015.18B**.

When empirical calibration [*see Alternative A*, section **D(d)**] is used, conversion of the percentage P_2O_5 in the certified or consensus material to milligrams per liter P in the calibration solution is obtained by using the following equation:

$$\mathrm{P, \mu g/mL} = \%\ P_2O_5 \times 10{,}000 \times \left(\mathrm{W}/250\right) \times \left(\left(31.0 \times 2\right)/142\right)$$

where P, µg/mL is the P concentration in the extracted standard solution; % P_2O_5 is the certified or consensus value, 10 000 is the conversion of percentage to micrograms per milliliter, W is the test portion weight in grams, 250 is the final volume in milliliters, 31 is the FW of P, 2 is the mole ratio of P_2O_5/P, and 142 is the FW of P_2O_5.

I. Comments

Relative to other AOAC Methods (**960.03**, **978.01**, and **993.01**), the ICP-OES method can produce lower P recoveries and/or greater data variability (http://www.magruderchecksample.org). Critical factors and common error sources are included here. For P, three issues are critical: addressing matrix challenges, implementing robust plasma conditions, and utilizing proper standards. Carbon in the citrate and EDTA will reduce the plasma efficiency, so it must be addressed. Diluting the matrix by using a smaller sample pump

tube and a larger internal standard/ionization buffer pump tube as listed in Table **2015.18D** is the approach used in this method. Other options include (*1*) the use of oxygen addition to the argon to help combust the carbon, (*2*) a separate manual dilution of the test solutions and standards in a 4% nitric acid solution, and (*3*) a complete destruction of the carbon with a secondary digestion of the extract solution in nitric acid. Other factors that can help improve P recoveries include configurations that decrease the volume of aerosol injected into the plasma, such as a slower pump speed, slightly lower nebulizer pressure, and/or a double-pass or baffled spray chamber. Lastly, the final matrix of the calibration standards and the test solutions must match closely. Standards prepared from salts, as provided in Table **2015.18A**, have the closest match and offer the best P recoveries. When commercial stock standards are used, a source of P as PO_4^x in a matrix that will not adversely change the pH-neutral ammonium citrate–EDTA matrix are desirable. Stock standards preserved in acid solution are not recommended.

Although ruggedness testing suggested no difference in P data when Sc or Be was used as an internal standard for most fertilizer materials (1), in the case of polyphosphates, Be may result in better P recoveries because bound polyphosphates present additional challenges to the plasma that may not be detected by Sc because it is more easily ionized.

Because K is easily ionized, it generally poses fewer problems than P. The greatest challenge with K is capturing the broad concentration range found in fertilizers, because it produces an intense signal, resulting in a limited linear dynamic range. If possible, K should be read in the radial mode, and it may benefit from slightly lower nebulizer pressures and pump speeds. As described in Table **2015.18C**, the use of multiple wavelengths (766, 769, and 404 nm) and/or multiple calibration segments to cover the dynamic concentration range is recommended. Quadratic curve fit can help expand the useful range of some of these wavelengths, but great caution should be exercised to ensure that the curve falls within the sensitive response range without excessive curvature. Also, secondary dilution of high concentration test solutions can help.

Deviation from this method is not recommended, but if small revisions are necessary to accommodate differences in ICP-OES types and design, then these revisions should be validated.

Within each analytical batch of samples, inclusion of one or more certified or consensus fertilizer materials for quality control purposes is recommended, especially for the fertilizer concentrates (i.e., P_2O_5 >40% and K_2O >50%). Some sources of these materials include LQSI (http://www.sgs.com/en/mining/Analytical-Services/Proficiency-Testing-Programs-LQSi.aspx) and the Magruder (http://www.magrudercheckssample.org) and AFPC (http://www.afpc.net) check sample programs. The presumed "best practice" methods for available phosphate and soluble potash are AOAC Methods **960.03E** and **958.02**, respectively, so these consensus values should serve as the preferred reference value.

Alternative B: Acid-Soluble P and K using ICP-OES

B. Apparatus (Alternative B)

(a) *Balance*.—Readability to 0.1 mg, Sartorius BP210S (Gottingen, Germany), or equivalent.

(b) *Hot plate*.—Model 53015, Lindburg/Blue M (Watertown, WI), or equivalent.

(c) *ICP-OES instrument*.—Thermo 6500 Duo View (Thermo Scientific, Cambridge, UK), or equivalent.

(d) *Gated riffle splitter*.—SP-177 Jones Standard Aluminum Splitter (Gilson Co., Inc.), or splitter with equivalent or improved splitting performance (such as a rotary splitter).

(e) *Grinding mill*.—Model ZM200 rotor mill (Retsch), with 0.5 mm screen, or equivalent. Grinding to a fineness of 0.420 mm corresponding to a U.S. standard sieve size No. 40 or Tyler No. 35 mesh is preferred.

C. Reagents (Alternative B)

(a) *Hydrochloric acid*.—HCl, 35–38%, trace metal grade, Cat. No. A508-500 (Fisher Scientific, Pittsburgh, PA).

(b) *Ammonium dihydrogen phosphate*.—$NH_4H_2PO_4$, FW 115.03, trace metal basis, purity >99.999%, Cat. No. 204005-100G (Sigma-Aldrich).

(c) *Potassium chloride*.—KCl, FW 74.55, trace metal basis, purity >99.99%, Cat. No. 204099-250G (Sigma-Aldrich).

(d) *Scandium oxide*.—SC_2O_3, FW 137.91, Item No. OX21-5N (Stanford Materials Corp., Irvine, CA).

(e) *Nitric acid*.—HNO_3, 69.2%, certified ACS plus grade, Cat. No. A200 C212 (Fisher Scientific).

(f) *Triton X-100*.—Polyethylene glycol *p-tert*-octylphenyl ether, 4-$(C_8H_{17})C_6H_4(OCH_2CH_2)_nOH$ (*n* approximately 10), FW 624, Cat. No. BP151-500 (Fisher Scientific).

(g) *Cesium chloride*.—CsCl, FW 168.36, trace metal basis, purity >99.999%, Cat. No. 203025-50G (Sigma-Aldrich).

(h) *Lithium nitrate*.—$LiNO_3$ ReagentPlus grade, FW 68.95, Cat. No. 227986-1KG (Sigma-Aldrich).

(i) *10 000 µg/mL Be stock standard*.—In 5% HNO_3, Cat. No. PLBE-10-500 (Exaxol Corp., Clearwater, FL).

(j) *10 000 µg/mL Sc stock standard*.—Weigh 15.3374 g SC_2O_3 (*see* **d** above) into a 600 mL beaker. Add 300 mL deionized water and slowly add 100 mL nitric acid (*see* **e** above). Heat solution on a hotplate to a gentle boil, and continue boiling until the solution becomes clear.

(k) *1% Triton X*.—Pipet 10 mL Triton X-100 solution (*see* **f** above) into a 1 L flask. Dilute to volume with deionized (or equivalent) water and mix.

(l) *Internal standard/ionization buffer (60 µg/mL Sc in 0.035 M CsCl and 2% HNO_3)*.—Add 6 mL 10 000 µg Sc/mL stock standard (*see* **j** above), 6 g CsCl (*see* **g** above), 20 mL HNO_3 (*see* **e** above), and 2 mL 1% Triton X (*see* **k** above) to a 1 L flask containing approximately 500 mL deionized (or equivalent) water. Dilute to volume with deionized water and mix. If $LiNO_3$ is used as the ionic buffer, replace the CsCl with 8 g $LiNO_3$ (*see* **h** above). If Be is used as an internal standard, add 1 mL 10 000 µg/mL Be stock standard solution (*see* **i** above) to obtain a 10 µg/mL Be internal standard concentration.

(m) *4 M Hydrochloric acid digestion solution*.—Add approximately 500 mL deionized (or equivalent) water to to a 1 L volumetric flask. Slowly add 333 mL concentrated hydrochloric acid (*see* **a** above) and dilute to volume with deionized water and mix.

D. Calibration (Alternative B)

(a) *Standard solution*.—Prepare calibration standards from ammonium dihydrogen phosphate [*see Alternative B: Acid-Soluble P and K using ICP-OES* (*Alternative B*), section **C(b)**] and potassium chloride [*see Alternative B*, section **C(c)**]

as recommended in Table **2015.18E**. As with Alternative A, many calibration standards are required because (*1*) multiple ICP-OES wavelengths are used, (*2*) some wavelengths are split into multiple calibration segments, and (*3*) a minimum of five points/curve is recommended. Table **2015.18E** provides the P and K concentrations expressed as micrograms per milliliter and their percentage of oxide forms. *Note*: Better P recoveries were obtained using weighed salts [*see Alternative B*, section **C(b)**], so commercially available stock standard solutions are not recommended.

(b) *ICP-OES calibration.*—Emission intensity for each of the calibration standards is plotted against concentration. A minimum of five calibration standards is used for each wavelength. Use an internal standard [*see Alternative B*, section **C(l)**] to adjust the concentration of the calibration standards and the test solutions. The wavelengths, standards used, concentration ranges, curve fit, and wavelengths that may require spectral deconvolution are listed in Table **2015.18F**. The data in Table **2015.18F** are based on a radial view for K. When linear regression to 1000 µg/mL K is not possible, one or more of the following will be necessary: selecting quadratic curve fit (provided the curvature is not excessive), utilizing a wavelength of 404.721 nm for the five highest K calibration standards listed in Table **2015.18E**, dropping one or more of the top K standards listed in Table **2015.18E**, and/or conducting dilutions of the test solutions using 0.16 M HCl.

The test solution and internal standard/ionic buffer solutions are blended using a T-connector (Part No. 116-0522-01; Bran+Luebbe) or Y-connector (Part No. 30703-90; Cole-Parmer) just before the nebulizer, using the conditions described in Table **2015.18G**.

E. Sample Preparation (Alternative B)

Collect a primary field sample using one of the recommended AOAC sampling procedures (i.e., Method **929.01**, **969.01**, or **992.33**) or other recognized protocol. Prepare solid materials by riffling [*see Alternative B*, section **B(d)**] the entire laboratory sample to select an approximate 100 g subsample. Grind the entire 100 g subsample [*see Alternative B*, section **B(e)**] to pass through a Tyler No. 35 mesh sieve (U.S. standard sieve size No. 40, 0.420 mm or 0.165 in. opening, Fisherbrand stainless steel; Fisher Scientific). Place the ground analytical sample into a one-quart (0.946 L) glass jar and mix by careful rotation and inversion. For liquid materials, shake the laboratory sample vigorously to thoroughly mix. Invert and rotate the container again (for solid materials) or shake (for liquids) immediately before selecting a test portion. Other validated sample preparation techniques that result in a representative test portion are also acceptable. When the analytical sample is split or the mass is reduced for any reason, the splitting process should be validated to not introduce unintended sampling error.

F. Extraction (Alternative B)

Weigh ~0.5 g test portion to the nearest 0.01 g and completely transfer to a 250 mL class A volumetric flask. Slowly add 30 mL deionized (or equivalent) water to each flask. Dispense 10 mL 4 M HCl digestion solution [*see Alternative B*, section **C(m)**] into each flask. Place flasks on a preheated hotplate and gently boil for 15 ± 1 min. Remove individual flasks that have boiled for 15 ± 1 min and allow them to cool to room temperature

Table 2015.18E. ICP-OES calibration standards from stock reagent salts for total P and K

Standard ID	Volume, mL	Acid, mL[a]	Weight $NH_4H_2PO_4$, g	Weight KCl, g	P concn, µg/mL	P_2O_5, µg/mL	P_2O_5, solution, %	P_2O_5, sample, %	K concn, µg/mL	K_2O, µg/mL	K_2O solution, %	K_2O sample, %
Blank	1000	40	0	0	0	0	0	0	0	0	0	0
1	1000	40	40 of Std 6[b]	0.6305	9.8	22.4	0.00224	1	332	400	0.0400	20
2	1000	36	100 of Std 10[b]	0.4748	47	108	0.01076	5	249	300	0.0300	15
3	500	12	100 of Std 10[b]	100 of Std 14[b]	94	215	0.02153	11	163	196	0.0196	10
4	1000	32	0.4539	200 of Std 12[b]	122	280	0.02802	14	116	140	0.0140	7
5	1000	36	0.6810	100 of Std 14[b]	184	420	0.04204	21	81	98	0.0098	5
6	1000	40	0.9079	50 of Std 13[b]	245	561	0.05605	28	34.9	42	0.0042	2
7	1000	40	1.1349	25 of Std 13[b]	306	701	0.07007	35	17.4	21	0.0021	1
8	1000	40	1.3619	NA[c]	367	841	0.08408	42	NA	NA	NA	NA
9	1000	40	1.5888	NA	428	981	0.09809	49	NA	NA	NA	NA
10	1000	40	1.7510	NA	472	1081	0.10811	54	NA	NA	NA	NA
11	1000	40	NA	0.7915	NA	NA	NA	NA	415	500	0.0500	25
12	1000	40	NA	1.1079	NA	NA	NA	NA	581	700	0.0700	35
13	1000	40	NA	1.3295	NA	NA	NA	NA	697	840	0.0840	42
14	1000	40	NA	1.5511	NA	NA	NA	NA	814	980	0.0980	49
15	1000	40	NA	1.7727	NA	NA	NA	NA	930	1120	0.1120	56
16	1000	40	NA	1.9943	NA	NA	NA	NA	1046	1260	0.1260	63
17	1000	40	0.9728	0.9497	262	601	0.06006	30	498	600	0.0600	30

[a] Acid = Volume of HCl–water (1 + 2, v/v) required to make the standard.

[b] Serial dilution from another standard (e.g., 40 of Std 6 = add 40 mL of Standard 6).

[c] NA = Not applicable.

Table 2015.18F. Calibration criteria for acid-soluble, or total, P and K

Element ID	Wavelength, nm[a]	Calibration range, µg/mL	Standards used[b]	Curve fit	Spectral deconvolution
P	213.618 (*1*)	0–245	Blank, 1, 2, 3, 4, 5, 6	Linear	Cu 213.598
P	213.618 (*2*)	184–472	5, 6, 7, 8, 9, 10	Linear	Cu 213.598
P	214.914 (*1*)	0–245	Blank, 1, 2, 3, 4, 5, 6	Linear	Cu 214.898
P	214.914 (*2*)	184–472	5, 6, 7, 8, 9, 10	Linear	Cu 214.898
K	766.485 (*1*)	0–332	Blank, 1, 2, 3, 4, 5, 6, 7	Linear	None
K	766.485 (*2*)	332–1046[c]	11, 12, 13, 14, 15, 16, 17	Linear	None
K	769.897 (*1*)	0–332	Blank, 1, 2, 3, 4, 5, 6, 7	Linear	Possible $LiNO_3$
K	769.897 (*2*)	332–1046[c]	11, 12, 13, 14, 15, 16, 17	Linear	Possible $LiNO_3$

[a] The designators (*1*) and (*2*) are used to distinguish between the same wavelength selected twice to cover two separate concentration ranges.

[b] The standards correspond to those listed in Table **2015.18E**.

[c] Potassium viewed in the radial orientation.

(20–25°C). Dilute flasks to volume with deionized (or equivalent) water. Filter any test solution containing suspended debris using P- and K-free filters. The final acid strength of the test solution is approximately 0.16 M HCl, so any test solutions requiring dilution should be prepared in 0.16 M HCl and stored in a glass container. Due to a limited shelf life, all analyses should occur within 2 weeks of digestion. After repeated heating and cooling cycles of the 250 mL volumetric flasks, check the calibration of the flasks by adding 250 g deionized (or equivalent) water and verify that the volume is at the meniscus. When a flask loses calibration, either use the corrected volume established by water weight, or discard it.

G. ICP-OES Conditions (Alternative B)

Limit the deviation of a test portion weight of 0.5 g to ± 0.025 g. Because K is sensitive to nebulizer pressure/flow, closely monitor the nebulizer condition, which can deteriorate over time. Instrument conditions used for method validation of acid-soluble/total P and K are listed in Table **2015.18G**. ICP-OES instruments differ in their design and options, so minor adjustment to the conditions listed in Table **2015.18G** may be necessary; however, any adjustments to these conditions should be performance based and validated. Special attention should be paid to the recovery of P in fertilizer concentrates or fertilizers containing ≥40% P_2O_5, because these materials pose the greatest need for optimal instrument performance.

Table 2015.18G. Final ICP-OES conditions used for acid-soluble or total P and K validation

Factor	Setting
Power, kW	1.15[a]
Plasma flow, L/min	15
Auxiliary flow, L/min	1.5
Nebulizer pressure, L/min	0.40
Nebulizer type	V-grove
Spray chamber	Scott's (baffled)
Sample pump tube	Orange/white (0.64 mm id)
Buffer/internal standard pump tube	Orange/white (0.64 mm id)
CsCl concentration, M	0.035
Internal standard and concn, µg/mL	6
Buffer matrix	2% Nitric acid
Exposure length, s	10
No. of exposures	3
Rinse time, s	30
Total analysis time, min	2.4

[a] A power of 1.20 kW is required for a Thermo 6500 (Thermo Scientific) radial view.

H. Calculations

For Alternative B calculations, *see Alternative A*, section **H**.

I. Comments (Alternative B)

The 0.16 M HCl matrix used in Alternative B poses fewer analytical challenges for the ICP-OES than does the citrate–EDTA solvent used in Alternative A. If minor method modifications are necessary to accommodate different ICP-OES types or designs and/or to correct for variable or low P recoveries, the following are likely watch areas: (*1*) increasing the plasma power often benefits P, and (*2*) decreasing the volume of the aliquot injected into the plasma can also help improve recoveries of materials containing high concentrations of P. The latter can be accomplished by using a smaller sample pump tube and/or larger internal standard/ionization buffer pump tube, and/or by slightly decreasing the pump speed and/or nebulizer pressure. The final matrix of the test solutions and standards should closely match. Standards prepared from salts as provided in Table **2015.18E** provide the greatest match and offer the best P recoveries. Stock standards preserved in acid solution are not recommended. The comments provided for K in *Alternative A*, section **H** also apply to K in Alternative B.

Deviation from this method is not recommended, but if small revisions are necessary to accommodate differences in ICP-OES types and design, then these revisions should be validated.

Discussion

The ERP recommended that before First Action method publication, the method protocol should be revised to state that system optimization is based on the instrument manufacturer's recommendation to allow for all manufacturer's equipment. They also suggested the author consider incorporating an alternative

for empirical calibration procedures. ERP recommendations have been fully incorporated into the method as presented (3).

The ERP requested that R data be generated on a variety of instrument manufacturers' equipment. The R data will establish whether the method is suitable as a screening method or as a confirmatory method (3).

Acknowledgments

Recognition for this AOAC First Action method goes to James Bartos, Office of the Indiana State Chemist, who coauthored the single-laboratory validation (1) and submitted the method to the ERP for fertilizers (3). He has championed this method throughout the long and tedious process. His expertise, diligence, input, and effort were essential to properly document the method into AOAC First Action format.

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AGRICULTURAL MATERIALS

Determination of Nitrogen, Phosphorus, and Potassium Release Rates of Slow- and Controlled-Release Fertilizers: Single-Laboratory Validation, First Action 2015.15

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A previously validated method for the determination of nitrogen release patterns of slow- and controlled-release fertilizers (SRFs and CRFs, respectively) was submitted to the Expert Review Panel (ERP) for Fertilizers for consideration of First Action *Official Method*℠ status. The ERP evaluated the single-laboratory validation results and recommended the method for First Action *Official Method* status and provided recommendations for achieving Final Action. The 180 day soil incubation-column leaching technique was demonstrated to be a robust and reliable method for characterizing N release patterns from SRFs and CRFs. The method was reproducible, and the results were only slightly affected by variations in environmental factors such as microbial activity, soil moisture, temperature, and texture. The release of P and K were also studied, but at fewer replications than for N. Optimization experiments on the accelerated 74 h extraction method indicated that temperature was the only factor found to substantially influence nutrient-release rates from the materials studied, and an optimized extraction profile was established as follows: 2 h at 25°C, 2 h at 50°C, 20 h at 55°C, and 50 h at 60°C.

Slow-release fertilizers (SRFs) and controlled-release fertilizers (CRFs) are designed to gradually release nutrients at rates that can more closely match nutrient demand by plants, while potentially reducing nutrient losses to the environment through leaching, volatilization, and/or runoff. Determining the nutrient-release patterns of SRFs and CRFs is essential in the agronomic evaluation of these materials (1). Although various field techniques had been used to investigate the agronomic effectiveness of SRFs and CRFs, a critical need existed for any laboratory method(s) that could be demonstrated to correlate with field data (1). In 1994, a Controlled-Release Fertilizer Task Force was established by the Association of American Plant Food Control Officials to address issues concerning the effective regulation and analysis of SRF and CRF materials (2, 3).

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The method was approved by the Expert Review Panel on Fertilizers as First Action.

The Expert Review Panel on Fertilizers invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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The Fertilizer Methods Forum is a meeting for stakeholders to establish and prioritize method needs, communicate and discuss method validation results, organize and coordinate collaborative studies, and support volunteers involved in method development and validation. The Forum stakeholders placed a high priority on the development of any method(s) for nutrient release in SRFs and CRFs, and provided a forum for the evaluation of methods brought forth (4).

With the need for such methods well established by agronomists, industry, and regulatory communities, Carolina Medina undertook the validation of the Sartain et al. 180 day soil extraction to estimate nutrient release and the optimization and validation of a 4–7 day accelerated extraction method that resulted from the efforts of the Controlled-Release Fertilizer Task Force (5). The work was done as requirements for doctoral research at the University of Florida under the guidance of Thomas Obreza (University of Florida), Jerry Sartain (University of Florida), and William Hall (The Mosaic Co.). Medina et al.'s work was published as an evaluation of a 180 day soil extraction method to characterize N release patterns of SRFs and CRFs (1), an optimization and validation of an alternative accelerated 74 h extraction method (2), and a statistical correlation of the two extractions (6).

Method Optimization and Validation

180 Day Extraction

The effect of changes in soil/sand ratio, incubation temperature, and soil type on the 180 day soil incubation method to characterize the N release rates of various SRFs and CRFs were studied by Medina et al. (1) to establish the robustness of the method. These variables were tested on sulfur-coated urea, resin-coated NPK, polymer-sulfur-coated urea, reactive layer-coated urea, polyolefin-coated NPK, isobutylidenediurea, three types of ureaform, and biosolids.

The 180 day soil incubation-column leaching technique was demonstrated to be a robust and reliable method for characterizing N release patterns from SRFs and CRFs. The method was reproducible, and the results were only slightly affected by variations in environmental factors such as microbial activity, soil moisture, temperature, and texture. The release of P and K were also studied, but at fewer replications than for N.

Accelerated Extraction

Medina et al. (1) investigated the effect of extraction temperature, test portion mass, and extraction time on the ability of the accelerated extraction to estimate N, P, and K

release rates. Ruggedness testing was also performed using a fractional multifactorial design. Fertilizer materials used for the optimization experiments were polymer-coated urea, three types of ureaform, and two types of polymer-coated NPK. Fertilizer materials used for the ruggedness testing were polymer-coated urea, polymer-sulfur-coated urea, polyolefin-coated NPK, reactive layer-coated urea, and isobutylidenediurea,

Optimization experiments indicated that temperature was the only factor found to substantially influence nutrient-release rates from the materials studied. The optimal extraction temperature sequence that produced the most consistent and highly correlated N, P, and K release rates and showed no abnormal nutrient release due to coating deformation or fertilizer caking was determined to be:

Extraction 1.—2 h at 25°C
Extraction 2.—2 h at 50°C
Extraction 3.—20 h at 55°C
Extraction 4.—50 h at 60°C

Overall, the optimized method proved to be rugged for measuring N release rates of CRFs. The release of P and K were also studied, but at fewer replications than for N.

Method Summary

With the 180 day soil method, a fertilizer test portion is exposed to ambient temperature extractions with 0.01% citric acid in a biologically active sandy soil medium. Extractions are designed to extract and isolate nutrients becoming available over time (e.g., 7, 14, 28, 56, 140, and 180 days). Each extract is analyzed using an appropriate AOAC method (or comparable validated method) for the nutrient of interest. Cumulative nutrient released over time is calculated and release plots are graphed.

The alternative 74 h accelerated method provides an estimate of the 180 day method cumulative nutrient-release and nutrient-release plot within a time frame amenable to laboratory testing for manufacturing process control and regulatory testing to verify manufacturer label claims. Extractions are made with 0.2% citric acid and temperatures increased in a step-wise manner to accelerate the release of nutrients: 2 h at 25°C, 2 h at 50°C, 20 h at 55°C, 50 h at 60°C, and if needed, 94 h at 60°C.

AOAC Official Method 2015.15
Nitrogen, Phosphorus, and Potassium Release Rates of Slow- and Controlled-Release Fertilizers
First Action 2015

(Applicable for the determination of extractable N, P (as P_2O_5), and K (as K_2O) and cumulative N, P or K release in slow release fertilizers (SRFs) and controlled release fertilizers (CRFs).)

A. Principle

In Alternative A, a representative unground test portion is exposed to ambient temperature extractions of a solvent in a biologically active sandy soil medium. In Alternative B, a representative unground test portion is exposed to increasingly aggressive solvent temperature extractions. Extractions are designed to extract and isolate nutrients becoming available over time. Each extract is analyzed by AOAC procedures for the nutrient of interest (total N, P, and K). Along with analyses of total nutrients and reference materials, data are used to develop information specific to the cumulative percentage of nutrient released over time.

Alternative A: 180 Day Extraction at Ambient Temperature

B. Apparatus

(a) *Extraction columns*.—Extraction columns (incubation lysimeters; *see* Figure **2015.15A**) are constructed of PVC pipe (30 × 7.5 cm) fitted with a fiberglass mat in the bottom held in place by a 7.5 in. id PVC cap. The cap is fitted with a barbed plastic fitting, and vacuum tubing attached for leachate collection. A PVC cap is used on the top with no hole, but with a coating of stopcock grease to cap the lysimeter. All columns are supported on a wood frame.

(b) *Beaker*.—A 50 mL beaker is placed in the headspace of each incubation lysimeter.

(c) *Filtering flasks*.—Filtering flasks with a one-hole stopper are placed beneath the leaching columns and attached to the vacuum tubing. A pinch clamp is used to prevent leaks when filtration and leachate collection is complete.

(d) *Vacuum manifold*.—Vacuum manifold and tubing connecting each flask to a standard laboratory vacuum pump.

(e) *Riffle*.— gated or rotary.

C. Reagents and Reference Materials

(a) *Extraction solution*.—0.01% (w/v) citric acid [2 g/20 L deionized water (DI)] prepared from reagent-grade citric acid.

(b) *Ammonia trap solution*.—0.2 M H_2SO_4 solution.

(c) *Loamy, siliceous, hyperthermic, Grossarenic Paleudult soil*.—Arredondo fine sand. Particle size analysis is shown in Table **2015.15A**.

(d) *Uncoated quartz sand United States Golf Association Greens* (*USGA Mix)*.—Topdress sand (noncoated quartz), 20/30 silica sand. Available from Edgar Minerals Inc. (Edgar, FL) and Standard Sand and Silica Co. (Lynne, FL). Particle size analysis is shown in Table **2015.15B**.

(e) *Soil media*.—Mixture of 1710 g uncoated quartz sand, **C(d)**, and 90 g loamy siliceous, hyperthermic, Grossarenic Paleudult soil, **C(c)**, or similar type of local soil acting as a microbial inoculum.

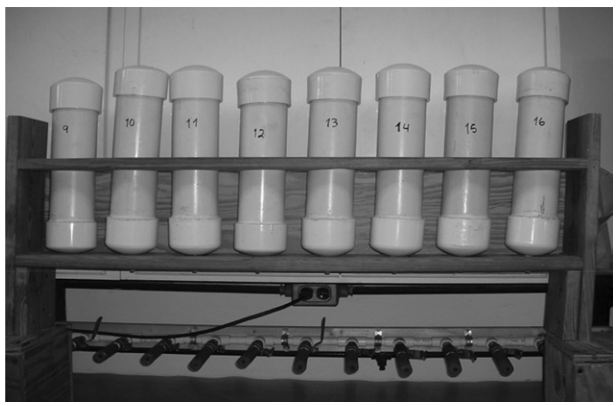

Figure 2015.15A. Incubation lysimeters.

Table 2015.15A. Particle size analysis of Arredondo fine sand[a]

Mesh (US)[b]	Opening, mm	Retained, %	Cumulative, %
5	4.000	0.0	0.0
10	2.000	0.4	0.4
20	0.850	1.2	1.6
40	0.425	12.4	14.0
100	0.150	68.7	82.7
200	0.075	14.5	97.2
−200		2.8	100.0

[a] Actual data, not specifications.

[b] United States Standard Mesh - ASTM E11:01.

D. Sample Preparation

(a) *For granular materials*.—Using a gated riffle splitter, reduce laboratory sample to yield an unground representative test portion containing approximately 450 mg of total N to mix thoroughly with the soil–sand mixture. If no N is present, a 3 (±0.1) g test portion should be used. Note: Quick release N must be limited to 600 mg N/test portion to prevent ammonia buildup in the column (thus preventing an active biological system); however, when doing so, replicates must be used to cumulatively measure at least 3.0 g total test portion mass and averaged to generate a single result. If soluble N is not limiting, 5–6 g of unground fertilizer should be used for the test portion.

(b) *For liquid materials*.—Assure the material is properly mixed and extract via pipet a representative test portion containing approximately 450 mg of total N. Mix thoroughly with the soil/sand mixture. Note: Quick release N must be limited to 600 mg N/test portion to prevent ammonia buildup in the column (thus preventing an active biological system); however, when doing so, replicates must be used to cumulatively measure at least 3.0 g total test portion mass and averaged to generate a single result . If soluble N is not limiting, 5–6 g of unground fertilizer should be used for the test portion.

E. Procedure

Test portions from each material to be tested are placed in incubation columns held at room temperature (20–25°C). The column preparation sequence is as follows: fiberglass mat, 100 g sand, then a mixture of remaining sand, soil, and test portion followed by placement of an acid trap. The sand–soil–test portion mixture is brought to 10% gravimetric moisture by adding 180 mL 0.01% citric acid. A 50 mL beaker containing 20 mL 0.2 M H_2SO_4 is placed in the headspace of the column as an ammonia trap. The solution in the ammonia trap is replaced and analyzed for NH_4-N by titration every 7 days. After 7, 14, 28, 56, 84, 112, 140, and 180 days, each column is leached at the same time of day with one pore volume (500 mL) 0.01% citric acid using a vacuum manifold. Vacuum is pulled for 2 min at 20–25" Hg vacuum (1.3 cfm) to ensure all free extraction solution is removed. Mix well and transfer to a 250 mL graduated cylinder. Record the leachate volume and remove aliquots to test for total N. In addition, measure the pH and electrical conductivity of the leachate. Retain the remaining leachate in reserve in case an additional or recheck analysis is required. Store in dark bottles and freeze if retained for more than 7 days. (*Note*: If no volatile N is detected in the ammonia trap during the first two sampling periods, the NH_4 trap can be removed and analysis for volatile N discontinued.)

Table 2015.15B. 20/30 particle size analysis of Topdress sand[a]

Mesh (US)[b]	Opening, mm	Retained, %	Cumulative, %
5	4.000	0.0	0.0
10	2.000	0.0	0.0
20	0.850	11.6	11.6
40	0.425	34.2	45.9
100	0.150	51.9	97.8
200	0.075	2.2	99.9
−200		0.1	100.0

[a] Actual data, not specifications.

[b] United States Standard Mesh - ASTM E11:01.

F. Analytical Determinations

(a) Determine total N in each of the extracts obtained using AOAC Method **993.13** (combustion), or **978.02** (modified comprehensive), or an equivalent applicable method validated in your laboratory. Use an applicable method-matched reference material in each run. Use at least three standards appropriate for the range of extract concentrations. Typically a combination of 10, 100, 1000, and 10 000 mg N/L cover the range of N in the extracts.

(b) Determine total phosphate (as P_2O_5) using AOAC Method **962.02** (gravimetric quinolinium) or AOAC Method **978.01** (automated spectrophotometric) or an equivalent applicable method validated in your laboratory. Use an applicable method-matched reference material in each run. Use internal reference standards appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg P_2O_5/L will cover the full range of P_2O_5 concentrations in the extracts.

(c) Determine soluble potash (as K_2O) using AOAC Method **958.02** sodium tetraphenylboron method or AOAC Method **983.02** (flame photometry) or an equivalent applicable method validated in your laboratory. Use an applicable method-matched reference material in each run of samples. Use internal reference standards appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg K_2O/L will cover the full range of K_2O concentrations.

Nomenclature for extraction calculation equations.—Time is measured in days and is expressed in the extract identifications as days; e.g., ex7 is the extract removed on the 7th day of incubation.

$$A_{(t)} = \%\ \text{Total nutrient/analyte}$$

where A can be N, P, or K.

ex *x* = An extract collected on a specific day (7, 14, 28, 56, 84, 140, or 180 days).

$$AC_{(\text{ex } x)} = \text{Analyte concentration (in mg/L)}$$

In extract *x* as determined in Section **F** above, where A can be N, P, or K.

$$\%AR_{(\text{ex } x)} = \%\ \text{Nutrient released during extraction } x$$

where A can be N, P, or K.

V = Volume (in mL) of respective extract collected.
W = Total unground test portion weight in g.
Calculations.—(An example calculation is provided in ref. 1.)

$$\%AR_{(ex\,x)} = \left(\frac{AC_{(ex\,x)},\ \frac{mg}{L} \times \frac{1\ g}{1000\ mg} \times V,\ mL/1000\ mL}{W,\ g \times \left(A_{(t)},\ \frac{g}{100\ g} \right)} \right) \times 100$$

G. Expression of Results

Results for each extraction are presented as cumulative percentage of total nutrient. Extraction 1 (7 days) is considered water-soluble and not an SRF. However, slowly available water-soluble materials (low-MW urea formaldehydes and methylene ureas) may be present. These materials can be analyzed directly from Extract 1.

Graphing release plots.—Plot the cumulative % of analyte (nutrient) released on the *y*-axis versus days of extraction on the *x*-axis as in Figure **2015.15B**. (The example calculations are provided in ref. 1.)

Alternative B: Accelerated 74 h Extraction at 25–60°C

H. Apparatus

(a) Covered water bath capable of maintaining a temperature of up to 60°C for extended periods. Ensure the mean temperature in the system is 50.0, 55.0, or 60.0 ± 1.0°C by monitoring incoming and exit temperatures to the manifold at comparable locations. Before Extractions 2–4 begin, it is necessary to preheat the bath several degrees (*see Extraction* section below) above the desired temperature to account for initial heat exchange and temperature equilibration with manifold and columns. The bath should be stabilized at the desired temperature within 10 min.

(b) Reversible peristaltic pump capable of delivering 4.0 (±0.1) mL/min continuously for 54 h. Pump heads capable of using 16–40 tubes are used for an 8–20 column apparatus, respectively (Lsmatc® No. 78006–00; Cole-Parmer, Vernon Hills, IL).

(c) Extraction apparatus consisting of two parts (illustrated in Figures **2015.15C**–**F**). Example equipment with sources can be found as a parts list in Appendix A of ref. 2 available on the *J. AOAC Int*. web site.

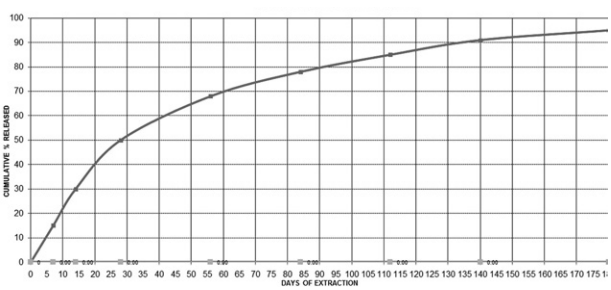

Figure 2015.15B. Example release plot showing % N released over 180 days.

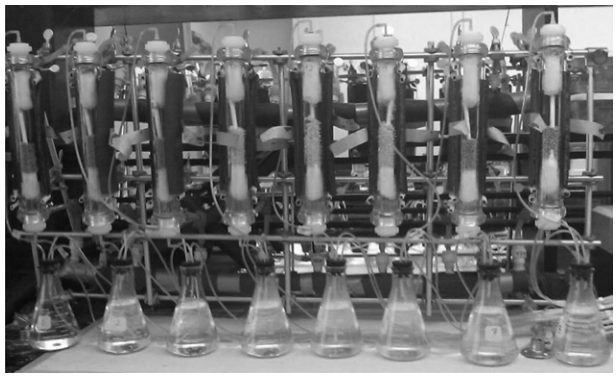

Figure 2015.15C. Extraction apparatus with eight jacketed chromatography columns.

(d) Vertical jacketed chromatography columns enclosing inner column of 2.5 × 30 cm (e.g., No. 5821–24, filter removed, with Teflon adapter No. 5838–51; Ace Glass, Vineland, NJ). PTFE rods (6 mm × 15 cm) should be used to avoid channeling of air or caking. Assure all fittings attaching column, pump tubes, and transfer tubing are secure to avoid leaks. Standardize the length of tubing for each column (typically about 75 cm). Example equipment with sources can be found as a parts list in Appendix A of ref. 2 available on the *J. AOAC Int*. web site.

(e) Constant temperature water circulation manifold and pump system capable of maintaining adequate (minimum 4 L/min) flow and stable temperature for each column. Insulation is typically required to maintain a stable temperature. Two inline, symmetrically placed thermometers (Figure **2015.15E**) are used to monitor temperature to input and outflow of manifolds. Attach roll clamps and flow monitors to column manifold tubing to ensure balanced flows and uniform temperatures. Example equipment with sources can be found as a parts list in Appendix A of ref. 2 available on the *J. AOAC Int*. web site.

(f) Solvent/extract reservoirs [500 mL volumetric flasks (e.g., Cat. No. 28100–500; Kimball Chase Life Science, Vinland, NJ)] with three-hole stoppers and properly placed rigid tubing attached to transfer tubing and to pump (*see* Figure **2015.15E**). Ensure return tube remains approximately 2 cm from the bottom of the flask to prevent pickup of any precipitates.

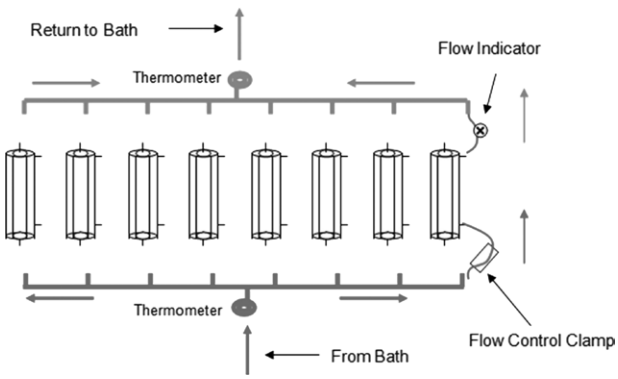

Figure 2015.15D. Schematic diagram of water manifold used in the extraction apparatus.

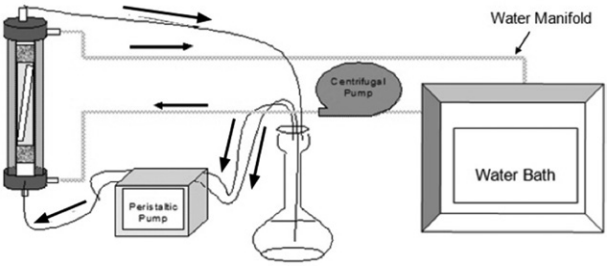

Figure 2015.15E. Schematic diagram of the extraction phase.

(g) Detection equipment capable of analyzing liquids at moderate to high (100–10 000 mg/L) nutrient levels. Analysis falling below the LOD or LOQ should be noted.

(h) 250 mL graduated cylinders (on an 8–20 column apparatus).

I. Reagents and Reference Materials

(a) *Extraction solution*.—0.2% Citric acid (w/v, 40 g/20 L DI water) prepared from reagent-grade citric acid.

(b) *Polyester fiber.*—A available in fabric or craft stores.

(c) *Wide mouth bottles*.—250 mL amber high-density polyethylene (HDPE) for sample storage.

(d) *0.08 M Cupric sulfate solution stabilizer*.—20 g $CuSO_4 \cdot 5\ H_2O$/L in 1 + 1 HCl.

(e) *Calibration standard*.—500 mg N/L, matrix-matched to the liquid extracts for AOAC **993.13**.

(f) *Matrix-matched internal reference material*.— –7 + 9 mesh IBDU.

(g) *HCl/DI water solution.*—2% for internal cleanup of equipment and tubing.

J. Sample Preparation

(a) *Homogeneous or blended materials (e.g., coated N-P-K fertilizers, granulations fertilzers, or blended fertilizers, etc.)*.—Reduce via rotary or gated riffle splitter (Jones Micro-Splitter SP-175X; Gilson Co., Inc., Lewis Center, OH) to 30.0 ± 1.0 g unground test portion. Place 3 (±0.2) g fiber [*see* Section **I(b)**] 2–3 cm above the bottom of column (do not pack), and insert PTFE rod (ThermoFisher Scientific, Pittsburgh, PA). Using powder funnel, add test portion and place 3 (±0.2) g fiber near the top of column below O-ring, but not directly on top of test portion. Ensure no test portion or fibers compromise O-ring seals. *Note*: A smaller test portion (e.g. 15g, but not less than 10g) may be used for homogeneous materials if column plugging occurs or if sample solubility constants dictate a lower sample solvent ratio to prevent solution saturation. If fine particles are escaping the column a syringe filter, type AP 20 glass fiber (2.0µm nominal pore size) in polyvinyl chloride (PVC) or polypropylene (PP) housing (e.g. EMD Millipore SLAP05010) may be added to the exit tubing just past the column to prevent material from being transferred to the reservoir.

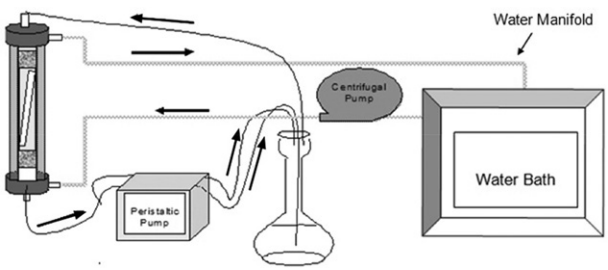

Figure 2015.15F. Schematic diagram of the collection phase.

(b) *Pellets, spikes, briquettes, etc*.—If larger than 2.5 cm, crack, crush, or break to yield pieces as large as possible that fit column (<2.5 cm). Use largest pieces equaling 30.0 ± 1.0 g and weigh to ±0.01 g. Place 3 (±0.2) g of fiber [*see* Section **I(b)**] approximately 2–3 cm above bottom of column (do not pack), insert polyethylene rod, add test portion, and place 3 (±0.2) g fiber near top of column, but not on top of test portion. Ensure no fibers compromise the O-ring seals.

(c) *For gelatinous or liquid materials*.—Assure the material is properly mixed and extract via pipet a representative test portion containing 30 ± 1.0g. Quantitatively add test portion to column, place 3g (± .2g) fiber 2–3 cm above the bottom of column (do not pack), insert PTFE rod. Add test portion, place 3g (± .2g) fiber near top of column below O ring, but not directly on top of test portion. Assure no test portion or fibers foul O ring seals. Note: A smaller test portion (e.g. 15g, but not less than 10g) may be used for homogeneous materials if column plugging occurs.

K. Extraction

Extraction sequence (examples in parenthesis).—Day 1.—Extraction 1.—2 h at 25°C (e.g., Monday 9:00 a.m.–11:00 a.m.). *Extraction 2*.—2 h at 50°C. Begin 1 h following Extraction 1 (e.g., Monday 12:00 p.m.–2:00 p.m.). *Extraction 3*.—20 h at 55°C. Begin 1 h following Extraction 2 (e.g., Monday 3:00 p.m.–11:00 am Tuesday).

Day 2.—Extraction 4.—50 h at 60°C. Begin 1 h following Extraction 3 (e.g., Tuesday 12:00 p.m.–2:00 p.m. Thursday).

Day 4.—Extraction 5 (if needed).—94 h at 60°C complete Extraction 4 (e.g., Thurs. 3:00 p.m.). Begin extraction 5, 1 h following Extraction 4 (e.g., Thursday 4:00 p.m.–2:00 p.m. Monday).

Day 7.—Complete Extraction 5; clean columns and system immediately.

(a) *Extraction 1*.—Adjust bath to maintain a temperature of 25 ± 1.0°C in columns and start circulation pump (Figure **2015.15E**). Add 475 mL extraction solution to each flask. Pump extraction solution and air from flasks to the bottom of the columns. Extract for exactly 2 h after solution reaches test portion. Swirl flask occasionally to mix solution during extraction. After 2 h, stop pump and reverse flow to top of column (Figure **2015.15F**); pump flows may be accelerated to hasten transfer process. Pump air for 1 min after liquid is emptied from column to ensure complete transfer of solution. Cool solution to 25.0°C, dilute to volume (500 mL) with 0.2% citric acid extraction solution, and mix. Transfer exactly 250 mL extract to a storage bottle; add exactly 5.0 mL stabilizing solution **I(d)**. Extracts should be stored frozen or analyzed within 21 days. Remainder of test solution can be discarded. Extract 1 is ready for analysis.

(b) *Extraction 2*.—Immediately after completion of Extraction 1, adjust bath to a temperature needed to maintain 50.0 ± 1.0°C in columns. Drain manifold(s) to preheat all

manifold water. Start circulation to stabilize temperature in entire system 15 min before beginning Extraction 2. Do not circulate water more than 5 min prior to Extraction 2. Begin Extraction 2 exactly 1 h after Extraction 1 is complete. Add 475 mL extraction solution to flasks. Pump extraction solution and air from the flasks at 4 mL/min to the bottom of columns at predetermined time. Extract for exactly 2 h after solution first reaches samples. Swirl occasionally to mix extract solution during extraction. After 2 h, stop pump and reverse flow to top of columns, pumping solution back into flasks. Pump air for 1 min after all liquid is emptied to ensure maximum transfer of solution. Cool extract to 20°C, dilute to volume with solution, and mix. Using a clean, dry graduated cylinder, transfer exactly 250 mL extract to amber HDPE bottles, and add exactly 5.0 mL stabilizing solution **I(d)**. Extract is now ready for analysis. Keep all remaining 250 mL of solution in flasks to be used in next extraction. Add approximately 225 mL freshly prepared extraction solution to flasks, bringing total volume to approximately 475 mL.

(c) *Extraction 3*.—Immediately after completion of Extraction 2, adjust bath to a temperature needed to maintain 55.0 ± 1.0°C in columns. Drain manifold(s) to preheat all manifold water. Start circulation to stabilize temperature in entire system 15 min before beginning Extraction 2. Do not circulate water more than 5 min prior to Extraction 3. Begin Extraction 3 exactly 1 h after Extraction 2 is complete. Remainder of Extraction 3 is identical to Extraction 2 except extraction time is exactly 20 h.

(d) *Extraction 4*.—Immediately after completion of Extraction 3, adjust bath to a temperature needed to maintain 60.0 ± 1.0°C in columns. 1 h after completion of Extraction 3, begin Extraction 4. Remainder of Extraction 4 is identical to Extraction 2 except extraction time is exactly 50 h.

(e) *Extraction 5 (if needed)*.—1 h after completion of Extraction 4, begin Extraction 5. Extraction 5 is identical to Extraction 4 except extraction time is exactly 94 h.

Following removal of the test portion, clean columns in place with a large brush. If there is buildup or precipitation in columns or tubing, flush by circulating 2% HCl through system for 5 min. Follow with two 5 min DI water washes. If there is no buildup, water washes are sufficient. Allow columns to dry before placing new packing and samples in column for next run.

L. Analytical Determinations

Determine nutrients of interest (e.g., N, P, and K) on each of the extracts obtained.

(a) Determine Total N using AOAC Method **993.13** (combustion) or AOAC Method **978.02** (modified comprehensive) or other equivalent applicable methods validated in your laboratory. Use an applicable method-matched reference material in each run. Use an internal reference standard appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg N/L will cover the full range of N concentrations.

(b) Determine total phosphate (as P_2O_5) using AOAC Method **962.02** or AOAC Method **978.01** or equivalent applicable methods validated in your laboratory. Use an applicable method-matched reference material in each run. Use an internal reference standard appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg P_2O_5/L will cover the full range of P_2O_5 concentrations.

(c) Determine soluble potash (as K_2O) using AOAC Method **958.02** (STPB) or AOAC Method **983.02** (flame photometry) or equivalent applicable methods validated in your laboratory. Use an applicable method-matched reference material in each run of samples. Use an internal reference standard appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg K_2O/L will cover the full range of K_2O concentrations.

M. Expression of Results

Results for each extraction are presented as a cumulative percentage of total nutrient. Extraction 1 is water-soluble and not a slow-release fraction. However, slowly available water-soluble materials (low-MW urea formaldehydes and methylene ureas) may be present. These materials can be analyzed directly from Extraction 1 and expressed according to their definition.

N. Calculations and Graphing the Release Plot

Nomenclature for extraction calculations.—$A_{(t)}$ = % Total nutrient/analyte determined as in the *Analytical Determinations* section above, where A can be N, P, or K.

$AC_{(ex1)}$ = Concentration (in mg/L) of nutrient/analyte in Extract 1, where A can be N, P, or K.

$\%AR_{(ex1)}$ = % Nutrient released during Extraction 1, where A can be N, P, or K.

$AC_{(ex2)}$ = Concentration (in mg/L) of nutrient in Extract 2, where A can be N, P, or K.

$\%AR_{(ex2)}$ = % Nutrient released during Extraction 2, where A can be N, P, or K.

$AC_{(ex3)}$ = Concentration (in mg/L) of nutrient in Extraction 3, where A can be N, P, or K.

$\%AR_{(ex3)}$ = % Nutrient released during Extraction 3, where A can be N, P, or K.

$AC_{(ex4)}$ = Concentration (in mg/L) of nutrient in Extraction 4, where A can be N, P, or K.

$\%AR_{(ex4)}$ = % Nutrient released in Extraction 4, where A can be N, P, or K.

$A_{(tr)}$ = % Total release of all extractions, where A can be N, P, or K.

$A_{(ne)}$ = % Controlled release (nutrient not extracted, calculated below), where A can be N, P, or K.

$A_{(cr)}$ = % Controlled release (nutrient released in Extractions 2–4), where A can be N, P, or K.

W = Total unground test portion weight in g.

%TAR = Total nutrient (analyte) released, where A can be N, P, or K.

WIN = Water-insoluble N.

1.02 = Dilution factor (due to addition of the preservative) = 255 mL/250 mL.

V = Total volume of respective extract in L.

Calculations.—Note: An example calculation can be found in Appendix B of ref. 2 available on the *J. AOAC Intl*. Web site.

$$\%AR_{(ex1)} = \left(\frac{AC_{(ex1)}, \frac{mg}{L} \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%AR_{(ex2)} = \left(\frac{AC_{(ex2)}, \frac{mg}{L} \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%AR_{(ex3)} = \left(\frac{\left(AC_{(ex3)}, \frac{mg}{L} - \left(\frac{AC_{(ex2)}, \frac{mg}{L}}{2} \right) \right) \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%AR_{(ex4)} = \left(\frac{\left(AC_{(ex4)}, \frac{mg}{L} - \left(\frac{AC_{(ex3)}, \frac{mg}{L}}{2} \right) \right) \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%TAR = \left(\%AR_{(ex1)} \right) + \left(\%AR_{(ex2)} \right) + \left(\%AR_{(ex3)} \right) + \left(\%AR_{(ex4)} \right)$$

$$\%WIN = \left(A_{(t)}\% \right) - \left(\left(A_{(t)}\% \right) \left(\frac{\%AR_{(ex1)}}{100} \right) \right)$$

Graphing the release plot.—Plot the cumulative % of analyte (nutrient) released on the *y*-axis versus the time in hours of extraction on the *x*-axis. The release plot will be similar to Figure **2015.15B**, except for a change in the units on the *x*-axis from cumulative days of extraction to cumulative hours of extraction. An example graph can be found in Appendix B of ref. 2 available on the *J. AOAC Int*. web site.

Discussion

Medina et al.'s validated methods, including alternatives for a 180 day extraction and an accelerated extraction (1, 2), were submitted by William Hall to the AOAC Fertilizer Expert Review Panel (ERP), and adopted as First Action. Extensive method development and validation data supporting both alternatives as documented in Medina et al.'s studies (1, 2, 6) was further reviewed by the ERP. The validation was deemed thorough by the ERP, demonstrated that the method was scientifically sound, and confirmed that such a method is needed by the community.

Method reproducibility for both alternatives is proposed to be determined via collaborative study. The ERP asked that in addition to solid fertilizers, reproducibility data for liquid fertilizers be generated. The ERP also asked that the 180 day ambient method be clarified for its application to coated fertilizers (not including sulfur-coated) as reproducibility data are generated. It was noted that it might be difficult to obtain reproducibility data on the 180 day extraction within 2 years due to the long analysis time.

The collaborative studies will provide reproducibility data for N, P, K, and possibly other nutrients.

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(1) Medina, L.C., Sartain, J.B., Obreza, T.A, Hall, W.L., & Thiex, N.J. (2014) *J. AOAC Int.* **97**, 643–660. http://dx.doi.org/10.5740/jaoacint.13-065

(2) Medina, L.C., Sartain, J.B., Obreza, T.A, Hall, W.L., & Thiex, N.J. (2014) *J. AOAC Int.* **97**, 661–676. http://dx.doi.org/10.5740/jaoacint.12-482

(3) Association of American Plant Food Control Officials, Inc. (1995) *AAPFCO Official Publication No. 48*, D.L. Terry (Ed.), West Lafayette, Indiana.

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(5) Sartain, J.B., Hall, W.L., Littell, R.C., & Hopwood, E.W. (2004) in *Environmental Impact of Fertilizer on Soil and Water*, W.L. Hall & W.P. Robarge (Eds), Oxford University Press, Washington, DC, pp 180–195

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FOOD COMPOSITION AND ADDITIVES

Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method: Collaborative Study

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Starch, glycogen, maltooligosaccharides, and other α-1,4- and α-1,6-linked glucose carbohydrates, exclusive of resistant starch, are collectively termed "dietary starch". This nutritionally important fraction is increasingly measured for use in diet formulation for animals as it can have positive or negative effects on animal performance and health by affecting energy supply, glycemic index, and formation of fermentation products by gut microbes. AOAC Method 920.40 that was used for measuring dietary starch in animal feeds was invalidated due to discontinued production of a required enzyme. As a replacement, an enzymatic-colorimetric starch assay developed in 1997 that had advantages in ease of sample handling and accuracy compared to other methods was considered. The assay was further modified to improve utilization of laboratory resources and reduce time required for the assay. The assay is quasi-empirical: glucose is the analyte detected, but its release is determined by run conditions and specification of enzymes. The modified assay was tested in an AOAC collaborative study to evaluate its accuracy and reliability for determination of dietary starch in animal feedstuffs and pet foods. In the assay, samples are incubated in screw cap tubes with thermostable α-amylase in pH 5.0 sodium acetate buffer for 1 h at 100°C with periodic mixing to gelatinize and partially hydrolyze α-glucan. Amyloglucosidase is added, and the reaction mixture is incubated at 50°C for 2 h and mixed once. After subsequent addition of water, mixing, clarification, and dilution as needed, free + enzymatically released glucose are measured. Values from a separate determination of free glucose are subtracted to give values for enzymatically released glucose. Dietary starch equals enzymatically released glucose multiplied by 162/180 (or 0.9) divided by the weight of the as received sample. Fifteen laboratories that represented feed company, regulatory, research, and commercial feed testing laboratories analyzed 10 homogenous test materials representing animal feedstuffs and pet foods in duplicate using the dietary starch assay. The test samples ranged from 1 to 70% in dietary starch content and included moist canned dog food, alfalfa pellets, distillers grains, ground corn grain, poultry feed, low starch horse feed, dry dog kibbles, complete dairy cattle feed, soybean meal, and corn silage. The average within-laboratory repeatability SD (s_r) for percentage dietary starch in the test samples was 0.49 with a range of 0.03 to 1.56, and among-laboratory repeatability SDs (s_R) averaged 0.96 with a range of 0.09 to 2.69. The HorRat averaged 2.0 for all test samples and 1.9 for test samples containing greater than 2% dietary starch. The HorRat results are comparable to those found for AOAC Method 996.11, which measures starch in cereal products. It is recommended that the dietary starch method be accepted for Official First Action status.

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The method was approved by the Expert Review Panel for Dietary Starch.

The Expert Review Panel for Dietary Starch invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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Starch is an important, frequently analyzed component of animal feedstuffs. It can have substantial positive effects on animal performance and potential undesirable effects on glycemic response and animal health (1). AOAC *Official Method*℠ **920.40** for starch in animal feeds (2) is no longer valid because of discontinued production of the enzyme "Rhozyme-S" (Rohm and Haas, Philadelphia, PA) specified in the procedure. Accordingly, another approved method for starch in animal feeds is needed. Additionally, new terminology is needed to define "starch" to more accurately describe the nutritionally relevant fraction of interest, and the definition can be used to specify the analysis.

Starch has long been defined as a natural vegetable polymer consisting of long linear unbranched chains of α-1,4-linked D-glucose units (amylose) and/or long α-1,6-branched chains of α-1,4-linked glucose units (amylopectin; 3). However, the amylases and amyloglucosidases that specifically hydrolyze the linkages in plant starch also hydrolyze those same linkages in glycogen from animal (4) or microbial (5) sources and in maltooligosaccharides that are breakdown products of starch

but are not polysaccharides. Accordingly, enzymatic starch methods do not measure plant starch alone (6), unless animal and microbial ingredients and the feedstuffs that contain them are excluded from analysis. From a nutritional standpoint, inclusion of glycogen, starch, and maltooligosaccharides more completely describes the pool of carbohydrate that is potentially available to digestion by salivary or small intestinal amylases or amyloglucosidases (7), but the pool can not be called "starch" because that term is well established as referring to a plant polysaccharide.

Recognizing the aim of nutritional characterization, the Laboratory Methods & Services Committee of the Association of American Feed Control Officials with involvement of researchers and industry arrived at a definition for "Dietary Starch": An alpha-linked-glucose carbohydrate of or derived from plants, animals, or microbes from which glucose is released through the hydrolytic actions of purified α-amylases and amyloglucosidases that are specifically active only on α-(1-4) and α-(1-6) linkages in feed materials that have been gelatinized in heated, mildly acidic buffer. Its concentration in feed is determined by enzymatically converting the α-linked glucose carbohydrate to glucose and then measuring the liberated glucose. This definition encompasses plant starch, glycogen, maltooligosaccharides, and maltose/isomaltose. The use of mildly acidic buffer for the gelatinization excludes the use of alkali or dimethyl sulfoxide and, thus, excludes resistant starch from inclusion in the dietary starch fraction.

The proposed dietary starch method avoids known analytical defects and allows handling of diverse physical forms of samples. It is based on an assay published by Bach Knudsen (8) that was slightly modified to improve use of laboratory resources, reduce run time, and maintain starch recovery (9). It is similar in chemistry to AOAC Method **996.11** (10), but differs in the buffer used and in sample handling procedures and gave a greater recovery of starch (9). Specific to the dietary starch assay, all enzymatic reactions are carried out in an acidic buffer that improves recovery by limiting the production of maltulose, an isomerization product produced at more neutral pH (11). Maltulose is resistant to enzymatic hydrolysis and reduces starch recovery. The use of a screw cap tube as a reaction vessel allows for more vigorous mixing, which is useful for all types of feed materials but may be essential for those that clump, are moist, or do not behave like dry, ground powders. Although enzymes used in development of the method will be listed, learning from the loss of AOAC Method **920.40** (2), this assay will not be set to use specific commercial enzymes but rather enzymes with specific activity that give desired results under the conditions of the method. The detection method specified is a colorimetric glucose oxidase-peroxidase method based on an assay developed by Karkalas (12), but recommendations are made to use other approved chromatographic analyses if interferences such as antioxidants are present.

Collaborative Study

Method Performance Parameters and Optimization

The performance parameters of the dietary starch procedure were investigated by the Study Director, who developed the method evaluated in this study. The following factors were evaluated:

(*1*) *Repeatability.*—As tested previously in a single laboratory, the SDs of within laboratory replicates for dietary starch analysis of food and feed substrates were low (dietary starch mean = 46.9%, s_r = 0.48%; dry matter basis; 9).

(*2*) *LOD.*—LOD for the dietary starch assay was calculated from absorbance values as the mean reagent blank value + 3 × SD (13). The means and SD were calculated for the absorbances of duplicate readings for seven undiluted with-enzyme reagent blanks from six separate assay runs. For each reagent blank, the value of the mean absorbance + 3 SD was used in the glucose standard curve determined for that run to calculate the detected glucose value. This value was multiplied by the final reaction volume (51.1 mL), by 162/180 to convert glucose to a starch basis, and converted to g. The calculated dietary starch LOD are 0.3% of sample weight based on analysis of a 100 mg test portion.

(*3*) *Accuracy/recovery.*—Recovery of pure corn starch was determined on samples analyzed singly in five separate analytical runs and in duplicate in an additional run. The average recovery ± SD was 99.3 ± 0.8% on a dry matter basis. In the collaborative study, the average dietary starch value for the control corn starch sample was 89.9 ± 3.7% on an as received basis with an estimated actual value of 89.4%.

(*4*) *Linearity.*—Linearity of the dietary starch assay was evaluated on a dry matter basis using purified corn starch samples weighing 25, 50, 75, and 100 mg analyzed on 3 separate days. The effect of starch amount tended to have a linear effect on recovery (P = 0.07), but the difference was small at a maximum of 2 percentage units between the highest and lowest recoveries. The least squares means ± SD for recovery were 101.9 ± 1.7, 99.9 ± 0.2, 100.3 ± 0.4, and 100.0 ± 0.7% for 25, 50, 75, and 100 mg of corn starch, respectively.

(*5*) *Specificity.*—The dietary starch method gave very low values (mean ± SD) for sucrose (0.17 ± 0.00% of sample dry matter), α-cellulose (0.03 ± .02% of air dried sample), and isolated oat beta-glucan (0.31 ± 0.09% of air dried sample), indicating that run conditions and enzyme preparations used did not appreciably hydrolyze these feed components. Sucrose, in particular, has been shown to interfere with starch analysis (14), likely due to side activity of the enzyme preparations used. Use of separate free glucose determinations allows correction for free glucose and background absorbance associated with each sample. The final detection method, the glucose oxidase − peroxidase (GOPOD) method, is specific for glucose, which limits interference from other carbohydrates.

(*6*) *Interference.*—Antioxidants can depress glucose detection in the GOPOD assay. Addition of ascorbic acid as a model antioxidant gave a linear decrease in absorbance at additions of greater than 10 μmoles of ascorbic acid (15). The effect was relatively small up to 10 μmol of ascorbic acid. Investigations into the antioxidant content of foodstuffs (16) showed that most of the high starch or leafy vegetable foods had hydrophilic antioxidant values that would be equivalent to less than 10 μmoles of ascorbic acid/0.1 g of dry matter. Exceptions included foods high in phenolic compounds (e.g., beets and red sorghum grain with antioxidant content approximately equivalent to 23 and 14 μmol ascorbic acid, respectively). Because of the interference in the GOPOD assay, another method for measuring glucose should be considered for feeds or foods exceeding 10 to 20 μmol of hydrophilic antioxidant/0.1 g of test sample dry matter.

(*7*) *Use of quadratic standard curves.*—The standard curves in the GOPOD assay are slightly nonlinear, and this is normal for this assay within the glucose concentrations commonly used (15). The linear equations describing glucose standard curves had R^2 of nearly 1.0 (0.9998 to 1.0) suggesting a very good fit to the linear form but the intercepts were not 0. Thus, when the standard curves were used to predict glucose concentrations of the standard solutions used to produce them, the predicted values frequently differed slightly from the expected values. It was determined that a quadratic form fit the standard curves better than a linear form based on significance of the quadratic term in the regression equation, the reduction in the root mean squared error of the standard curve and the relative decrease in residual sums of squares (residual = observed minus predicted) between the linear and quadratic equations, and evaluation of the residual versus predicted value plots (15). Other nonlinear forms were not explored.

(*8*) *Determination of final volume by summation of liquid additions.*—The method uses summing of added reagent volumes or use of volumetric flasks to give the final volume of test solutions before dilution. Total volumes of test solutions and dilutions can be determined by summing of added volumes if accurately quantitative volumetric pipets and dispensers are used to add reagents. An evaluation of summation of volumes and determination of final volume by weight and density showed no difference in recovery of glucose ($P = 0.21$) or of corn starch ($P = 0.62$) analyzed with the dietary starch assay. The density of test sample solutions and reagent blanks appears to be quite consistent (0.999 g/mL, SD = 0.002, $n = 120$ from 16 analysis runs over 16 months). Accuracy of reagent additions can be determined by the final weight of total added liquid [(weight of tube + test sample + liquid) minus (weight of tube + test sample)]. The weights of total added liquid are 49.9 and 51.0 g for the portions of the assay run without or with enzyme additions, respectively. The deviations from these values should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. Alternatively, after the addition of water, test solutions can be quantitatively transferred with filtration through Whatman (Florham Park, NJ) 54 or equivalent paper into 100 mL volumetric flasks and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

(*9*) *Ease of use/efficiency.*—The method has the advantage that all reagent additions are made to samples in tubes that can be handled in racks. It does not require transfer of sample until the final dilution and measurement of glucose. Vortexing of the sealed tubes rinses the entire interior of the tube with solution, thus minimizing the possibility that test samples will escape contact with reagents. Studies verified the acceptability of using the same temperature for the amyloglucosidase digestion and glucose analysis incubations (15), which allowed more economic use of laboratory resources.

(*10*) *Use of control samples.*—The use of glucose and corn starch as control samples allows evaluation of quantitative recovery, and starch allows evaluation of quantitative recovery and efficacy of the assay.

(*11*) *Evaluation of enzymes for suitability.*—It is essential that the enzymes and run conditions used release only glucose bound by α-1,6- and α-1,4-linkages and give close to 100% recovery of corn starch. Sucrose is the most common interfering carbohydrate encountered in feedstuffs (14) typically due to its hydrolysis through side activity of the enzyme preparations used. Though the run conditions used will not hydrolyze sucrose, commonly available enzyme preparations have activity that can and are thus unsuitable for this assay. Analysis of glucose, corn starch, and sucrose with candidate enzymes should give values (mean ± SD) of glucose 90 ± 2%, starch 100 ± 2%, and sucrose 0.7 ± 0.3% on a dry matter basis. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%) or background absorbance readings will interfere with test sample measurements.

(*12*) *Method of glucose detection.*—The dietary starch protocol specifies use of an enzymatic-colorimetric assay that has been found to be very precise (15). However, it also allows use of other AOAC-approved glucose-specific assays that have been proven in laboratory validation to be appropriate for the dietary starch assay. On this basis, qualifying assays that are devoid of interference and are, thus, more suitable for use on specific matrixes, or are preferred in a given laboratory may be used.

Collaborating Laboratories

The 15 laboratories that participated in the study represented eight regulatory laboratories, three commercial feed testing laboratories, two feed company laboratories, and two research laboratories. One each of research, commercial feed testing, and regulatory laboratories that expressed interest in participating did not complete the study. Participating laboratories received no compensation. Collaborators were provided with blind test samples, control glucose and corn starch, thermostable α-amylase (Multifect AA 21L, Genencor International, Rochester, NY), amyloglucosidase (E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland), glucose standards, electronic data sheets, and larger reaction tubes if needed. They were required to prepare the GOPOD reagent, perform the dietary starch assay as written, analyze test samples in duplicate, and provide comments and detailed result forms containing both raw and calculated data describing their analyses of three blind familiarization test materials, 10 blind collaborative study test materials, and control samples for dietary starch.

Materials

Test materials selected for the collaborative study covered a wide range of dietary starch contents, ranging from 1 to 69% on an as-received basis and derived from single batches of manufactured and commodity feedstuffs used with different animal species. The test sample grinding and homogenizing methods used were designed to produce materials that would pass a 40 mesh screen. By virtue of their diverse handling characteristics, a number of different methods were used to prepare the samples for analysis. Corn silage, poultry feed, low starch horse feed, and alfalfa pellets were ground through the 6 mm screen of a cutting mill (Pulverisette 19, Fritsch GmbH, Idar-Oberstein, Germany) and then processed through the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife, Retsch GmbH, Haan, Germany). Dry corn, soybean meal, and distillers grains were ground to pass the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife), as

Table 1. Homogeneity of dietary starch for four sample sets of each test material[a]

Material	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	2.8 × s_r	2.8 × s_R	HorRat
Moist canned dog food	4	1.58	0.01	0.02	0.86	1.06	0.04	0.05	0.29
Low starch horse feed	4	7.17	0.06	0.11	0.85	1.56	0.17	0.31	0.53
Dry ground corn	4	72.70	0.34	0.34	0.46	0.46	0.95	0.95	0.22
Complete dairy feed	4	28.38	0.10	0.40	0.34	1.40	0.27	1.11	0.58
Soybean meal	4	1.17	0.05	0.05	3.94	3.94	0.13	0.13	1.01
Distillers grains	4	4.23	0.06	0.06	1.37	1.37	0.16	0.16	0.43
Pelleted poultry feed	4	28.50	0.32	0.32	1.14	1.14	0.91	0.91	0.47
Corn silage	4	41.15	1.06	1.06	2.58	2.58	2.98	2.98	1.13
Dog kibble, dry	4	27.82	0.95	1.01	3.43	3.64	2.67	2.83	1.50
Alfalfa pellets	4	1.46	0.04	0.05	3.06	3.18	0.12	0.13	0.84

[a] s_r = SD of repeatability within sample; s_R = SD within and among sample sets; RSD_r = repeatability SD; RSD_R = reproducibility SD.

was the textured dairy complete feed but with dry ice used in the grinding of this sample. Dog kibble was ground with a kitchen processing mill (Assistent, MagicMill, Upper Saddle River, NJ) and further processed through a blending mill (1095 Knifetec sample mill, Foss Tecator, Höganäs, Sweden). The moist, canned dog food was homogenized with a commercial blender (Waring laboratory blender, 14-509-66, Fisher Scientific, Pittsburgh, PA). Dry ground test samples were subsampled using a rotary splitter (Laborette 27, Fritsch GmbH) and stored at –20°C in vacuum sealed bags (3.5 mil nylon polyethylene standard barrier vacuum bag, DCE, Inc., Springville, CA) until shipment. Homogenized moist dog food was transferred to individual sealed plastic bags (Whirl-Pak 58 mL, B01009WA, Nasco, Fort Atkinson, WI) and stored at –20°C. Test sample weights/bag were approximately 20 g for dried ground samples and 25 g of homogenized moist dog food.

For the collaborative study, individual test samples were labeled with a letter. Dry test samples and control samples were packed together in a sealed plastic bag. The homogenized moist dog food test sample and enzymes were packaged in an insulated container with a frozen ice pack. Materials were shipped overnight to the laboratories with directions to place the homogenized moist dog food test sample in the freezer until analysis. That sample was to be thawed overnight at 4°C, and all analyses in the dietary starch procedure were to be performed on it on the following day; no such limitations were placed on analyses of the dry test samples.

As per the example of Mertens (17), dietary starch analyses in duplicate of four randomly selected samples of each test material were used to evaluate random variation within and among samples. In this application, the SD of repeatability within sample (s_r) and SD of reproducibility among laboratories (s_R) calculated using the AOAC spreadsheet designed for evaluating collaborative studies represent the variation within and between separate samples of test materials as tested in the Study Director's laboratory. The s_r and s_R were similar within each sample, indicating that the prepared test samples were homogenous (Table 1). The HorRat values for corn silage and dog kibble were greater than 1.1. As concluded in a similar evaluation (17), these results suggest that these samples were less homogenous or for some reason more difficult to analyze for dietary starch than the other samples. For the dog kibble test sample, small dark particles that did not dissolve or degrade and had the coloration of one form of kibble present in the original unground material were visible in the acetate buffer during incubations.

Statistical Analyses

Data from all laboratories were reviewed for data entry and calculation errors before statistical evaluation, and results were reverified if values were identified as outliers. Ranking scores (18) were used to identify laboratories that were outliers across all materials. Data from the one such identified laboratory were excluded from further data analysis.

The AOAC INTERNATIONAL Interlaboratory Study Workbook for Evaluation of Blind Duplicates (Version 2.0, 2006) spreadsheet was used to evaluate data from the collaborative study and from the homogeneity test performed in the Study Director's laboratory.

AOAC Official Method 2014.10
Dietary Starch in Animal Feeds and Pet Food
Enzymatic-Colorimetric Method
First Action 2014

(Applicable for the determination of dietary starch in forages, grains, grain by-products, dry, semi-moist, and moist pet food products, and mixed feeds that range in concentration from 1 to 100%.)

Caution: Acetic acid is flammable in both liquid and vapor forms. It can cause severe skin burns and eye damage and is toxic if inhaled. Avoid breathing fumes. Wear protective gloves, clothing, and eye and face protection.

α-Amylase and glucose oxidase are respiratory sensitizers, which may cause allergy or asthma symptoms. Avoid breathing dust. Amylase preparations can cause allergic reactions in hypersensitive individuals. Avoid inhaling aerosols or dusts.

Benzoic acid causes serious eye damage and respiratory irritation. Avoid breathing dust and mist. Wear eye protection.

Phenol can be toxic and cause severe burns and eye damage. It is suspected of causing genetic defects and may cause damage to organs. Do not breathe dust or fumes. Wear protective gloves, clothing, eye protection, and face protection.

See Table **2014.10** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Ground or homogenized animal feed and pet food test portions are mixed with acetic acid buffer and heat-stable α-amylase and incubated at 100°C for 1 h with periodic mixing to gelatinize and partially hydrolyze the starch. After cooling, amyloglucosidase is added and the test mixture is incubated for 2 h at 50°C. The digested mixture is clarified, diluted as needed, and glucose detected in the resulting test solution using a colorimetric glucose oxidase-peroxidase (GOPOD) method that is sensitive and specific to glucose. Free glucose is measured simultaneously or in a separate analytical run for each test sample by carrying a second test portion of each test sample through the procedure omitting enzymes and incubating at 100°C for 1 h with periodic mixing. Dietary starch is determined as 0.9 times the difference of glucose in the digested test portion minus free glucose in the undigested test portion.

B. Apparatus

(**a**) *Grinding mill*.—Mills such as an abrasion mill equipped with a 1.0 to 0.5 mm screen, or a cutting mill with 0.5 mm screen, or other appropriate device to grind test samples to pass a 40 mesh screen.

(**b**) *Homogenizer, blender, or mixer*.—To provide homogenous suspension of canned pet food, liquid animal feed, semi-moist pet food, and other materials containing less than 85% dry matter.

(**c**) *Bench centrifuge or microcentrifuge.*—Capable of centrifuging at 1000 × *g* to 10 000 × *g*.

(**d**) *Water bath.*—Capable of maintaining 50 ± 1°C.

(**e**) *Vortex mixer.*

(**f**) *pH meter.*

(**g**) *Stop clock timer (digital).*

(**h**) *Top-loading balance*.—Capable of weighing accurately to ±0.01 g.

(**i**) *Analytical balance.*—Capable of weighing accurately to ±0.0001 g.

(**j**) *Laboratory ovens.*—With forced-convection; capable of maintaining 100 ± 1°C for carrying out incubations.

(**k**) *Spectrophotometer*.—Capable of operating at absorbances of 505 nm.

(**l**) *Pipets.*—Capable of delivering 0.1 and 1.0 mL; with disposable tips.

(**m**) *Positive-displacement repeating pipet.*—Capable of accurately delivering 0.1, 1.0, and 3.0 mL.

(**n**) *Dispenser.*—1000 mL or greater capacity; capable of accurately delivering 20 and 30 mL.

(**o**) *Glass test tubes*.—16 × 100 mm.

(**p**) *Glass tubes.*—25 × 200 mm, with polytetrafluoroethylene (PTFE)-lined screw caps or comparable tubes to hold 51.1 mL and allow for adequate mixing when sealed.

(**q**) *Plastic film.*—Or similarly nonreactive material.

(**r**) *Magnetic stir plate.*

(**s**) *Glass fiber filter.*—With 1.6 µm retention.

(**t**) *Hardened filter paper.*—With 22 µm retention.

C. Reagents

Note: Use high-quality distilled or deionized water for all water additions.

(**a**) *Acetate buffer (100 mM, pH 5.0)*.—Weigh 6.0 g or pipet 5.71 mL glacial acetic acid and transfer immediately to a flask; quantitatively transfer weighed acid with H_2O rinses. Bring volume to ca 850 mL. While stirring solution on a magnetic stir plate, adjust pH to 5.0 ± 0.1 with 1 M NaOH solution. Dilute to 1 L with H_2O. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**b**) Heat-stable α-amylase solution.—Liquid, heat-stable, α-amylase (examples: Product Termamyl 120 L, Novozymes North America, Franklinton, NC; Product Multifect AA 21L, Genencor International, Rochester, NY; origin: Bacillus licheniformis, or equivalent). Should not contain greater than 0.5% glucose. pH optima must include 5.5–5.8.

Based on Bacterial Amylase Unit (BAU) method.—Approximately 83000 BAU/mL of concentrated enzyme (1 BAU is defined as the amount of enzyme that will dextrinize starch at the rate of 1 mg/min at pH 6.6 and 30 ± 0.1°C; 19). If modifications of volume delivered are necessary due to enzymatic activity of the enzyme used, the volume used per test portion should deliver approximately 8300 ± 20 BAU (19).

Table 2014.10. Method performance for determination of dietary starch in feeds

Material	No. of labs	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	r[a]	R[b]
Moist canned dog food	11	1.54	0.03	0.09	2.21	5.99	0.10	0.26
Low starch horse feed	13	7.02	0.23	0.36	3.32	5.19	0.65	1.02
Dry ground corn	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54
Complete dairy feed	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48
Soybean meal	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31
Distillers grains	13	4.11	0.11	0.20	2.67	4.94	0.31	0.57
Pelleted poultry feed	13	28.24	0.73	1.34	2.58	4.76	2.04	3.76
Corn silage	13	39.04	0.80	1.88	2.05	4.82	2.24	5.27
Dog kibble, dry	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46
Alfalfa pellets	13	1.38	0.12	0.13	8.61	9.69	0.33	0.38

[a] $r = 2.8 \times s_r$.

[b] $R = 2.8 \times s_R$.

The enzymes should be of a purity meeting the specifications listed in *Official Method*SM **991.43** (20), but as modified below for application in the assay for dietary starch. The enzyme preparation used must be validated within laboratory to verify efficacy, as well as lack of interference. Recommended validation: analyze 0.1 g test portions of purified glucose, sucrose, and purified corn starch with the enzymatic portion of the dietary starch assay and using a free glucose value of zero in calculations. Analyses with candidate enzyme should give values of [mean ± standard deviation (SD)] glucose: 90 ± 2%, starch: 100±2%, and sucrose: 0.7 ± 0.3% on a dry matter basis. To test for interference from release of glucose from fiber carbohydrates, analyze 0.1 g test portions of α-cellulose and barley β-glucan that are not contaminated with free glucose with the enzymatic portion of the dietary starch assay. Recovery of these substrates should be less than 0.5% on a dry matter basis (20). Use AOAC approved methods for determination of dry matters of the samples. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%), or background absorbance readings will interfere with test sample measurements.

(**c**) *Diluted amyloglucosidase solution.*—Dilute concentrated amyloglucosidase with 100 mM sodium acetate buffer, **C**(**a**), to give 1 mL of solution per test portion with 2 to 5 mL excess. Add 1/3 of needed buffer to an appropriately sized graduated cylinder. Pipet concentrated amyloglucosidase into buffer, rinsing tip by taking up and expelling buffer in the graduated cylinder. Bring to desired volume with additional buffer. Cap cylinder with plastic film and invert cylinder repeatedly to mix. The concentrated amyloglucosidase used should not contain greater than 0.5% glucose, and should have a pH optimum of 4.0 and pH stability between 4.0–5.5 (example of concentrated amyloglucosidase: Product E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland; origin: *Aspergillus niger*, or equivalent).

(*1*) *Based on release of glucose from soluble starch or glycogen.*—200 U/mL (1 unit of enzyme activity is defined as the amount of enzyme required to release 1 μmole glucose/min at pH 4.5 and 40°C; 21).

(*2*) *Based on p-nitrophenyl-β-maltoside method.*—13 units/mL (1 unit is defined as the amount of enzyme required to release 1 μmole *p*-nitrophenol from *p*-nitrophenyl-β-maltoside/min at pH 4.5 and 40°C; 22).

The enzyme used must be validated within laboratory to verify efficacy as well as lack of interference. Use the same validation procedure as described for heat-stable α-amylase, **C**(**b**).

(**d**) *Benzoic acid solution (0.2%).*—Weigh 2.0 g benzoic acid (solid, ACS reagent, >99.5% purity) and add to a flask. Bring flask to 1 L volume with H_2O. Add magnetic stir bar, stopper flask, and allow to stir overnight to dissolve benzoic acid. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**e**) *GOPOD reagent.*—(*1*) *Mixture of glucose oxidase, 7000 U/L, free from catalase activity; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74 mM.*—Prepare by dissolving 9.1 g Na_2HPO_4 (dibasic, anhydrous) and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with H_2O, and swirl gently to dissolve without causing excessive foaming. Bring to 1 L volume with H_2O. Seal and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 μm retention, **B**(**s**). Store in a sealed amber bottle at ca 4°C. Reagent life: 1 month. Before use in test sample determinations, determine a standard curve for the reagent using a 5-point standard curve using **C**(**e**) and **C**(**f**) according to **D**(**b**).

(*2*) Alternatively, use another AOAC-approved glucose-specific assay that has passed in laboratory validation to accurately determine glucose concentrations of glucose standard solutions and give values equivalent to the values listed for determination of efficacy of enzymes. Recommended validation: analyze all five glucose working standard solutions and 100 mg test portions of purified glucose, purified sucrose, and purified corn starch that have been processed through the enzymatic hydrolysis portion of the dietary starch procedure and using a free glucose value of zero in calculations. The glucose values of the working standard solutions should be predicted ±6 μg glucose/mL. On a dry matter basis, the control sample glucose should give a dietary starch value (mean ± SD) of 90 ± 2%, corn starch at 100 ± 2%, and sucrose 0.7 ± 0.3%.

(**f**) *Glucose working standard solutions.*—0, 250, 500, 750, and 1000 μg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥99.5%) by an AOAC-approved method. Weigh approximately 62.5, 125, 187.5, and 250 mg portions of glucose and record weight to 0.0001 g. Rinse each portion of glucose from weigh paper into a separate 250 mL volumetric flask with 0.2% benzoic acid solution, **C**(**d**), and swirl to dissolve. Bring each standard to 250 mL volume with 0.2% benzoic acid solution, **C**(**d**), to give four independent glucose standard solutions. The 0.2% benzoic acid solution, **C**(**d**), serves as the 0 μg/mL standard solution. Multiply weight of glucose by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divide by 250 mL to calculate actual glucose concentrations of the solutions. Prepare solutions at least one day before use to allow equilibration of α- and β- forms of the glucose. Standard solutions may be stored at room temperature for 6 months.

(**g**) *Internal quality control samples*.—Powdered crystalline glucose (purity ≥99.5%) and isolated corn starch. For the corn starch sample, crude protein as nitrogen content × 6.25 and ash should be determined to determine the nonprotein organic matter content of the sample. For use in recovery calculations, actual starch content of the corn starch control sample is estimated as 100% minus ash% and minus crude protein%, all on a dry matter basis. Analyze 100 mg of each sample with each batch of test samples. Glucose will allow evaluation of quantitative recovery, and starch will allow evaluation of quantitative recovery and efficacy of the assay.

(**h**) *Determination of accuracy of volume additions for use of summative volume approach*.—The method as described relies on accurate volumetric additions in order to use the sum of volumes to describe test solution volume. Accuracy of volume additions can be evaluated before the assay by the following procedure: Using 1–2 L distilled water at ambient temperature, determine the g/mL density of the water by recording the weight of three empty volumetric flasks (volumes between 50 and

100 mL), add the water to bring to volume, and weigh the flasks + water. Calculate water density g/mL as:

$$\text{Water density g/mL} = [(\text{flask + water, g}) - (\text{flask, g})]/\text{water volume mL}$$

Record the weights of five empty tubes used for the dietary starch assay. Using the ambient temperature water and the devices used to deliver the liquid volumes for the enzymatic hydrolysis portion of the assay, deliver the 30, 0.1, 1, and 20 mL volumes to each tube (total of 51.1 mL in each tube). Record the weight of each tube + water. Calculate the grams of water in each tube as:

$$\text{Water in each tube, g} = (\text{tube + water, g}) - (\text{tube, g})$$

Divide the weight of water in each tube by the determined average density of water to give the volume of water in each tube. The deviation should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. If the deviations are greater than these, after the addition of 20 mL water during the dietary starch assay, individual samples should be quantitatively transferred with filtration through hardened filter paper with a 22 µm retention, **B**(**t**), into a 100 mL volumetric flask and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

D. Preparation of Reagent Blanks, Standard Curves, and Test Samples

(**a**) *Reagent blank.*—For each assay, two reaction tubes containing only the reagents added for each method are carried through the entire procedure. Reagent blanks diluted to the same degree as samples (no dilution or diluted to the same degree as control and test samples) are analyzed. Absorbance values for the reagent blanks are subtracted from absorbance values of the test solutions prepared from test and control samples.

(**b**) *Standard curves.*—Pipet 0.1 mL of 0.2% benzoic acid solution, **C**(**d**), and nominal 250, 500, 750, and 1000 µg/mL working standard glucose solutions, **C**(**f**), in duplicate into the bottoms of 16 × 100 mm glass culture tubes. Add 3.0 mL GOPOD reagent, **C**(**e**), to each tube using a positive displacement repeating pipet aimed against wall of tube, so it will mix well with the sample. Vortex tubes. Cover tops of tubes with plastic film. Incubate in a 50°C water bath for 20 min. Read absorbance at 505 nm using the 0 µg glucose/mL standard to zero the spectrophotometer. All readings should be completed within 30 min of the end of incubation; avoid subjecting solutions to sunlight as this degrades the chromogen. Calculate the quadratic equation describing the relationship of glucose µg/mL (response variable) and absorbance (abs) at 505 nm (independent variable) using all individual absorbances (do not average within standard). The equation will have the form:

$$\text{Glucose, µg/mL} = \text{abs x quadratic coefficient} + \text{abs} \times \text{linear coefficient} + \text{intercept}$$

Use this standard curve to calculate glucose µg/mL in test solutions. A new standard curve should be run with each glucose determination run.

(**c**) *Test samples.*—Feed and pet food amenable to drying should be dried at 55°C in a forced-air oven. Dried materials are then ground to pass the 0.5 or 1.0 mm screen of an abrasion mill or the 0.5 mm screen of a cutting mill or other mill to give an equivalent fineness of grind (to pass a 40 mesh screen). Ground, dried materials are transferred into a wide mouthed jar and mixed well by inversion and tumbling before subsampling. Semi-moist, moist, or liquid products may be homogenized, blended, or mixed to ensure homogeneity and reduced particle size (23).

E. Determination of Dietary Starch

The analyses for free glucose and enzymatically released glucose + free glucose may be performed in separate analytical runs. For flow of assay, *see* Figure **2014.10**.

(*1*) Accurately weigh two test portions (W_E, W_F) of 100 to 500 mg each of dried test samples or 500 mg semi-moist, moist, or liquid samples (for all samples, use ≤500 mg, containing ≤100 mg dietary starch; use 500 mg for samples containing <2% dietary starch) into screw-cap glass tubes. Test portion W_E is for the analysis of enzymatically released glucose and W_F is for the determination of free glucose. In addition to unknowns, weigh test portions (W_E, W_F) of D-glucose and purified corn starch, which serve as quality control samples **C**(**g**). Also include two tubes with no test portion to serve as reagent blanks per each analytical run for free glucose or enzymatically released glucose + free glucose.

(*2*) Dispense 30 mL of 0.1 M sodium acetate buffer, **C**(**a**), into each tube.

(*3*) To tubes with test portions designated W_E and to each of the reagent blanks to be used with analysis of enzymatically released glucose + free glucose, add a volume of heat-stable, α-amylase, **C**(**b**), to deliver ca 1800 to 2100 liquefon units or 8200 to 8300 BAU of enzyme activity (typically 0.1 mL enzyme as purchased); do not add the amylase to W_F and to the reagent blanks to be used with free glucose determinations. Cap tubes and vortex to mix.

Note: Vortex tube so that the solution column extends to the cap, washing the entire interior of the tube and dispersing the test portion.

(*4*) Incubate all tubes for 1 h at 100°C in a forced-air oven, vortexing tubes at 10, 30, and 50 min of incubation.

(*5*) Cool tubes at ambient temperature on bench for 0.5 h. At this point, separate tubes designated for free glucose analysis (tubes containing W_F test portions and reagent blanks with no enzyme) from the rest of the run. Those designated for free glucose should skip steps (*6*) and (*7*) and continue with steps (*8*)–(*13*).

(*6*) Add 1 mL of diluted amyloglucosidase solution, **C**(**c**), to W_E test and quality control samples and reagent blanks. Vortex tubes.

(*7*) Incubate tubes for 2 h in a water bath at 50°C, vortexing at 1 h of incubation.

(*8*) Add 20 mL water to each tube. Cap and invert at least 4 times to mix completely. Proceed immediately through steps (*9*)–(*13*).

(*9*) (*a*) *Volume by sum of volume additions.*—Transfer ca 1.5 mL test sample solutions to microcentrifuge tubes, and centrifuge at 1000 x *g* for 10 min. If the sample remains cloudy after centrifugation, centrifuge an additional 10 min at

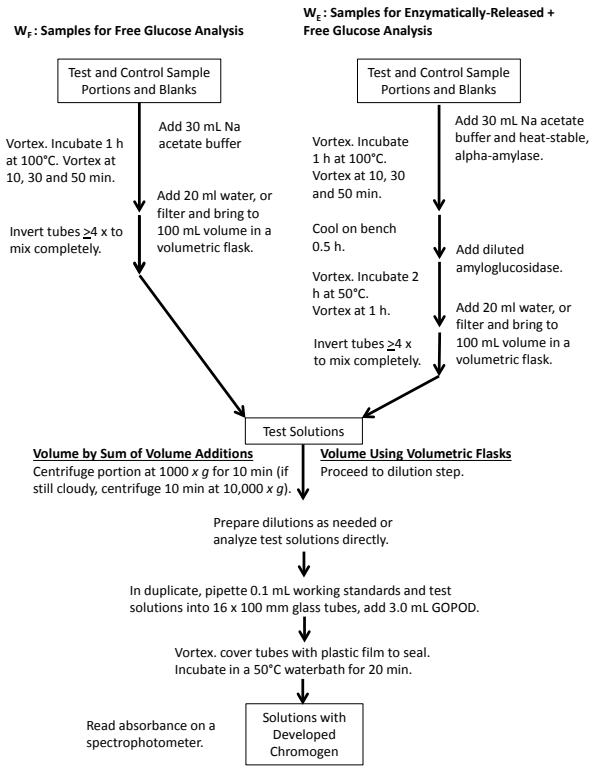

Figure 2014.10. Flow chart of the dietary starch assay.

10 000 × *g* to clarify the solution before proceeding. Solutions may increase in temperature during centrifugation; allow centrifuged solutions to come to room temperature before preparing dilution.

(*b*) *Volume using volumetric flasks*.—Quantitatively transfer test sample solutions with filtration through a hardened paper filter with 22 µm retention and rinses with water to 100 mL volumetric flasks.

(*10*) Prepare dilutions as needed with distilled or deionized water. Solutions from control samples and test samples estimated to give greater than 1000 µg glucose/mL concentrations of free and released glucose should be diluted 1 in 10 if processed as in (*9*)(*a*) or 1 in 5 if processed as in (*9*)(*b*). Reagent blanks should be diluted to provide solutions with the same dilutions as used with the test solutions, so that the diluted reagent blank solutions can be used to make corrections for similarly diluted test solutions. Dilutions may be prepared using volumetric flasks or by accurate pipetting. If done by pipetting, use a minimum of 0.5 mL test sample or control solution to minimize the impact of variation in pipetting small volumes.

(*11*) Pipet 0.1 mL in duplicate of glucose working standard solutions (0, 250, 500, 750, and 1000 µg/mL glucose), **C**(**f**), and reagent blank, quality control sample, and test sample solutions into the bottoms of 16 × 100 mm glass test tubes using two tubes/solution. Add 3.0 mL GOPOD reagent, **C**(**e**)(*1*), to each tube. Vortex tubes. Place tubes in a rack and cover with plastic film to seal.

Note: Alternative to the use of the GOPOD method, proceed with alternate glucose determination method, **C**(**e**)(*2*), for measurement of glucose in working standards, reagent blank, control sample, and test sample solutions.

(*12*) Incubate in a 50°C water bath for 20 min.

(*13*) Set spectrophotometer to measure absorbance at 505 nm. After the incubation is complete, zero the spectrophotometer with the GOPOD-reacted 0 µg/mL working standard solution. Read absorbances of remaining GOPOD-reacted working standard solutions, and reagent blank, control sample, and test sample solutions. All reacted solutions must be read within 30 min of the end of the GOPOD incubation. The duplicate absorbance values are averaged for each reagent blank, test sample, and control sample solution and used in *Calculations*.

F. Calculations

Determine the quadratic equation that fits the absorbances of the working standard solutions. The absorbance values, A_{CF} or A_{CE}, are the independent variables (X), and actual glucose concentrations are the dependent variables (Y). Individual absorbance values of the working standard solutions, not averages, are used. The equation has the form:

$$\mu g\ Glucose/mL = (A_{CF\ or\ CE}^{2} \times Q + A_{CF\ or\ CE} \times S + I)$$

Calculate dietary starch content in test sample as received as follows:

$$\text{Free glucose, \%} = (A_{CF}^{2} \times Q + A_{CF} \times S + I) \times V_F \times DF_F \times 1/1\,000\,000 \times 1/W_F \times 162/180 \times 100$$

$$\text{Dietary starch, \%} = [(A_{CE}^{2} \times Q + A_{CE} \times S + I) \times V_E \times DF_E \times 1/1\,000\,000 \times 1/W_E \times 162/180 \times 100] - \text{free glucose \%}$$

where subscript *F* represents values for samples analyzed for free glucose and subscript *E* represents values for samples treated with amylase and amyloglucosidase; A_{CF}, A_{CE} = absorbance of reaction solutions minus the absorbance of the appropriately diluted reagent blank, values are averages of the two replicates for each test solution; Q = quadratic slope term, S = linear slope term, and I = intercept of the standard curve to convert absorbance values to µg glucose/mL; V_F, V_E = final sample solution volume, ca 50.0 mL for V_F and 51.1 mL for V_E if done by summation of volumetric additions, otherwise, by size of volumetric flask used; DF = dilution factor, e.g., 0.5 mL sample solution diluted into 5.0 mL = 5.0/0.5 = 10; 1 g/1 000 000 µg = conversion from µg to g; W_E, W_F = test portion weight, as received; 162/180 = factor to convert from measured glucose as determined, to anhydroglucose, as occurs in starch.

If test samples are run in duplicate portions, the free glucose % in the dietary starch equation is the average free glucose % value determined for the test sample.

Results and Discussion

Evaluation of the Dietary Starch Method

Initial evaluation of data from all laboratories showed that most outliers occurred in two laboratories (Table 2). Laboratory 14 had significant Cochran's tests for five of the test materials, indicating suspect replicate results within this laboratory. Unlike the other laboratories, Laboratory 14 ran duplicate portions of test materials on separate days, rather than together within the same run. Based on laboratory ranking scores (18), this laboratory was designated as an outlier and its data were

Table 2. Results of collaborating laboratories for dietary starch individual replicate values on an as-received basis

		Collaborating laboratory														
Material	Duplicate	0	1	2	3	4	5	6	7	8	9	10	11[a]	12	13	14[b]
Moist canned dog food	1	1.58	1.42	1.34	1.56	1.58	1.58	1.57	1.64	1.59	1.44	**2.47**[c]	**1.94**[d]	1.55	**1.84**[c]	**0.22**[c]
	2	1.57	1.46	1.36	1.67	1.62	1.59	1.47	1.62	1.60	1.44	**1.59**[c]	**1.94**[d]	1.53	**1.61**[c]	**0.32**[c]
Low starch horse feed	1	7.03	6.29	7.01	7.30	6.78	7.21	6.88	7.33	7.27	6.47	6.68	8.32	7.15	7.02	**5.76**[c]
	2	7.21	6.50	7.44	7.60	6.43	7.61	7.02	7.33	6.98	6.74	7.37	7.87	7.08	6.68	**6.50**[c]
Dry ground corn	1	70.80	63.08	71.80	**58.85**[c]	71.27	68.13	70.18	71.22	71.52	71.25	67.97	**5.84**[d]	70.39	68.98	**60.19**[c]
	2	69.24	63.14	72.89	**26.64**[c]	70.23	67.33	71.47	73.29	71.08	70.04	65.82	**5.93**[d]	70.53	68.74	**65.42**[c]
Complete dairy feed	1	29.19	26.86	28.53	28.90	26.88	28.27	28.39	29.33	29.07	27.59	**26.89**[c]	**37.21**[d]	28.41	25.42	27.85
	2	29.79	26.69	28.49	30.02	26.11	28.70	28.19	29.10	28.89	27.49	**30.90**[c]	**35.45**[d]	28.01	26.10	27.28
Soybean meal	1	1.01	1.04	**1.09**[c]	1.10	0.97	1.13	0.94	1.04	1.06	0.87	1.02	**2.35**[d]	0.82	1.00	**0.02**[c]
	2	1.03	1.11	**1.42**[c]	1.19	0.93	1.11	0.90	0.93	1.09	0.78	1.16	**2.38**[d]	0.84	1.02	**0.82**[c]
Distillers grains	1	4.02	3.90	4.23	4.27	4.05	4.55	4.05	4.16	3.99	4.10	3.81	**4.82**[e]	4.19	3.98	**3.16**[c]
	2	4.07	3.90	4.09	4.30	4.08	4.49	3.94	4.14	4.06	4.06	4.09	**4.85**[e]	4.58	3.79	**3.00**[c]
Poultry feed	1	28.67	28.12	28.57	28.71	26.47	27.99	27.44	29.59	28.78	27.67	27.9	26.50	29.07	25.06	27.51
	2	29.25	27.35	27.95	30.26	28.00	28.27	28.52	29.43	28.83	27.65	30.39	25.18	29.45	24.80	26.56
Corn silage	1	41.10	37.44	39.20	40.92	37.54	39.18	38.08	39.17	40.91	37.00	37.26	36.03	43.50	36.59	37.99
	2	40.34	36.84	39.02	41.59	37.71	38.58	37.65	39.83	40.22	37.34	40.23	35.72	41.31	36.40	36.55
Dog kibble, dry	1	29.87	25.50	24.58	27.73	29.23	27.53	27.37	24.10	27.32	**17.99**[f]	25.73	27.55	28.68	26.30	24.31
	2	27.92	26.45	27.52	24.21	26.57	27.33	25.64	28.00	25.19	**18.35**[f]	27.25	26.93	29.34	25.70	26.25
Alfalfa pellets	1	1.29	1.17	1.56	1.32	1.56	1.59	1.61	1.35	1.33	1.58	1.42	1.31	1.13	1.25	0.60[c]
	2	1.36	1.43	1.43	1.41	1.32	1.61	1.31	1.24	1.34	1.38	1.35	1.13	1.38	1.27	**1.01**[c]

[a] Data for this laboratory was omitted from analysis based on a 7% change in glucose standard absorbances between runs for detection of free glucose and free + enzymatically released glucose. When data were included, four of 10 samples were identified as outliers by the single Grubbs' test, and one by the double Grubbs' test.

[b] Outlier laboratory detected by laboratory ranking.

[c] Outlier detected by the Cochran's test.

[d] Outlier detected by the single Grubbs' test.

[e] Outlier detected by the double Grubbs' test.

[f] Data omitted from analysis because the large test portion used (0.5 g) exceeded the 100 mg α-glucan limit for this assay.

not used in calculation of the study statistics. Laboratory 11 had four outlier values detected by the single Grubbs' test, which would indicate that this laboratory's values for these test samples were substantially higher or lower than those generated by the other laboratories. The very low value for dry ground corn appeared to be a possible error in recording the dilution of the sample, but laboratory records indicated that that was not the case. The basis for the high values for dairy feed, soybean meal, and moist canned dog food was not immediately obvious. The distillers grains results for Laboratory 11 was designated as an outlier based on results of the double Grubbs' test.

Laboratory 11 was not designated as an outlier by the ranking procedure, but test material results were generally higher for this laboratory. A likely basis for the higher dietary starch values was that the absorbances of the glucose standards were lower in the analytical run with the test samples treated with enzyme than were those reported for two other standard curves run for the dietary starch assay in that laboratory. The decrease in absorbance was on the order of 0.029 to 0.089 for 500 and 1000 mg glucose/mL standard solutions. To put this in perspective, the difference in absorbance values between runs represents an almost 8% lower absorbance value for the 1000 mg glucose/mL standard in the assay with enzyme-treated test samples. Standard curves produced from lower absorbance values will give higher calculated glucose and dietary starch values if the absorbances of the test samples are not similarly depressed. Absorbance values for glucose standards are not expected to be identical among analytical runs. However, the glucose oxidase-peroxidase assay used tends to be very consistent. For example, in the Study Director's laboratory, eight glucose standard curves run with dietary starch assays on 4 separate days showed RSD values (SD/mean) of less than 0.8% for absorbance values determined across runs within glucose standard (Table 3). Data from 12 collaborating laboratories that provided absorbance data for more than one standard curve showed the RSD of the absorbances calculated for individual glucose standards and then averaged across all standards were less than 1% for five laboratories, less than 2% for eight,and more than 2% for four (Table 4). Replicate absorbance readings for glucose standards within analytical run showed overall good repeatability for all laboratories. Laboratory 14, which was excluded from the study based on a ranking test, had the

Table 3. Absorbance values for glucose standards analyzed in repeated runs and in the collaborative study

Repeated analyses of glucose standard solutions: values by standard[a]						
Glucose standard, µg/mL	Runs[b]	Mean[c]	SD[d]	CV%[d]	Minimum value	Maximum value
249.4	8	0.285	0.0020	0.69	0.282	0.289
499.4	8	0.568	0.0028	0.49	0.563	0.574
748.7	8	0.848	0.0031	0.36	0.841	0.852
998.7	8	1.125	0.0045	0.40	1.116	1.133
Collaborative study: means across standards of values calculated for individual standards						
Laboratory	Runs	Overall mean[e]	Mean SD[f]	Mean CV, %[g]	Replicate SD[h]	
Study Director	3	0.704	0.0023	0.35	0.001	
7	2	0.688	0.0031	0.46	0.002	
8	4	0.712	0.0040	0.62	0.003	
13	2	0.658	0.0034	0.68	0.003	
2	2	0.855	0.0068	0.79	0.007	
1	6	0.827	0.0083	1.41	0.004	
12	3	0.684	0.0092	1.47	0.004	
3	2	0.736	0.0073	1.49	0.005	
6	2	0.723	0.0121	1.56	0.009	
4	3	0.682	0.0143	2.22	0.008	
5	4	0.727	0.0160	2.28	0.007	
11	3	0.709	0.0287	3.55	0.009	
14	2	0.667	0.0531	8.78	0.004	

[a] Glucose standards were analyzed in the Study Director's laboratory in eight separate analytical runs for the dietary starch assay. Glucose standards were analyzed in duplicate in two separate runs/day on 4 days. Two separate batches of GOPOD reagent were each used for four runs. The same preparations of glucose standards were used for all eight runs.

[b] Number of separate analytical runs in which the glucose standards were analyzed in duplicate.

[c] The mean value of the 16 replicates for each glucose standard.

[d] SD = standard deviation; RSD = 100 × (SD/mean).

[e] Mean of all absorbance values generated by the laboratory.

[f] The mean of all SD of absorbance values calculated for individual glucose standards.

[g] The mean of all RSD of absorbance values calculated for individual glucose standards.

[h] The mean of all SD of absorbance values for replicate pairs of glucose standards.

largest average RSD for absorbances of the glucose standards. Given the good replication for duplicates in this laboratory, the large RSD reflects differences in glucose standard absorbances between analytical runs. The difference this variation would generate in the standard curves could explain the variation detected in test sample replicates for this laboratory, because test sample duplicates were analyzed singly in separate runs, each of which used a different standard curve. Laboratory 11 had the second highest average RSD for absorbances of the standards. Discussions with Laboratory 11 did not uncover the basis for the variation between analytical runs. The dietary starch assay relies on the soundness of the standard curves to give reliable results. For Laboratory 11, because the glucose standard results used with the enzyme-treated samples deviated from two other standard curves they performed, and because the lower absorbances gave a standard curve that appears to have inflated the dietary starch values, the data are suspect and has been omitted from the statistical analysis of this study.

It is important to control the run to run and between replicate variation in analysis of the glucose standards because of the impact these have on accuracy of results. This GOPOD glucose detection assay is highly sensitive to pipetting accuracy. Samples should be read within 30 min of the end of incubation with GOPOD. It is also recommended that the incubated GOPOD-reacted samples be kept out of sunlight as this can degrade the chromagen. In addition to evaluating standard curve data for obvious changes in response, it is recommended that for each batch of GOPOD a log be kept of absorbance data for glucose standards from all runs. Within a glucose standard, calculate the SD of all absorbances. The mean of these SDs across all standards should not be greater than 0.016. Even lower levels of variability in absorbances can be readily achieved with this assay.

Another factor that likely affected accuracy was exceeding the 100 mg of starch limit/test portion in the assay, which was the case for Laboratory 9 when dry dog kibble was analyzed using 0.5 g test portions. The resulting low dietary starch values were likely the result of the enzyme no longer being in the excess required for complete hydrolysis of the dietary starch.

Variability of results may also have been affected by the approach to sample dilution. Laboratory 3 used 0.1 mL of test sample solution and 0.9 mL of water to make a 1 in 10 dilution

Table 4. Statistical data for dietary starch results

Material	Outlier	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	2.8 × s_r	2.8 × s_R	HorRat	Largest within-lab variance	Largest average lab result	Smallest average lab result
Moist canned dog food	10, 13	11	1.53	0.03	0.09	2.21	5.99	0.10	0.26	1.60	0.01	1.63	1.35
Low starch horse feed		13	7.02	0.23	0.36	3.32	5.19	0.65	1.02	1.74	0.24	7.45	6.40
Dry ground corn	3	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54	1.83	2.31	72.34	63.11
Complete dairy feed	10	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48	1.83	0.64	29.49	25.76
Soybean meal	2	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31	2.79	0.01	1.15	0.83
Distillers grains		13	4.11	0.11	0.20	2.67	4.94	0.31	0.57	1.53	0.08	4.52	3.88
Pelleted poultry feed		13	28.24	0.73	1.34	2.58	4.76	2.04	3.76	1.97	3.10	29.51	24.93
Corn silage		13	39.04	0.80	1.88	2.05	4.82	2.24	5.27	2.09	4.41	42.40	36.49
Dog kibble, dry	9	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46	2.43	7.61	29.01	25.97
Alfalfa pellets		13	1.38	0.12	0.13	8.61	9.69	0.33	0.38	2.54	0.05	1.60	1.25

for the ground corn sample. Even with small differences in pipetted amounts, such an approach could result in the between duplicate difference noted for that sample. Test solutions from the enzymatic hydrolysis procedure can be "sticky", i.e., they do not pipet exactly like water, and require care to pipet accurately. If dilutions are made by pipetting, prewetting of pipet tips and use of larger volumes, such as 0.5 mL of test solution and 4.5 mL of water, are recommended.

The quantity of test material used also may have affected assay variability. Test samples with starch contents of less than 2% generally showed greater variability than test samples that contained more starch (Figure 1 and Table 4) in a pattern nearly identical to that described by Wehling and DeVries (24) for dietary fiber assays. However, among the low starch materials, the moist dog food had RSD values for repeatability and reproducibility that were approximately half those of soybean meal and alfalfa pellets (Table 4); these latter two samples also had the highest HorRat values in the study. In addition to being the only moist, homogenized sample, laboratories were directed to use 0.5 g of the moist dog food as compared to 0.1 g of other samples. The one case in which dietary starch values for the moist dog food were identified by the Cochran test as suspect replicates within laboratory was where Laboratory 10 reported values determined on 0.10 g test samples for this material (Table 2). In the collaborative study, the 0.1 g sample size was used for most samples to minimize the likelihood that the 100 mg limit of dietary starch/test portion would be exceeded, based on the laboratories' prestudy results with the assay; however, it also greatly reduced the concentration of glucose to be detected in low starch test samples. Final glucose concentrations of test sample solutions for 0.1 g enzyme-treated test portions of soybean meal and alfalfa pellets were 22 and 30 µg/mL, respectively as compared to 167 µg/mL for the moist dog food using 0.5 g test portions. These glucose concentrations of the low starch feeds equate to absorbance values of 0.035, 0.054, and 0.221, respectively, as determined in the Study Director's laboratory. Although the glucose detection assay is sensitive and precise, small variations in absorbances of test solutions with very low glucose concentrations will give more variability in calculated glucose values than the same amount of variation will with test solutions with higher glucose concentrations. This can result in greater within and between laboratory variability for low starch test samples for which smaller test portions are used. In the case of the dietary starch assay, as with gravimetric dietary fiber analyses, the increase in RSD as concentrations of the analyte approaches zero may be related to limits of precision of the detection methods themselves. The absorbances and glucose concentrations noted for soybean meal, alfalfa pellets, and moist dog food represent 1.0, 1.4, and 7.7 mg of dietary starch in the respective test portions. It is notable that the distillers grains, for which the 0.1 g test portion would provide approximately 4 mg of dietary starch, had a HorRat value below 2, possibly suggesting a level of dietary starch at and above which precision is improved.

A viable approach to decreasing RSD values for low starch test samples analyzed with the dietary starch method is to increase the size of the test portion in order to increase the amount of analyte to be detected. The idea of increasing the amount of test sample analyzed in order to improve precision by having a greater amount of analyte to measure has been raised (25). Unlike the dietary fiber analyses that may need to restrict test portion size to assure that the extractant remains in excess, starch assays will primarily be restricted by the need to maintain an excess of enzyme to assure complete hydrolysis of the α-glucan. The approach of allowing a range of test portions but a limit on the amount of starch added to the reaction vessel is used by two current AOAC starch methods: AOAC Method **948.02** for starch in plants (26) specifies a use of 0.1–1.0 g of test portion containing approximately 20 mg of starch, and AOAC Method **979.10** for starch in cereals (27) indicates use of a 0.5 g test portion and then specifies "≤1.0 g containing ≤0.5 g starch". In the present method, a limit of 100 mg of dietary starch in each reaction vessel leaves latitude to increase the size of the test portion to that upper limit. Although 0.1 g test portions may be generally adequate, increasing the amount of substrate within the bounds of the assay for feedstuffs with low starch contents may reduce variability of results. The remaining caveat is that as sample quantity is increased, attention must be paid to increasing amounts of interfering substances also brought into the reaction (e.g., antioxidants if the GOPOD assay is used).

With the exceptions of dry ground corn, dairy feed, poultry feed, and corn silage, s_r and s_R were similar within materials (Table 3). The HorRat values obtained in the present study compared favorably to those obtained with AOAC Method **996.11** (10; Table 3). In the collaborative study for that method, starch analyses performed without dimethyl sulfoxide (DMSO)

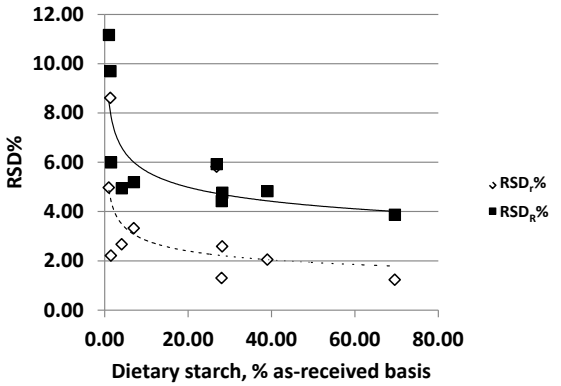

Figure 1. Relationship of dietary starch concentration and RSD values for repeatability within laboratory (RSD_r) and reproducibility between laboratories (RSD_R) obtained in the collaborative study. Equations for the regression lines are RSD_r, % = $4.8616x^{-0.236}$ (R^2 = 0.35; dashed line), and RSD_R,% = $8.4397x^{-0.176}$ (R^2 = 0.66; solid line), where x = dietary starch concentration.

had a starch content of 59.8% as received, and an average HorRat of 2.1 with one value below 2. For the dietary starch collaborative study, the HorRat was less than 2 for six of 10 materials, with an overall average of 2.0 on test materials that averaged 20.7% dietary starch on an as-received basis. Alfalfa pellets and soybean meal had HorRat values of greater than 2.5. As previously discussed, the high RSD_R for these test materials may relate to the combination of their low starch content and the small test portion amount used. Test samples with very low concentrations of the analyte have been reported to give elevated HorRat values (17). The high HorRat value for the dry dog kibble may reflect an issue with homogeneity of the sample, as described previously.

Collaborators' Comments

The collaborators all reported that the assay was not very complicated and was easy to do. They particularly liked additions of all reagents to a single vessel, performing reactions in screw cap tubes, determining total liquid volume as the sum of quantitative volume additions, and making sample solution dilutions by accurate pipetting of volumes. They indicated that they had to work within their laboratories to find tools of acceptable accuracy to make the volume additions, as some of the tools they worked with for other purposes were not adequate. They did report issues with screw cap tube adequacy to hold the needed volume; this was apparently related to differing amounts of glass used by the manufacturers while maintaining the same exterior dimensions of the tubes. That was addressed by describing the screw cap tubes by the volume they needed to contain while allowing adequate room for mixing. With the number of sodium phosphate chemicals available, it was noted that it was crucial to verify and use the exact chemicals specified for the GOPOD reagent. It was also raised that the only extended period to take a break from the assay was during the amyloglucosidase incubation; taking a break after adding water to the fully digested samples resulted in reduced recovery. Development of an approved assay for glucose detection that could be used on a plate reader or automated system was recommended as a way to increase throughput of the assay, which is currently limited by the 30 min period within which samples must be read after incubation in the GOPOD glucose detection assay. Some laboratories had issues with calculating quadratic glucose standard curves; this was resolved by graphing all individual glucose standard solution absorbances data with absorbance on the X-axis and glucose concentration on the Y-axis. Then, a quadratic or second order polynomial regression or "trend" line was graphed through the data. The regression line equation was used for calculation of glucose in test solutions. Collaborators gave extensive input on the method protocol writeup and recommended development of a flow chart for the assay

Recommendations

Based on the results of the collaborative study, the Study Director recommends that the enzymatic-colorimetric method for measurement of dietary starch in animal feeds and pet foods be adopted as Official First Action.

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