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# Development and Analytical Evaluation of a Spectrophotometric Procedure for the Quantification of Different Types of Phosphorus in Meat Products

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**ABSTRACT:** Phosphorus is an important natural nutrient, but high dietary phosphorus intake, including that sourced from added preservatives, is of great concern in renal patients. In this context a reliable analytical method able to quantify differential phosphorus in food could be a valuable tool for monitoring diet composition This paper presents a novel analytical procedure to quantify the following kinds of phosphorus in cooked ham: total (TP), inorganic (IP), from phospholipids (PL), and from phosphoproteins (PP). This technique is based on a suitable sample preparation followed by spectrophotometric analyses. Analytical performances of each method were evaluated, taking advantage also of food industry certified material and in-house reference samples. Limit of detection and limit of quantification values for TP, IP, PP-derived, and PL-derived phosphorus were 13 and 37, 11 and 33, 2 and 20, and 6 and 16 mg P/100 g fresh mass, respectively. Similar results were obtained when this procedure was used to quantify different types of phosphorus present in cooked ham samples. In conclusion, this procedure is effective for quantifying the content of different types of phosphorus present in cooked ham, which can be contributed by different phosphorus-containing ingredients and additives. To the best of the authors' knowledge, this is the first time that simultaneous determination of TP, IP, PL, and PP in cooked ham has been reported.

**KEYWORDS:** *meat products, cooked ham, total phosphorus, spectrophotometric analysis, food additives, polyphosphate, phospholipid, phosphoprotein*

## INTRODUCTION

Phosphorus is an important natural nutrient, but high dietary phosphorus intake, including that sourced from added preservatives, is of great concern for health, in particular in patients with kidney disease. Dietary phosphorus (P) exists as inorganic salt or bound to organic molecules. Organic P is present in foods as phosphoproteins (PP), phospholipids (PL), and other sources such as phytate or starch phosphate monoester in vegetable foods. Inorganic P is naturally present as phosphate anions in small amount.[1−3] Moreover, a contemporary diet that includes processed or enhanced foods provides significant amounts of inorganic P from preservatives.[3,4] This may be a concern to health because the inorganic P of food preservatives is almost 100% absorbable through the gastrointestinal tract as compared to natural P, which is usually only 40−60% absorbable. Previously, we and others used spectrophotometric determination to differentially measure total P (TP) and inorganic P (IP) in common foods.[5,6] Whereas it is a comparatively easy task to measure total P, differential measurements of diverse types of P in food and, in particular, quantification of the added inorganic P are difficult tasks.[7] The amount of added P is usually estimated by the difference between the measured total P and the P expected on the basis of the protein content estimates.[8,9]

In a recent study we described a novel biochemical differential measurement of natural versus added P capable of detecting the amount of the different P-containing molecules in meat products such as cooked ham.[10] The focus of the said study was the determination of the extra burden of dietary P due to P-containing additives used in meat products.

The present study provides a detailed description of the previously reported methods,[10] as well as an evaluation of the analytical performance of these methods, and the latter is verified either by using materials certified by the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre or in-house testing. It should be pointed out that the procedures for the quantification of the different kinds of P were based on well-known methods. We have optimized and refined them to minimize the number of needed steps, thus making the quantification of the different types of P more convenient and less expensive. These refined methods were then applied to commercial brands of cooked ham to investigate the contribution of the P-containing ingredients, such as milk proteins, plant protein extracts, soy protein isolates, and wine, to the total P content.

## MATERIALS AND METHODS

**Cooked Ham Samples.** Twenty-four cooked ham items were purchased at common markets in Pisa, Italy. Products were arbitrarily

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and randomly chosen. A portion of each item was minced three times with a meat grinder, packed into polyethylene bags, frozen, and stored at −20 °C until it was submitted to the specific sample preparation procedure preceding the assay. All commercial food labels were maintained and their data collected; food labeling in Italy is enforced by Council Directive 2000/13/EC on labeling: in the European Union the list of ingredients is compulsory on the labeling of food products, where under the term "Ingredient" there should be the list of any substance, including additives, used in the manufacture or preparation of a food and present in the final product, even in altered form. In this investigation, after examining the label information, we divided cooked ham items into three different groups that were characterized by a different use of P-containing ingredients and additives during the production processes. We classified them as follows: "regular", when no evidence of P-containing ingredients and additives was present on the package label ($n$ = 8); "enhanced with P-containing additives" (EWP), when the words "containing polyphosphates" or the coding "E338−E341, E450−452" was used on the package labelling ($n$ = 8); "enhanced with P-containing ingredients and additives" (EWPIA), when the words "containing polyphosphates" or the coding "E338−E341, E450−452" together with the key words "milk proteins", "plant protein extracts", "soy protein isolates", "wine" were mentioned on the package labeling ($n$ = 8).

**Chemicals, Reagents, and Certified Reference Materials.** Potassium dihydrogen phosphate ($KH_2PO_4$), sodium hydroxide (NaOH), sulfuric acid 96% ($H_2SO_4$), sodium sulfate ($Na_2SO_4$), trichloroacetic acid 99%, chloroform, and methanol were used as analytical grade and provided by Sigma-Aldrich (St. Louis, MO, USA). Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$), hydrogen peroxide solution 30% ($H_2O_2$), and L-ascorbic acid were purchased from Carlo Erba (Milano, Italy). $KH_2PO_4$ and $Na_2SO_4$ were dried in an oven (110 °C for 2 h) and cooled in a desiccator overnight before use. Meat reference material, lyophilized pork muscle powder (ERM-BB384), was supplied by LGC Standards (Teddington, UK), whereas a 1 g/L phosphorus standard solution (Certified Reference Material, CRM) was supplied by Carlo Erba. Calibrated class A glassware and bidistilled water (specific conductivity < 18 mS) were used in the preparation of all solutions. In particular, the following solutions were prepared: 4 M sulfuric acid, 100 g/L trichloroacetic acid solution, P working standard solution at a concentration of 5 mg/L by using the 1 g/L P standard solution, 18 g/L ammonium molibdate solution by dissolving ammonium heptamolybdate tetrahydrate and 25 g/L ascorbic acid, these latter freshly prepared.

**Instrumentation.** Absorbance was measured by a UV−visible spectrophotometer (CP/2300, CGA Strumenti Scientifici, Firenze, Italy) in a 1 cm quartz cell, against reagent blank. Mineralization of samples and solutions was performed using a heating digester with programmable temperature controller (DK 20/26, Velp Scientifica Ertec, Poland). Samples were centrifuged by a laboratory centrifuge (4235A, ALC, Milano, Italy) with an angle rotor (maximum speed 6000 rpm, RCF 4800$g$, angle 45°, Falcon tubes 50 mL). Minced samples were obtained by using a manual meat grinder having a plate with 3 mm diameter holes. Homogenization of samples and solutions was performed using an Ultra-Turrax T 25 homogenizer with an S25N-18G dispersing tool (IKA-Werke GmbH, Staufen, Germany). For solvent evaporation a Rotavapor R-114 (Büchi Labortechnik AG, Flawil, Switzerland) with a water bath B-480 (Büchi Labortechnik AG) was used.

**Total and Different Subtypes of Phosphorus.** Total P, inorganic P, P from phospholipids, and P from phosphoproteins were detected by molybdenum blue method on wet ashing samples, preceded by a specific sample preparation.

**Total Phosphorus (TP): Sample Preparation Method and Wet Ashing Procedure.** Total P was determined as previously described.[11] In brief, the sample (0.4 g fresh mass, fm) was wet mineralized with 96% sulfuric acid and 30% hydrogen peroxide by using the temperature digestion program shown in Table 1. The cool digest was then transferred to a 100 mL volumetric flask and made up to the mark with bidistilled water, and it underwent a final measurement of P by using the P quantification procedure.

**Table 1. Wet Digestion Program for TP, IP, PL, and PP Measurement Analyses**

| step | temperature (°C) | digestion time (min) |
|---|---|---|
| 1 | 120 | 45 |
| 2 | 260 | 60 |
| 3 | 320 | 90 |
| 4[a] | 420 | 180 |

[a]PP and PL sometimes needed an extra 60 min at step 4.

**Inorganic Phosphorus (IP): Sample Preparation Method and Wet Ashing Procedure.** Soluble phosphate ions, which usually include either natural or, to a greater extent, added inorganic P, were extracted according to the method described by Jastrzębska et al. modified in our laboratory: the samples (5 g fm) were extracted with 10 mL of 1 mM NaOH, using an orbital shaker for 60 min.[6,12] The extracts were separated by centrifugation at 6000 rpm (RCF 4800$g$) for 30 min, and supernatant was filtered under vacuum with a Whatman no. 1 filter paper. Filtrate was transferred to a 50 mL volumetric flask, where it was made up to the mark with bidistilled water. Afterward, a 5 mL aliquot of the sample solution was moved to a 125 mL digestion flask where, after the addition of 5 mL of 96% sulfuric acid and 5 mL of 30% $H_2O_2$, it was digested until the mixture was clear, by using the temperature program reported in Table 1. The so-obtained cool digest was transferred to a 100 mL volumetric flask and made up to the mark with bidistilled water, and it underwent a final measurement of P by using the P quantification procedure.

**Phosphorus from Phospholipids (PL): Sample Preparation Method and Wet Ashing Procedure.** P derived from phospholipid content was determined in the polar lipid extract of samples. On the basis of the results reported by Pérez-Palacios et al., lipid extraction was performed according to the method of Folch.[13,14] In practice, the samples (2 g fm) were mixed with 20 mL of a chloroform/methanol (2:1, v/v) mixture and immediately homogenized for 2 min at medium speed (motor speed set to 13500 rpm) in an Ultra-Turrax homogenizer. The apparatus was rinsed twice with 5 mL of the above-mentioned chloroform/methanol mixture, and the extracts were then combined. The pooled extract was centrifuged (10 min, 4000 rpm, RCF 2000$g$) and filtered through Whatman no. 1 filter paper. The residue in the filter paper was washed with 5 mL of chloroform/methanol (2:1, v/v), and all of the filtrates were then combined. Subsequently, 4 mL of bidistilled water was added, and the new mixture was shaken vigorously. The final biphasic system was separated by centrifugation (10 min, 4000 rpm, RCF 2000$g$). The upper aqueous layer was eliminated, 1 g of anhydrous sodium sulfate was added to the organic phase, and the mixture was shaken vigorously. The chloroform phase was filtered by Whatman no. 1 filter paper and collected. The residue in the filter paper was washed with 5 mL of chloroform, and the filtrates were then combined. The solvent was further evaporated with a rotary evaporator under vacuum and, finally, the glass vessels with the residue, which contained the lipids, were dried in an oven (100 °C for 30 min). Cool lipid extract content was then weighed, and half of it (from 40 to 80 mg) was then placed into a 125 mL digestion flask, to which 5 mL of 96% sulfuric acid and 5 mL of 30% $H_2O_2$ were added, and digested until the mixture was clear by using the temperature program reported in Table 1. The cool digest was transferred to a 100 mL volumetric flask and made up to the mark with bidistilled water, and it underwent a final measurement of phosphorus by using the phosphorus quantification procedure.

**Phosphorus from Phosphoprotein (PP): Sample Preparation Method and Wet Ashing Procedure.** The quantification method for P from phosphoprotein was strictly derived from that published by Dušek et al.:[9] samples (1.5 g fm) were homogenized in 36.5 mL of bidistilled water for 2 min at medium speed (motor speed set to 13500 rpm) by an Ultra-Turrax homogenizer. The apparatus was rinsed with 1 mL of bidistilled water, and the solutions were then combined. The so-obtained mixture was boiled for 30 min on a hot plate. After cooling, 10 mL of a 100 g/L trichloroacetic acid solution was added. The precipitate was filtered through Whatman no. 1 filter paper and

dried in an oven (100 °C for 30 min). After cooling, the filter was weighed, and about 50% of it (equivalent to 0.75 g fm) was placed in a 125 mL digestion flask and digested as described above. The cool digest was transferred to a 100 mL volumetric flask and made up to the mark with bidistilled water, and it underwent a final measurement of phosphorus by using the P quantification procedure.

**Phosphorus Quantification Procedure: Molybdenum Blue Method on Wet Ashing Sample.** Orthophosphate ions contained in wet mineralized sample solutions from TP, IP, PP, and PL were determined by using the Molybdenum Blue method according to Soderberg[15] and Italian Dairy Product Official Methods of Analysis. In brief, the mineralized sample solutions (800 μL) were transferred to a 10 mL volumetric flask and then were added the following reagents: 7 mL of bidistilled water, 390 μL of 4 M sulfuric acid solution, 400 μL of an 18 g/L ammonium molibdate solution, and 400 μL of a 25 g/L ascorbic acid solution. Each flask was gently swirled and finally placed in boiling water for 30 min to form characteristic molybdenum blue species. The solutions were then cooled to room temperature and made up to the mark with bidistilled water, and absorbance was measured by spectrophotometry at 650 nm against a blank.

**Reference Samples.** The TP method was tested by the ERM-BB384 Certified Reference Material (lyophilized pork muscle), the mean value and expanded uncertainty of which were provided by the certificate of analysis.

For IP analysis, one of the regular samples was spiked at two different levels: 100 mg P/100 g fresh sample or 150 mg P/100 g fresh sample. Because hydrolysis of polyphosphates leads to orthophosphate anions,[12] 443.8 and 665.7 mg of $KH_2PO_4$ were respectively added to 100 g of minced cooked ham, which was then passed twice through a meat grinder. Spiked samples were stored at −20 °C until assayed. The measurement uncertainty in spiked samples was calculated by type B evaluation of uncertainty. For PP and PL methods suitable meat certified reference materials were not available on the market; hence, in-house reference samples were prepared from "regular" cooked ham samples as reported below.

The PP in-house reference sample was obtained using the following procedure. Bidistilled water was added to a cooked ham sample and homogenized. The mixture thus obtained was boiled on a hot plate and, after cooling, trichloracetic acid was added. The precipitate was filtered and then was dried in a laboratory oven and allowed to cool in a desiccator overnight. Amounts of this dried filtrate were used as reference samples for PP analysis.

The PL in-house reference sample was prepared using the method developed by Folch.[14] In brief, the sample of cooked ham was mixed with a solution of chloroform/methanol (2:1, v/v) and homogenized. The extract was centrifuged and filtered. Bidistilled water was added to the filtrate and was shaken vigorously. The final biphasic system was allowed to separate by centrifugation. The upper aqueous phase was discharged, and anhydrous sodium sulfate (about 2 g) was added to the mixture to dry the organic phase. Then the chloroformic phase was filtered, the solvent was further evaporated under vacuum, and, finally, the glass vessel with the residue, which contained phospholipids, was dried in a laboratory oven. Cool lipid extract content was stored at −20 °C until assayed.

Reference values of P in-house reference samples of PP and PL were determined by the TP method as the averages of 10 measurements spread over three weeks. The standard deviations of average divided by the square root of the number of measurements were used as estimation of uncertainty of measurements. Coverage factors $k$, accordingly based on Student's test to a level of confidence of 95%, were applied to calculate the expanded uncertainties from the uncertainties of in-house reference samples used in PP and PL methods and of two spiked levels in IP method.

**Calibration Curve.** The linearity of the calibration curve was established for the concentration range of 0.1−1.2 mg P/L. The calibration curve was built using nine calibrators obtained by appropriate dilution of the 5 mg P/L working standard solution by bidistilled water. Their concentration values were 0.10−0.20−0.30−0.40−0.65−0.90−1.00−1.10−1.20 mg P/L. Calibration solutions underwent a P quantification procedure, after adjustment of the $H_2SO_4$ concentration by adding 190 μL of 4 M $H_2SO_4$, as required by the Molybdenum Blue quantitation method.[16,17] Notably, the amount of $H_2SO_4$ added to the calibrators was for matching its concentration in the samples from wet ashing. The instrumental limit of detection (LOD) and limit of quantification (LOQ) were calculated by the formulas $(y + 3s_{y/x})/b$ and $(10s_{y/x})/b$, respectively, where $y$ is the intercept of the calibration line, $s_{y/x}$ is the standard deviation in the $y$-direction of the calibration line, and $b$ is the slope of the calibration line.[18] Both values were expressed as mg P/L.

**Analytical Performances of TP, IP, PP, and PL Measurement Methods.** Precision and bias of TP, PP, and PL methods were estimated by using both Certified Reference Material ERM-BB384 and in-house reference standards. Sample spiking at two P addition levels, as previously described, was used for the IP method. Results were calculated as an average of five measurements spread over three weeks. All data were expressed as a mean value ± $\mu$ (confidence interval, $p$ = 95%). Intermediate precisions were evaluated as the between-day coefficient of variation (CV) and the standard error of the mean ($M$). Measurement bias of each TP, IP, PP, and PL analytical procedure was determined by evaluating the deviation between the average value obtained from a series of measurements and the theoretical reference value. It includes the percent method recovery (REC), calculated as 100(obtained concentration/theoretical concentration).[19] Each method's LOD and LOQ, expressed as mg P/100 g fm, were calculated from instrumental LOD and LOQ values taking into account weights, dilutions, and percent method recovery.

**Assay Quality Control in Cooked Ham Analysis.** Each sample batch of cooked ham samples for the quantification of TP, PP, or PL included two reference (certified or in-house) samples, whereas two spiked and two unspiked samples were used for the IP determinations. Set measurements were rejected and repeated when the absolute difference between measured average values and reference (certified, in-house, or spiked) values were outside the range ± expanded uncertainty reported in the certificate of analysis for CRM and ± estimation of expanded uncertainty for in-house or spiked values. All sample analyses were performed in duplicate. Notably, food label nutritional content was blinded to all laboratory technicians to have no influence on the final results.

**Statistical Analysis.** Two relationships, the first between the concentration of P and absorbance at 650 nm in the calibration curve and the second between TP and the total differential P (DfP), which was the sum of the subtypes of sample P in form of IP plus PP and PL (DfP = IP + PP + PL) in cooked ham samples, were evaluated by paired $t$ test for the significance of the Pearson product−moment correlation coefficient. The experimental $t$ test value ($t_{exp}$) was compared to its tabulated value ($t_{crit}$). A linear relationship between variables was accepted at a significance level of $p < 0.001$. The unbiased measurement of any TP, IP, PL, and PP methods was verified with Student's paired test, when REC was not significantly different from 100 at a significance level of $p < 0.01$. Differences in P values from TP, IP, PL, and PP in each group of cooked ham were compared by one-way ANOVA post hoc tests. The null hypothesis was rejected at a significance level of $p < 0.05$. When a significant effect was detected, the comparison of means was done using Fisher's least significant difference (LSD) test.

## RESULTS AND DISCUSSION

**Calibration Curve of TP, IP, PP, and PL.** The regression parameters of the calibration curve are shown in Table 2. The calibration line exhibits both a coefficient of determination close to 1 and a nearly 0 intercept, which suggests a satisfactory linearity. The calculated experimental $t$ test value ($t_{exp}$) was higher than the theoretical values ($t_{crit}$), indicating that there was a significant linear relationship ($p < 0.001$) between the concentration of P and absorbance at 650 nm in the selected calibration solution range. The standard deviations (SD) of either slope and intercept were $1.3 \times 10^{-3}$ and $9 \times 10^{-4}$, respectively. Instrumental LOD and LOQ values were 41 and


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**Table 2. Analytical Parameters of Calibration Curves**

| parameter | value |
|---|---|
| slope | 0.2841 |
| standard deviation of slope | ±0.0013 |
| intercept | 0.0016 |
| standard deviation of intercept | ±0.0009 |
| coefficient of determination, $R^2$ | 0.9991 |
| LOD[a] ($\mu$g P/L) | 41 |
| LOQ[b] ($\mu$g P/L) | 118 |
| molar absorptivity, $\varepsilon$/(L mol$^{-1}$ cm$^{-1}$) | $8.80 \times 10^3$ |

[a]LOD, instrumental limit of detection. [b]LOQ, instrumental limit of quantification.

118 $\mu$g P/mL, respectively. In our study instrumental LOQ value was a few units higher than the lowest concentration of standard solutions. These data were in agreement with the data in the literature for P determination by phosphomolybdic acid with different reducing agents methods.[20−22]

**Analytical Performances of the Proposed Procedures for TP, IP, PP, and PL.** The amounts of TP, PL, and PP, as an average of the five replicates above-mentioned (certificate and in-house references), are reported in Table 3. The estimated amounts of IP in two spiked samples, in five replicates, are reported in Table 4. In the TP analysis, REC, between-day CV, and *M* were 100.7%, 1.3%, and 5.1, respectively. For the assessment of the TP method performance, the measured value of CRM lyophilized pork muscle was compared to the certified value, confirming that no significant difference ($p < 0.05$) was present and REC was not significantly different from 100 at a significance level of $p < 0.01$. Therefore, the TP method showed a satisfactory precision and was unbiased. A previous study comparing several spectrophotometric procedures for total P determination in meat samples reported between-day CV from 0.34 to 1.48%, *M* from 0.11 to 0.66, and REC in the range of 99.6−101.5% by using Standard Meat Reference Material.[22] Indeed, the present study provides analytical performances for TP method in agreement with those reported in the paper by Jastrzębska.[22] In addition, LOD and LOQ values were 13 and 37 mg P/100 g fm, respectively.

For the assessment of the IP method performance, the measured REC values for the two spiked levels were compared by using Student's paired test, and no significant difference ($p < 0.01$) was present, thus indicating that REC was stable in the range of 100−150 mg P added to 100 g of cooked ham samples (Table 4). Lower between-day CV and *M* in the 100 mg P spiking level with respect to those measured in the 150 mg P spiking level showed that the IP method precision worsens when the inorganic phosphate content is increased. The measured values of IP spiked samples were compared to the reference values, and significant differences ($p > 0.05$) were present. Values of between-day CV and *M* ranged from 0.4 to 2.6% and from 0.2 to 1.6, respectively, and thus indicate satisfactory precision; data from REC showed an acceptable method bias. Therefore, these results are in agreement with data present in the literature. In particular, Lee et al.[23] reported within-day relative standard error (RSD) values of 2.39 and 9.49% in raw and cooked beef meats, respectively, and Jastrzębska et al.[12] a within-day RSD of 9.9% in fresh pork meat and a within-day RSD ranging form 6.01 to 11.7% in polyphosphate-spiked fresh pork meat, whereas Jastrzębska et al.[24] reported between-day CV and REC ranging from 2.22 to 4.25% and from 95 to 97%, respectively, in polyphosphate-spiked pork meat. LOD and LOQ values for IP were 11 and 33 mg P/100 g fm, respectively.

**Table 4. Results of Phosphorus Determination in Spiked Sample IP Methods (Number of Samples = 5)**

| | | level 1 | level 2 |
|---|---|---|---|
| spiked sample | av (mg P/100 g sample) | 100 | 150 |
| | uncertainty[a] (mg P/100 g sample) | 1 | 2 |
| $(w \pm \mu)^b$ (mg P/100 g sample) | | 90 ± 1 | 135 ± 4 |
| standard error of mean, *M* | | 0.2 | 1.6 |
| coefficient of variation, CV% | | 0.4 | 2.6 |
| recovery %[c] ($w \pm \sigma$) | | 90 ± 1 | 90 ± 2 |
| LOD[d] (mg P/100 g sample) | | 11 | |
| LOQ[d] (mg P/100 g sample) | | 33 | |

[a]Expanded uncertainty reference value. [b]Measured average phosphorus content with the confidence limits for $p = 95\%$. [c]Average % recoveries with standard deviations. [d]LOD, LOQ from calibration curve.

In the PP analysis, REC, between-day CV, and *M* were 97.5%, 1.2%, and 0.7, respectively. For the assessment of the PP method performance, the measured value of PP in-house reference samples was compared to the reference values, and a significant difference ($p > 0.05$) was present. Therefore, the PP method showed a satisfactory precision and an acceptable method bias. In addition, LOD and LOQ values were 2 and 20 mg P/100 g fm, respectively. To the best of the authors' knowledge, no data about the analytical performance of the PP method are present in the literature.

In the PL analysis, REC, between-day CV, and *M* were 93.1%, 1.2%, and 2.1, respectively. For the assessment of the PL

**Table 3. Results of Phosphorus Determination in Reference Materials by TP, PP, and PL Methods (Number of Samples = 5)**

| | | methods | | |
|---|---|---|---|---|
| parameter | | TP | PP | PL |
| reference | av (mg P/100 g sample) | 870 | 131 | 433 |
| sample | uncertainty[a] (mg P/100 g sample) | 50 | 2 | 4 |
| $(w \pm \mu)^b$ (mg/100 g) | | 876 ± 14 | 128 ± 2 | 404 ± 6 |
| standard error of mean, *M* | | 5.1 | 0.7 | 2.1 |
| coefficient of variation, CV% | | 1.3 | 1.2 | 1.2 |
| recovery %[c] | | 100.7 ± 1.3 | 97.5 ± 0.1 | 93.1 ± 1.1 |
| LOD[d](mg P/100 g sample) | | 13 | 2 | 6 |
| LOQ[d](mg P/100 g sample) | | 37 | 20 | 16 |

[a]Expanded uncertainty certified value for TP, reference value for PP and PL. [b]Measured average phosphorus content with the confidence limits for $p = 95\%$. [c]Average % recoveries with standard deviations. [d]LOD − LOQ from calibration curve.


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Table 5. Results of Phosphorus Determination in Cooked Ham Samples (mg/100 g Fresh Samples; Number of Samples = 24)

| | TP | IP | PP | PL | DfP |
|---|---|---|---|---|---|
| $(w \pm \sigma)^a$ (mg P/100 g sample) | 251 ± 59 | 154 ± 50 | 39 ± 14 | 37 ± 16 | 229 ± 57 |
| $w$ %[b] | 100 | 61 | 16 | 14 | 91 |
| RSD%[c] | 24 | 33 | 35 | 43 | 25 |
| range min−max[d] (mg P/100 g sample) | 174−331 | 87−238 | 22−67 | 16−60 | 155−311 |

[a]Average phosphorus content with standard deviations. [b]% average phosphorus content. [c]Relative standard deviation of average phosphorus content. [d]Values range min−max.

method performance, the measured value of PL in-house reference samples was compared to the reference values, and a significant difference ($p < 0.05$) was present. Therefore, the PL method showed a satisfactory precision and an acceptable method bias. In addition, LOD and LOQ values were 6 and 16 mg P/100 g fm, respectively. Quantification of phospholipids by using total P content in lipid extracts from biological samples is currently used, but only a few data regarding the validation of these procedures are reported in the literature.[25−29] In particular, Kirkpatrick et al.[17] reported a recovery range from 95 to 101% and a relative standard deviation of 1.7% by using organophosphate and phosphonate pure standards, and Xi et al.[30] found recoveries within 93−103% and CV from 0.6 to 6.1% by using phospholipid pure standards. The present study provides analytical performances for the PL method in agreement with those published in the literature.

In conclusion, the analytical performances of the proposed procedures for differential measurement of TP, IP, PP, and PL prove their suitability for routine analyses. Whereas our innovative measurements are derived from well-known methods and make use of instrumentations present in routine analytical laboratories, our novel combinations and refinements of these techniques enable accurate and differential quantification of the different types of P more practical and hence more attractive.

**Determinations of Phosphorus Type and Content in Different Brands of Cooked Ham Samples.** The proposed analytical methods were applied to cooked ham samples of 24 different brands. Table 5 shows the results obtained for TP, IP, PP, and PL, as well as DfP.

TP average content and value range were 251 and 174−331 mg P/100 g fm, respectively. These results are in agreement with data present in the literature. In particular, Dušek et al.[9] reported values from cooked hams with listed phosphorus-containing additives ranging in the interval 204−366 mg P/100 g fm and Hrynczyszyn et al.[31] values between 141 and 197 mg P/100 g fm from tinned pork foods, between 106 and 111 mg P/100 g fm from luncheon pork meats, and 100 mg P/100 g fm from a single smoked pork ham sample, whereas Jastrzębska[7] reported values of 222 and 286 mg P/100 g fm, respectively, from single samples of cooked ham and smoked ham with a reported content of orthophosphate additives. Moreover, the obtained values for TP content in cooked ham samples were in close agreement with both previously published results[6] and data reported in the Food Composition Table of the National Research Institute for Food and Nutrition, Italy.[32]

IP average content and value ranges were 154 and 87−238 mg P/100 g fm, respectively. These results are in agreement with data present in the literature. In particular, Dušek et al.[9] reported an average content of 212 mg P/100 g fm and values ranging in the interval of 136−292 mg P/100 g fm from cooked hams with listed phosphorus-containing additives and Hrynczyszyn et al.[31] values between 124 and 173 mg P/100 g fm from tinned pork foods, between 73 and 88 mg P/100 g fm from luncheon pork meats, and 75 mg P/100 g fm from a single smoked pork ham sample.

PP average content and value ranges were 39 and 22−67 mg P/100 g fm, respectively. To the best of our knowledge, no data about PP contents in meat products were present in the literature. Only Dušek et al.[9] reported values ranging in the interval of 22−43 mg P/100 g fm in fresh meat from different species.

DfP average content and value ranges were 229 and 155−311 mg P/100 g fm, respectively. In our series of experiments the average DfP value was slightly lower than the measured average TP (229 vs 251 mg/100 g), indicating that we succeeded in measuring 91% of the constituents of the TP accurately. This gap of approximately 9%, by TP with respect to DfP, may be due to the technical difficulties of completely extracting the inorganic P from the food matrix, as reported by Jastrzębska et al.,[12] or due to yet other unmeasured types of P present in cooked ham items. Figure 1 shows the scatter plot of

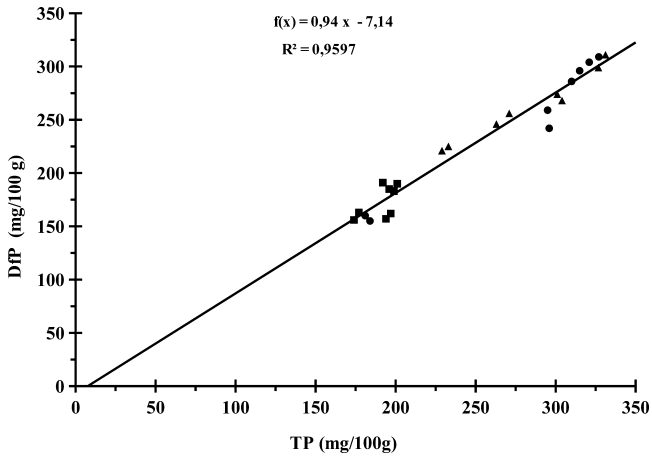


**Figure 1.** Scatter plot of the correlation between the sum of the subtypes of sample P and the measured total P: EWPIA (▲), EWP (●), and regular (■).

the correlation between the measured TP ($X$ axis) and DfP ($Y$ axis). The relationship between DfP and TP was evaluated by $t$ test for the significance of the Pearson product−moment correlation coefficient. The calculated experimental $t$ test value ($t_{exp}$) was higher than the theoretical values ($t_{crit}$), indicating that there was a significant linear relationship (Figure 1) for all intervals of TP measured (174−331 mg P/100 g) that include both EWPIA and EWP regular cooked ham items. Large RSD values in TP as well as in IP, PL, and PP content in cooked ham (ranged from 23 to 43%) demonstrate a wide variability in P contents. To explain this, we classified TP, IP, PL, and PP contents into three different groups of cooked ham items: EWPIA, EWP, and regular. Table 6 shows the results of TP as

Table 6. Results of Phosphorus Determination into Three Different Groups of Cooked Ham Brands (mg P/100 g Fresh Samples; Number of Samples = 8)

| group | TP | IP | PP | PL | DfP |
|---|---|---|---|---|---|
| EWPIA $(w \pm \sigma)^a$ | 282 ± 40 | 159 ± 18 | 55 ± 7 | 48 ± 11 | 263 ± 32 |
| EWP $(w \pm \sigma)^a$ | 279 ± 60 | 199 ± 50 | 27 ± 4 | 25 ± 14 | 251 ± 62 |
| regular $(w \pm \sigma)^a$ | 191 ± 10 | 103 ± 12 | 35 ± 8 | 36 ± 13 | 173 ± 15 |

[a]Average phosphorus content with standard deviations.

well as IP, PP, and PL contents classified into the three groups of cooked ham items. As expected, TP content was significantly higher in EWPIA and EWP than regular but no significant difference was observed for EWPIA with respect to EWP. Differences in inorganic phosphorus content were found not only between the regular group as compared to both EWPIA and EWP, but also in IP content, being higher in the EWP group than in regular products. Therefore, obtained results of TP and IP contents showed that the addition of P-containing ingredients, such as milk proteins and plant protein extracts or isolates, decreased the amount of functional additives, such as polyphosphates and phosphate salts, used in the production processes of EWPIA with respect to EWP.

PP content was higher in EWPIA than in EWP and regular samples, and no significant difference was observed for EWP with respect to regular, although the average value of PP content of EWP was lower with respect to those of regular. The key reason for higher measured PP contents in EWPIA with respect to EWP and regular was the addition of ingredients, such as milk proteins and plant protein extracts or isolates, in which there was a large increase of P from phosphoprotein with respect to total phosphorus content.

PL content was higher in EWPIA than EWP, and no significant difference was observed from EWP with respect to regular, although the average value of PL content of EWP was lower with respect to those of regular items. The dilution effect due to inclusion of water in ingredients (reported on the package label) of each EWPIA and EWP with respect to regular (in which water was not listed on the product label) may explain even lower PL and PP values measured in EWP with respect to those of regular items.

## AUTHOR INFORMATION

### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS USED

CRM, Certified Reference Material; CV, coefficient of variation; DfP, differential P; EWP, enhanced with phosphorus-containing additives; EWPIA, enhanced with phosphorus-containing ingredients and additives; IP, inorganic phosphorus; LOD, limit of detection; LOQ, limit of quantification; *M*, standard error of mean; P, phosphorus; PL, phosphorus from phospholipids; PP, phosphorus from phosphoprotein; RCF, relative centrifugal force; REC, recovery; RPM, rounds per minute; RSD, relative standard deviation; TP, total phosphorus

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