Measurement of amorphous ferric phosphate to assess iron bioavailability in diets and diet ingredients

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A method of measuring amorphous ferric phosphate in the presence of crystalline ferric phosphate is described. This procedure is important because there appears to be a big difference in availability between the amorphous and crystalline forms of ferric phosphate. This difference has been proven for two situations. In the first situation, when amorphous ferric phosphate is used as the source of iron for gypsy moths, growth is normal, but if the crystalline form is used, results are identical to the case when no iron is used. In the second situation, several plant species have been reported to grow much better when amorphous ferric phosphate is used instead of crystalline ferric phosphate as the source of phosphate in fertilizer. Differentiation of the amorphous from the crystalline uses citrate solutions that extract the amorphous form but not the crystalline form. The procedure was optimized for three different sample forms: agar based artificial diet, Wesson salt† (a salt mixture containing all recommended minerals for insect diets), and pure ferric phosphate. A method for overcoming a problem with turbidity that occurs when analyzing some prepared diets is also described.

The essentiality of iron in diets has been known since ancient times and it must be included in artificial diets and often supplemented in natural diets. Many iron sources have been tested and found to have different levels of bioavailability as well as other advantages or disadvantages.[1] Ferric phosphate is one source of iron that is often used as a food supplement and it has one very important advantage. It is relatively unreactive and, therefore, does not cause problems of discoloration, rancidity, or unpleasant taste.[2] Unfortunately, it has a very critical disadvantage. In order to be available, it must be present in the amorphous form (AFP), not the crystalline form (CFP). This has been proven true for plants.[3–6] The only animal for which this has been proven to be true is the gypsy moth.[7] In 1994, Willis and Montgomery, using data from several literature references, speculated that this requirement is also true for humans.[8] To date, there has been no report of anyone testing this hypothesis using standard procedures for measuring iron bioavailability, so any difference in their availability for humans is still speculation.

Because the amorphous form (AFP) is necessary when using ferric phosphate as the source of iron supplement in certain animal diets, it is necessary to specify AFP when purchasing the ferric phosphate. To date, chemical suppliers have been unreliable when supplying the amorphous form and, even if it is obtained, it can deteriorate and change to the crystalline form (CFP).[9–12] An analytical technique is needed to measure AFP in the presence of CFP. A method to do this has been reported for artificially prepared diet and the salt mixture, Wesson salt, which is a mixture of several salts that meet the mineral requirements of insects.[8] To date, there has been no report of an analytical procedure to measure the amorphous content of pure ferric phosphate. This paper describes such a method and also describes improved methods for evaluating iron in artificial diets and salt mixtures. We discuss amorphous and crystalline ferric phosphate. In actuality, there are two crystalline forms: strengite and phosphosiderite.[8] Strengite has an orthorhombic crystal structure and phosphosiderite has a monoclinic crystalline structure.[13] We have studied only phosphosiderite because it is the only crystalline form that we have found in commercially available preparations.

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Experimental

Test material

Ferric phosphate. At the beginning of this project, samples of ferric phosphate were secured from several commercial sources (Table 1) and tested to determine the amorphous and crystalline composition. Those samples that were mostly crystalline were additionally tested to determine whether the crystalline form was strengite or phosphosiderite. The only source of ferric phosphate with a high amorphous content was Riedel de Haen AG, Seelze, Hanover, Germany. The chemical was purchased from a US distributor, Crescent Chemical Co., Inc., located in Hauppauge, NY. As of December, 1998, it is no longer available from Crescent but is available from Aldrich Chemical Co., Milwaukee, WI (ID 04241). The ferric phosphate from all other sources tested at least 95% crystalline and, based on X-ray diffraction and electron microscopy, every crystalline sample was phosphosiderite. The source that came closest to being pure crystalline, and the one we used for any test that called for crystalline ferric phosphate, was ICN Biochemicals,

Table 1 Sources of ferric phosphate that were tested

Company	Location	Lot #	Amorphous (%)
Crescent Chemical Company	Hauppauge, NY	04241	96.6
Johnson-Matthey	Ward Hill, MA	A11D11	0.6
Ingredients Int.	Commerce, GA	—	5.1
Spectrum	Gardena, CA	JB162	0.6
Strem Chemicals	Newburyport, MA	130758-S1	0.6
ICN Biochemicals	Cleveland, OH	39856	0.3
		67653	0.3

Cleveland, OH (Lot 67653). Using the procedure described in this paper, we found it was 99.7% crystalline.

Wesson salt. Wesson salt is a mixture of twelve different salts (Table 2)[14] used in artificial diet for insects and other animals. It was obtained from ICN (catalog No. 902851), Purina Mills, Inc., Richmond, IN, and United States Biochemical Corporation, Cleveland, OH (USB). The salt preparations labeled USB were purchased from USB, which is now owned by Purina Mills, Inc. Two of the salt formulations identified as artificial AFP or artificial CFP were prepared in this laboratory using commercially obtained Wesson salt (from USB and ICN) with ferric phosphate deleted from the formula by the vendor. AFP was added to one and CFP to the other.

Diet. Three laboratories contributed samples of diet for this study. The diet from two of the sources was Bell Diet, an agar based, high wheat germ diet[15] used for growing gypsy moths. The two laboratories preparing this diet were the Northeastern Center for Forest Health Research at Hamden, Connecticut, and the Otis Plant Protection Center at Otis ANGB, MA. Diet from the third laboratory (Pink Boll Worm Rearing Facility, Phoenix, AZ) was an agar based soy flour diet used for raising pink boll worm.[16] Two other diets that were analyzed were experimental diets that have not been described in the literature and are being tested for rearing *Lymantria monacha*. These two contain alfalfa, cellulose, linseed oil, and 37 mg g^{-1} of sucrose, in addition to the ingredients in the Bell diet.

Reagents

1,10-Phenanthroline. A 0.25% solution was prepared by dissolving 0.25 g of 1,10-phenanthroline monohydrate (also called *o*-phenanthroline) in approximately 80 ml of water. The solution was heated and stirred until dissolved, and diluted to 100 ml.

Hydroxylamine. A 10% solution was prepared by dissolving 10 g of anhydrous hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$) (also called hydroxylammonium chloride) in water and diluted to 100 ml.

Citrate solutions. Citrate solutions with a concentration of 0.2 M but with varying pH were prepared by dissolving 38.42 g of citric acid (anhydrous) in 900 ml water. If this was diluted to 1 l with water and no acid or base added, the pH would be 1.8. Ammonium hydroxide (50%) was added for solutions that required a higher pH, and 3 M HCl was added for solutions that required a lower pH. Methylparaben (0.1%) was added as a preservative. The final volume was brought to 1 l with water.

Table 2 Salt mixture, Wesson modification[a]

Compound	Amount (%)
Calcium carbonate ($CaCO_3$)	21.00
Calcium phosphate, tribasic [$Ca_3(PO_4)_2$]	14.9
Cupric sulfate ($CuSO_4{\cdot}5H_2O$)	0.039
Ferric phosphate ($FePO_4{\cdot}2H_2O$)	1.47
Magnesium sulfate ($MgSO_4{\cdot}7H_2O$)	9.0
Manganous sulfate ($MnSO_4$)	0.02
Potassium aluminum sulfate [$KAl(SO_4)_2{\cdot}12H_2O$]	0.009
Potassium chloride (KCl)	12.0
Potassium iodide (KI)	0.005
Potassium phosphate, monobasic (KH_2PO_4)	31.0
Sodium chloride (NaCl)	10.5
Sodium fluoride (NaF)	0.057

[a] This mixture is 4.14% of the dry ingredients that make up the gypsy moth diet.

Sodium acetate. 20 g or 100 g of sodium acetate trihydrate dissolved in 1 l of water for a 2% or a 10% solution, respectively.

Experimental tests

Calibration curves were prepared by using commercially available iron standard solution with a concentration of 1000 μg ml^{-1}. This was diluted to various concentrations using citrate solution, and three or more diluted solutions were analyzed using each volume listed in Table 3 and following the recommended procedure.

The test to measure the time required for pure ferric phosphate to completely dissolve was measured by extracting the mixture for three different lengths of time not exceeding the time required to completely dissolve the sample or 8 h, whichever came first. For each sampling time, the percentage of iron dissolved was calculated. These three measurements were used to extrapolate to the amount of time required to dissolve 100% of the ferric phosphate.

Other experimental tests were conducted following recommended procedures described below, but varying the one parameter being tested.

Recommended procedure

Pure ferric phosphate. This procedure is used to analyze pure ferric phosphate. The amorphous or crystalline content may vary but based on elemental analysis, it is pure. Place 0.15 g of ferric phosphate in a test tube that fits a heater–stirrer device such as the 'Reacti Therm' (Pierce Chemical Co., Rockford, IL). Add 10 ml pH 4 citrate and a small triangular stirring bar (called 'stirring vane' by Fisher Scientific, Pittsburgh, PA). Cap the tubes loosely and place in a heater–stirrer that has been preequilibrated at 75 °C and heat and stir for 20 min. Remove the samples from the heater–stirrer. Remove the stirring vane and cap, and centrifuge at 2400*g* (4400 rpm for 10.8 cm radius rotor) for 5 min. The supernatant, or citrate solution, contains iron from the AFP that dissolved in the solution. Analyze this solution for iron using colorimetry or any preferred analytical method. The following recommendations use colorimetry, but it is assumed that atomic absorption or any other analytical technique will give equally acceptable results.

There are two modifications of the colorimetric method: the amorphous procedure, used if the amorphous content is 10 to 20% or higher, and the crystalline procedure, used if the amorphous content is less than 10 to 20%. If using the amorphous procedure, take 0.2 ml of citrate extract and place it in a 25 ml calibrated flask. Add 1 ml of 3 M HCl and dilute to 25 ml with water. Take 0.5 or 1.0 ml of this solution, as indicated in Table 3. Add 1 ml of hydroxylamine, 1 ml of

Table 3 Experimental details for measuring concentration of AFP in either pure ferric phosphate or Wesson salt

Sample type	Procedural method	Vol. of sample	Concentration range (%)[c]	Waiting time/min
P[a]	A[b]	0.5 ml	20 to 100	2
P	A	1.0 ml	10 to 80	2
P	C	50 μl	2 to 15	1
P	C	200 μl	0.5 to 5	2
P	C	1.0 ml	0.1 to 1	6
W		200 μl	20 to 100	3
W		1.0 ml	4 to 33	8
W		5.0 ml	0.07 to 5	80

[a] P = Pure ferric phosphate; W = Wesson salt. [b] A = Amorphous procedure; C = crystalline procedure. [c] Units for concentration range are the percentage of the iron that is AFP compared to the total iron.

1,10-phenanthroline, and 5 ml of 2% sodium acetate. Color develops in less than 2 min. Measure the absorbance at 510 nm.

If using the crystalline procedure, take 50 μl, 200 μl, or 1 ml of citrate extract, depending on the AFP content (see Table 3). Add 0.2 ml of 6 M HCl, 1 ml of hydroxylamine, 1 ml of 1,10-phenanthroline, and 5 ml of 10% sodium acetate. Wait the minimum time as shown in Table 3 and measure the absorbance at 510 nm. Calculate the concentration of iron by comparing to a calibration curve. Our results are expressed as a percentage that is the concentration of iron present as AFP compared to the total iron concentration (measured by completely dissolving the sample in 6 M HCl).

Artificial diet. Place 0.4 g of undried artificial diet in a test tube that fits a heater–stirrer device. Add 5 ml of pH 4 citrate. Homogenize the sample using a homogenizer or similar device. Do not wash the homogenizer after each sample, but clean the surface with a paper wipe to reduce contamination or dilution of succeeding samples as much as possible. Insert a stirring vane, and loosely cap the tube. Place the tube in the heater–stirrer that has been preequilibrated at 75 °C and heat and stir for 15 min. Remove the cap and stirring vane, refrigerate for 30 min and centrifuge at 2400*g*. The clear solution (citrate extract) can be analyzed for iron by any of the accepted techniques for analyzing iron. If using colorimetry, combine 2 ml of the citrate extract, 1 ml of hydroxylamine, 1 ml of 1,10-phenanthroline, and 2 ml of 2% sodium acetate. Wait a minimum of 5 min and measure the absorbance at 510 nm. Compare to standards containing 0 to 12 ppm iron dissolved in citrate.

We recommend using undried diet because it homogenizes completely whereas dried diet does not. We eliminated drying by placing the containers of diet in plastic bags and storing in a refrigerator so that the humidity remained constant.

Wesson salt mixture. Place 0.2 g of salt in a test tube. Add 10 ml of pH 1 citrate and a stirring vane. Cap the tubes loosely and place in a heater–stirrer that has been preequilibrated at 75 °C. Heat and stir for 15 min. Remove the caps and stirring vanes and centrifuge for 5 min at 2400*g*. The clear citrate extract can be analyzed for iron by any of the usual techniques. If using colorimetry, the recommended procedure depends on the AFP content. Take the quantity of clear citrate extract indicated in Table 3. Add 1 ml of hydroxylamine and 1 ml of 1,10-phenanthroline. If the volume of sample is 5 ml, add 1.0 ml of 10% sodium acetate. If the volume of sample is less than 5 ml, add 5 ml of 2% sodium acetate. Wait until the color is fully developed (Table 3) and measure the absorbance at 510 nm.

Cleaning recommendation. In the analysis of both Wesson salt and ferric phosphate, soak the stirring vanes in hot dilute HCl and fill the test tubes with hot HCl for several minutes.

Results and discussion

Pure ferric phosphate

Ferric phosphate, pure in the sense that no other compound was present, but varying in the percentage of amorphous or crystalline form, was tested for the amount of iron extracted as a function of pH, temperature, heating time, and concentration of citrate in the extracting solution. The goal was to find extracting conditions that extracted all of the amorphous form and none of the crystalline form. In 1994 Willis and Montgomery reported that citrate was the preferred reagent for this procedure.[8] Based on the work of Lehrecke,[11] a pH of 9.1 was recommended. In this investigation, it was found that pH 4 was the optimum for achieving the goal described above (Fig. 1). At pH 4, essentially 100% of the amorphous form was extracted, whereas less than 1% of the crystalline form was extracted.

Another reagent that has shown potential for differentiating the amorphous and crystalline forms is hydrochloric acid, which was predicted based on the work of Harrison *et al.*[17] It was evaluated by measuring the amount of iron extracted using different concentrations of hydrochloric acid instead of citrate solutions and following the recommended procedure as described above. The test indicates that the optimum concentration of HCl to use is about 0.3 to 0.4 M (Fig. 2). With 0.3 M HCl, 96% of AFP is extracted and 0.9% of CFP is extracted. This indicates very good discrimination between amorphous and crystalline ferric phosphate, but is not as good as citrate, for which 100% of the amorphous form was extracted. Therefore, citrate is recommended as the preferred extractant.

The concentration of citrate required for quantitative extraction depends on the sample size (Fig. 3). For a sample size of 0.15 g of AFP, the minimum concentration of citrate needed to quantitatively extract AFP is 0.2 M. For a sample size of 0.3 g of AFP, the minimum concentration is about 0.4 M. Because solutions with a high salt content tend to present more difficulties, it was decided to use a concentration of 0.2 M and keep the sample size at 0.15 g.

Completely dissolving 0.15 g of AFP ranges from 2 h when heated at 45 °C to 8 min when heated at 85 °C (Table 4). CFP also dissolves at all temperatures tested but at a much slower

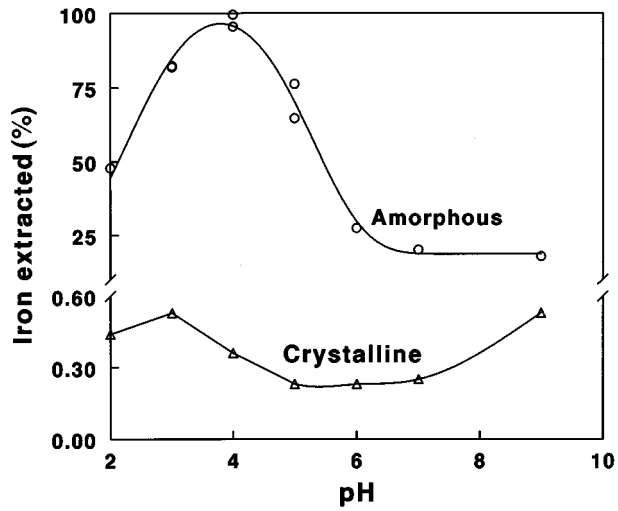

Fig. 1 Extraction of iron from pure ferric phosphate (amorphous or crystalline) using 0.2 M citrate as extractant.

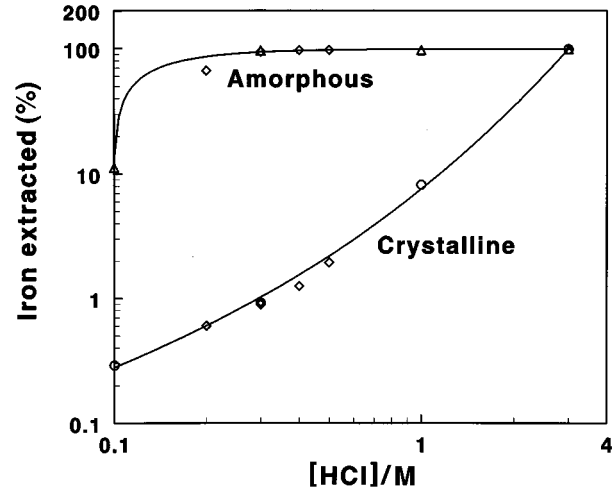

Fig. 2 Extraction of iron from either pure amorphous or pure crystalline ferric phosphate as a function of HCl concentration.

rate. The optimum heating time and temperature is 20 min at 75 °C (Table 4), which dissolves AFP completely, but only 0.7% of CFP.

Extraction of AFP from ferric phosphate samples that are all or nearly all amorphous is quantitative for sample weights less than 0.15 g. Thus, the weight of a sample can be lower than 0.15 g without affecting the results (Fig. 4). For samples that are mostly crystalline, there is an inverse relationship between extraction of iron and the weight of the sample, necessitating the use of the same weight each time and indicating the method is not truly quantitative. However, results are still useful because the apparent AFP content varies by less than 1% as the sample weight varies from 0.1 g to 0.3 g. The weight of 0.15 g was recommended earlier and is still appropriate.

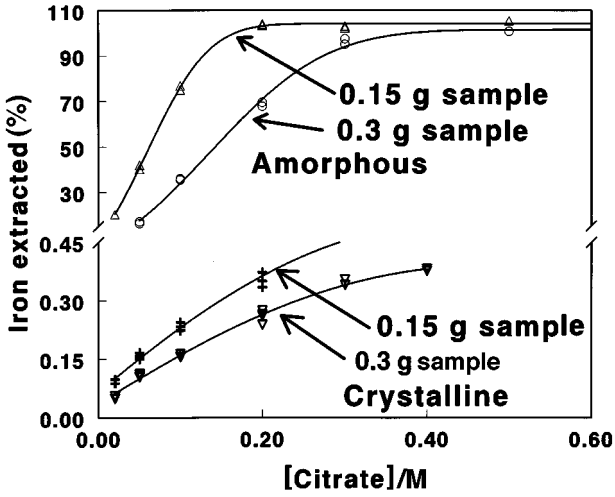

Fig. 3 Extraction of iron from pure ferric phosphate (amorphous or crystalline) as a function of citrate concentration.

Table 4 Time for 0.15 g sample of amorphous or crystalline ferric phosphate to completely dissolve in 10 ml citrate when heating in a heater–stirrer

	Time[a]	
Temp/°C	C	A
45	17 d	120 min
55	6 d	60 min
65	53 h	30 min
75	7 h	15 min
85	4 h	8 min

[a] The procedure for estimating total time required is described in Recommended procedure.

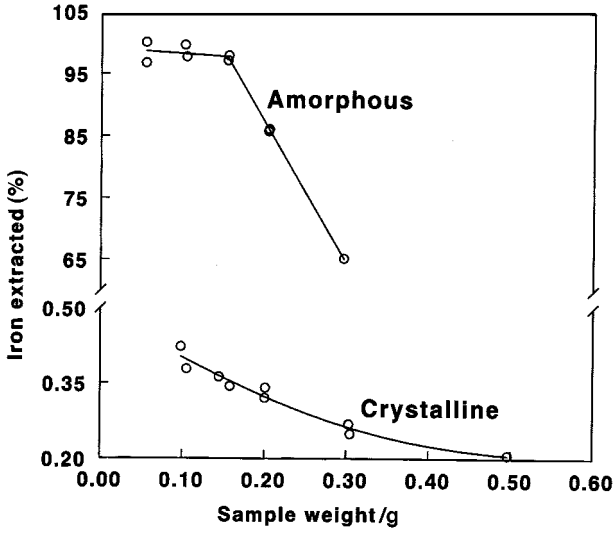

Fig. 4 Extraction of iron from pure ferric phosphate (amorphous or crystalline) as a function of sample weight.

After the extraction is complete, the sample is centrifuged and the supernatant is analyzed for iron. Any of the usual techniques such as atomic absorption or colorimetry can be used. With the colorimetric procedure, it is necessary to add a strong acid such as hydrochloric acid before the actual colorimetric step. The necessity for acid is somewhat surprising because the pH required for 1,10-phenanthroline to react quantitatively with iron ranges from 2 to 9.[18] Adding acid lowers the pH taking it out of the desired pH range. After the addition of acid, hydroxylamine, and 1,10-phenanthroline, the pH is brought back to the desired range by the addition of sodium acetate. With this procedure, the reaction is completed in less than 6 min. If acid is not used, the reaction time is very slow, approximately 5 h. The reaction takes 5 h whether the original sample of ferric phosphate that was dissolved is crystalline or amorphous. However, some samples react very fast for a minute or two and then very slow. For example, a ferric phosphate sample with ID: USB, 5-93 was extracted and the iron content measured using the crystalline procedure using 50 μl of citrate extract. No acid was added. A small portion of the iron in the citrate extract (3.8% of the total iron) reacted with 1,10-phenanthroline in less than 1 min. The remainder of the iron that was extracted by citrate (5.6%) reacted very slowly requiring about 5 h to complete. Based on the criteria that both species of iron dissolved in the citrate, they would be reported as AFP for a reported value of 9.4%. However, the iron that reacted immediately is probably contamination and so the true AFP content is assumed to be 5.6%.

In summary, the recommended procedure of first adding acid to the citrate extract results in a colorimetric reaction that goes to completion in less than a minute rather than 5 h which would be the case if acid was not added. There is a disadvantage in that iron compounds other than ferric phosphate are measured and would be reported as AFP. If it is necessary to exclude any iron contamination, eliminate the addition of acid from the procedure and measure only the increase in absorbance between 2 min and 5 h. To obtain the concentration of CFP, measure the total iron content by completely dissolving the sample in a strong acid such as 6 M HCl and measuring the iron content of this solution. This value minus the AFP content is the CFP content.

Artificial diet

Previous tests using pure ferric phosphate led us to assume that pH 4 would be optimum for extracting AFP from diet. This assumption was tested by extracting iron with citrate solutions with pH ranging from 3 through 9. One difficulty observed was the presence of turbidity that could not be removed by filtering or centrifugation. It seems the primary cause of turbidity is casein. The intensity of the turbidity varied with pH and was lowest at pH 4. It was reduced further by high-speed centrifugation and refrigeration. Table 5 shows the results of three diets containing different concentrations of casein and subjected to different centrifugation speeds and refrigeration; the results are measured as apparent AFP but, in reality, are turbidity.

For diet containing 25 mg g^{-1} or less casein, we recommend refrigeration for 0.5 h followed by centrifugation at 2400*g*. This gives adequate results and increasing the centrifugation speed to 48 000*g* does not significantly change the apparent AFP content. A pH of 4 is recommended for the citrate extract because this produces the minimum turbidity, and also was found to be the optimum pH based on extraction of AFP from pure ferric phosphate.

Temperature is a very critical parameter but, within the range tested, does not seem to have any effect on the quantity of AFP extracted. Different diets having AFP in varying quantities from 0 to 60 μg of iron per g of diet were extracted at 75 °C and 95 °C and the apparent AFP measured. Results for the two temperatures were equal (paired *t*-test, *P*-value = 0.567). However, even though temperature does not affect the apparent iron content, there are two very important parameters that are affected by temperature: turbidity and the amount of puffy or gelatinous material. At temperatures lower than 70 °C, turbidity forms regardless of the pH. At temperatures greater than 80 °C, a puffy or gelatinous material is formed due to agar, a normal component of the diet. The quantity of gelatinous material increases as the temperature increases. So, considering both of these effects of temperature we chose a temperature of 75 °C. At that temperature, there is very little turbidity and no gelatinous material. This assumes the casein content of the diet is 25 mg g^{-1} or less. If the casein content is higher, turbidity becomes a problem and it is necessary to use more stringent reaction conditions heating the samples at 95 °C rather than 75 °C. This produces more gelatinous material that is packed down tight on the bottom of the tube if a higher centrifugation speed of 20 000 to 27 000*g* with simultaneous refrigeration is used. At the same time, the particles that cause turbidity are embedded in the gelatinous material and turbidity is essentially eliminated.

There is an inverse relationship between the apparent iron concentration and the sample weight (Table 6). For this reason, it is important to use the same weight of diet each time. We recommend 0.4 ± 0.03 g samples.

When investigating the effect of heating time, we found that the time required to completely extract the iron when the diet contained the amorphous form of ferric phosphate depended on the method of preparing the diet. For both the Otis diet and the Phoenix diet, all iron was extracted immediately after homogenization and no heat was required. For the Hamden diet, only half of the AFP was extracted immediately after the homogenization. Because all three of these diets were prepared following the same recipe,[15] it is assumed the difference in amount of iron extracted was due to some physical difference in the preparation technique such as the stirring speed. It required about 15 min at 75 °C to completely extract the iron from the Hamden diet. For all diet preparations, CFP extracted much more slowly. At 95 °C, the extraction of CFP was about 18% per hour. At 75 °C, there was no evidence that any CFP was extracted when tested up to 1.5 h. Iron from all ingredients other than ferric phosphate (wheat germ and iron contamination in the Wesson salt) dissolved in the citrate buffer immediately after the diet and citrate buffer mixture was homogenized, even with no heat applied. This was verified by measuring the iron content for the mixture, which had been homogenized but received no heat, and comparing the iron content to that found when heated at 75 and 95 °C for times up to 1.5 h. Even if the iron is extracted without any heat applied, it is still necessary to heat the mixture to eliminate turbidity as discussed earlier. Considering all the effects just described, we recommend heating the mixture for 15 min. This is enough to extract any AFP that is present regardless of the method of diet preparation, and turbidity is not a significant interference.

When analyzing colorimetrically, any dye or colored reagent in the diet is a concern. Three different dyes were present in the diets we tested. The first was Calco Red Dye, which was present in the diet used for pink boll worm at a concentration of 298 μg g^{-1} (wet weight). This dye was not a problem because it is nonpolar and was not extracted into the citrate extract. Two dyes used in each of the two experimental diets were blue food coloring, 3 drops l^{-1}, and green food coloring, 2 drops l^{-1} (both from McCormick & Co., Inc., Hunt Valley, MD). The apparent AFP content due to the presence of these two dyes was 1.5 μg g^{-1} as iron. This is a relatively small amount causing only a 2% positive error in the apparent iron content. It is also constant, so it can be subtracted from the final value.

HCl, which showed good promise as the extractant when measuring the AFP content of pure ferric phosphate, was tested as a potential solvent for extracting AFP from diet. We found it to be unacceptable because of high levels of turbidity in the extracted solution.

Wesson salt

The suitability of citrate for measuring AFP in Wesson salt mixtures was tested by measuring the amount of iron extracted as a function of pH from Wesson salt, containing either AFP or CFP (Fig. 5). The optimum pH appears to be 1.0, which is significantly different than that found using pure ferric phosphate which was pH 4. One possible reason for the difference is that other components of the Wesson salt cause a large change in the pH of the mixture. This theory was tested using pH 1 and pH 4 extracts. After combining the pH 1 extract and Wesson salt, the pH changed to 1.47. Doing the same test using pH 4 extract and Wesson salt resulted in a pH of 4.55. Thus, Wesson salt changes the pH of the extract but not enough to explain the large difference in optimum pH between Wesson salt and pure ferric phosphate. Why pH 1 is the optimum pH rather than pH 4 is unknown.

Hydrochloric acid was also evaluated as a potential extractant of AFP using a concentration of 0.3 M, which was the optimum concentration when analyzing pure ferric phosphate. Wesson salt preparations made by different companies or different batches made by the same company were chosen to obtain the maximum possible variation in AFP content. These samples were analyzed and the resulting concentration was compared to that using citrate. In every case except one, the results were statistically equal. The one exception had a value of 7.64 ± 0.02% using citrate and 9.47 ± 0.05% using acid, where the percentage is the iron in the AFP compared to the total iron. This exception was studied more intensely and it was found that if the citrate extract was allowed to sit before being analyzed the apparent iron content increased until it eventually reached the same value as that obtained if extracted with HCl. Specifically,

Table 5 Effect of turbidity on apparent AFP[a] content of diets containing different concentrations of casein and subjected to different levels of centrifugation and refrigeration

		Apparent AFP/μg g^{-1}		
Sample ID	Casein/mg g^{-1}	No refrig 2400*g*	Refrig[b] 2400*g*	Refrig[c] 48 000*g*
I81	0	3.98 ± 0.40	1.31 ± 0.12	0.97 ± 0.06
I68	25	6.87 ± 0.30	4.35 ± 0.03	3.44 ± 0.40
Q339	30	9.03 ± 1.28	4.74 ± 0.36	3.31 ± 0.94
Q340	35	10.6 ± 2.9	4.87 ± 0.24	2.37 ± 0.61

[a] Apparent iron content was measured by using the recommended colorimetric procedure but substituting 1 ml of water for 1 ml of 1,10-phenanthroline. Weight of diet is wet weight. [b] Samples were refrigerated at 4 °C for 0.5 h followed by centrifugation. [c] Samples were centrifuged at 48 000*g* while being simultaneously cooled at 1–4 °C.

the apparent AFP measured immediately after extraction using citrate was 75% of the value measured using acid. After 3 h, it was 78% and after 24 h, it was 97%. When the same salt extracted with HCl was allowed to sit up to 24 h, the apparent iron did not change. Nor did the iron content change for either the citrate or acid extract of the other salt preparations. Thus, it appears that the salt in question had an iron impurity that, when dissolved in HCl, reacted immediately but, when dissolved in pH 1 citrate, did not initially react with 1,10-phenanthroline but slowly changed to a form that did react. Because this iron impurity is probably not AFP, it should not be measured. For this reason, the preferred solvent to extract Wesson salt is pH 1 citrate.

The sample weight is important in analyzing Wesson salt mixtures. If the iron that is present in the salt is 99% or more AFP, results are equal for sample weights of 0.2 g or less. If the AFP content is less than 99%, the apparent AFP content varies slightly with the weight of the sample. The amount of variation can be illustrated using two examples, one with an apparent AFP content of 46% and the other with 4%. Both had an apparent decrease of about 2% when the sample weight increased from 0.12 g to 0.3 g. We recommend a weight of 0.2 g which gives quantitative results for salt samples having a high AFP content and only an error of about 1% for samples having less than 99%.

The effect of temperature was examined by measuring the amount of iron extracted from various salt samples with AFP content varying from 3% to 99%. There was no statistical difference (paired *t*-test, *P*-value = 0.703) in the apparent AFP content when extracting at 75 °C compared to 95 °C. We recommend 75 °C because it was the preferred temperature when extracting pure ferric phosphate.

Table 6 Extraction of iron from diet containing either AFP, CFP, or no ferric phosphate as a function of sample weight

Sample wt/g	AFP[a]	CFP[a]	No ferric phosphate[a]
0.2	35.5	17.7	14.8
0.4	32.3	14.7	12.0
0.6	31.9	12.8	11.0

[a] Results are expressed as μg g^{-1} as iron. Concentration of both AFP and CFP added to the diet was 25.4 μg g^{-1} as iron. The iron that was measured was greater than the iron added because wheat germ contained iron and there was iron contamination from compounds other than ferric phosphate in the Wesson salt.

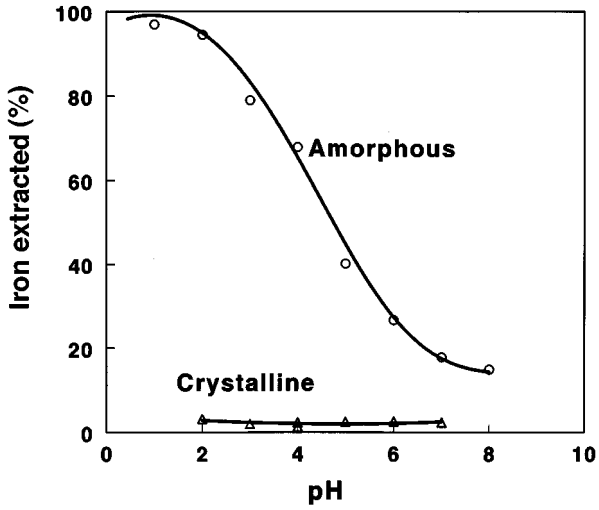

Fig. 5 Extraction of iron from Wesson salt containing ferric phosphate (amorphous or crystalline) as a function of pH using 0.2 M citrate as extractant.

Conclusion

In order for ferric phosphate to be utilized as a source of iron for gypsy moths and as a source of phosphate for certain plants, it must be amorphous. If this is true, there must be a method to measure AFP in the presence of CFP. The procedures described herein have been successfully used to measure the AFP content of artificial diet, Wesson salt mixture, and relatively pure ferric phosphate. This is the first publication of any method describing the measurement of the AFP content of pure ferric phosphate. A method for measuring the AFP content of artificial diet and Wesson salt was published in 1994 by Willis and Montgomery.[8] This method was compared to the 1994 method in the analysis of diet to which 35.6 μg g^{-1} as iron of either AFP or CFP had been added. When CFP was added, the results were statistically equal to those where nothing was added using either this method or the 1994 method. When AFP was added, 61% was recovered using the 1994 method and 83% was recovered using this method. Thus, this method is a considerable improvement over the 1994 method.

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