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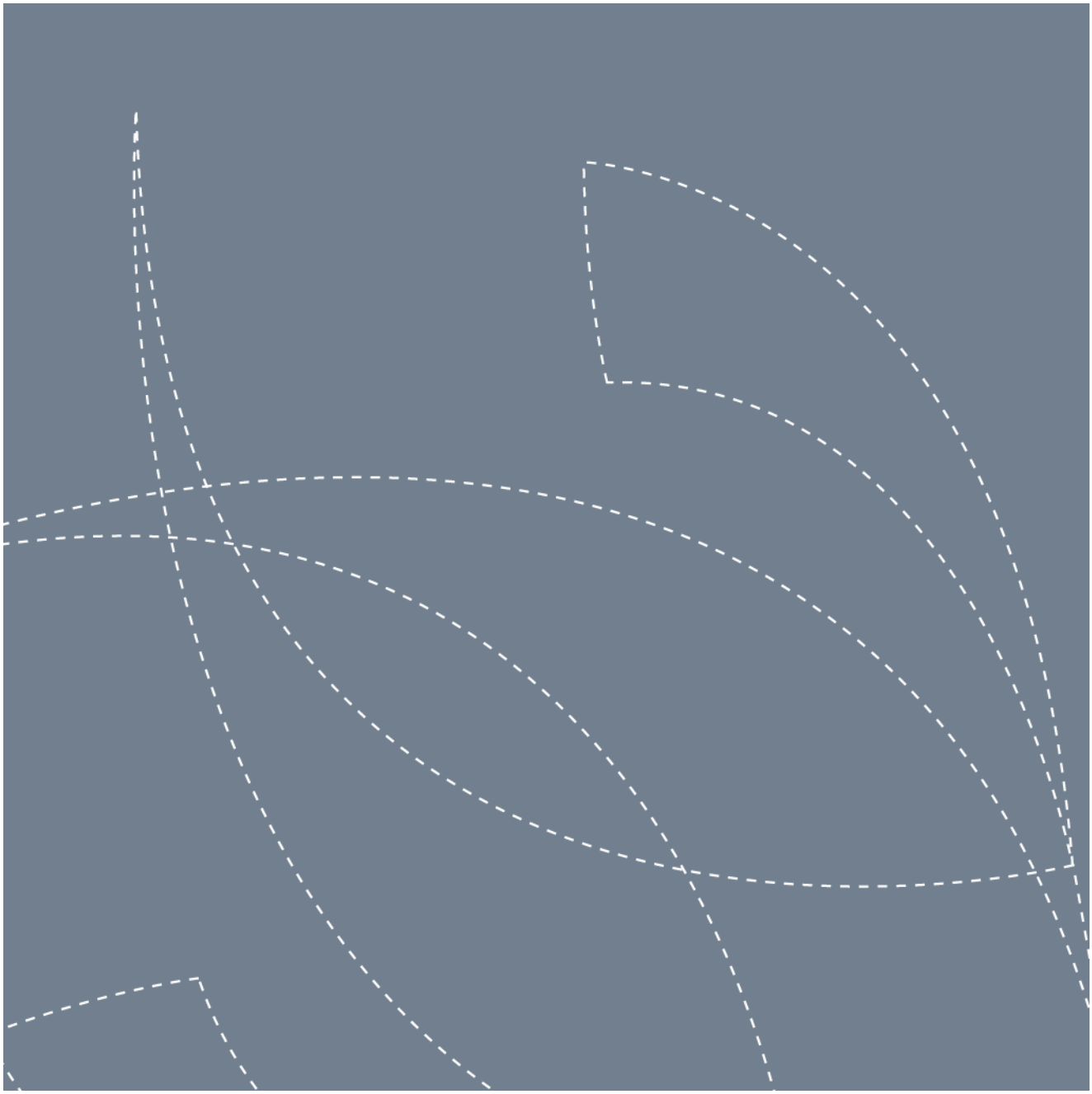
Validation of a method for analysis of soluble phosphorus by use of alkaline extraction and spectrophotometric determination

Gunnhild Hovde



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Main office in Tromsø
Muninbakken 9–13
P.O. box 6122
NO-9291 Tromsø
Norway
Tel.: +47 77 62 90 00
Fax: +47 77 62 91 00
E-mail: nofima@nofima.no

Internet: www.nofima.no

Nofima AS
P.O. box 6122
NO-9291 Tromsø, Norway
Visiting address:
Muninbakken 9–13,
Tel.: +47 77 62 90 00
Fax: +47 77 62 91 00
nofima@nofima.no
www.nofima.no

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Summary:
The aim of this project is to validate Nofima BioLab's method for analysis of soluble phosphorus. The definition of soluble phosphorus in this context is phosphorus that is not bound in bone material. Validating a method means investigating and establishing the method's quality parameters. The tested method parameters will include recovery, bias, precision, ruggedness, limit of detection, limit of quantification, and uncertainty.

The recovery test/spiking showed how important it is to have knowledge about the properties of the phosphate salts, especially the solubility. The repeatability of the method is similar to the repeatability of the well-established method for analysis of total phosphorus (ISO 6491). The combined measurement uncertainty of the method is low where the largest uncertainty contribution comes from diluting the sample.

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1 Introduction

The aim of this project is to validate Nofima BioLab's method for analysis of soluble phosphorus. The definition of soluble phosphorus in this context is phosphorus that is not bound in bone material. The method is hence called "not bone bound phosphorus" at Nofima BioLab.

Phosphorus is an essential nutrient in fish. Its metabolic role includes being a constituent in bone, scales, adenosine triphosphate (ATP), cell membranes and nucleic acids (Skonberg et al., 1997). Phosphorus not bound in bone material includes inorganic phosphate salts and phospholipids. In fish bone, phosphorus is tightly bound in a mineral complex with low solubility (Sugiura et al., 2004). Phosphorus not bound in bone material indicates what is available to fish. The exception is phosphorus in phytate form, which is not bioavailable because fish lack the digestive enzyme phytase required to remove phosphate from the phytate molecule (Storebakken et al., 1998).

Validating a method means investigating and establishing the method's quality parameters. The tested method parameters will include recovery, bias, precision, ruggedness, limit of detection, limit of quantification, and uncertainty. Validation performed by one laboratory is called internal validation (NMKL, 2009). Validation determines the suitability of an analysis for providing the desired information (Skoog et al., 2004).

2 Validation

As mentioned, validation of a method means investigating and establishing the method's quality parameters (NMKL, 2009). This chapter describes the degree of validation and the validation plan for soluble phosphorus.

2.1 Degree of validation and previous external validation

The method of soluble phosphorus can be divided into two steps: In step 1, soluble phosphorus is extracted with an alkaline solution (sodium hydroxide) before an exact aliquot is transferred into a crucible. The aliquot is evaporated until dryness. In step 2, the exact same procedure as in "total phosphorus" is followed (ISO 6491). The courses of the methods are shown in Figure 1.

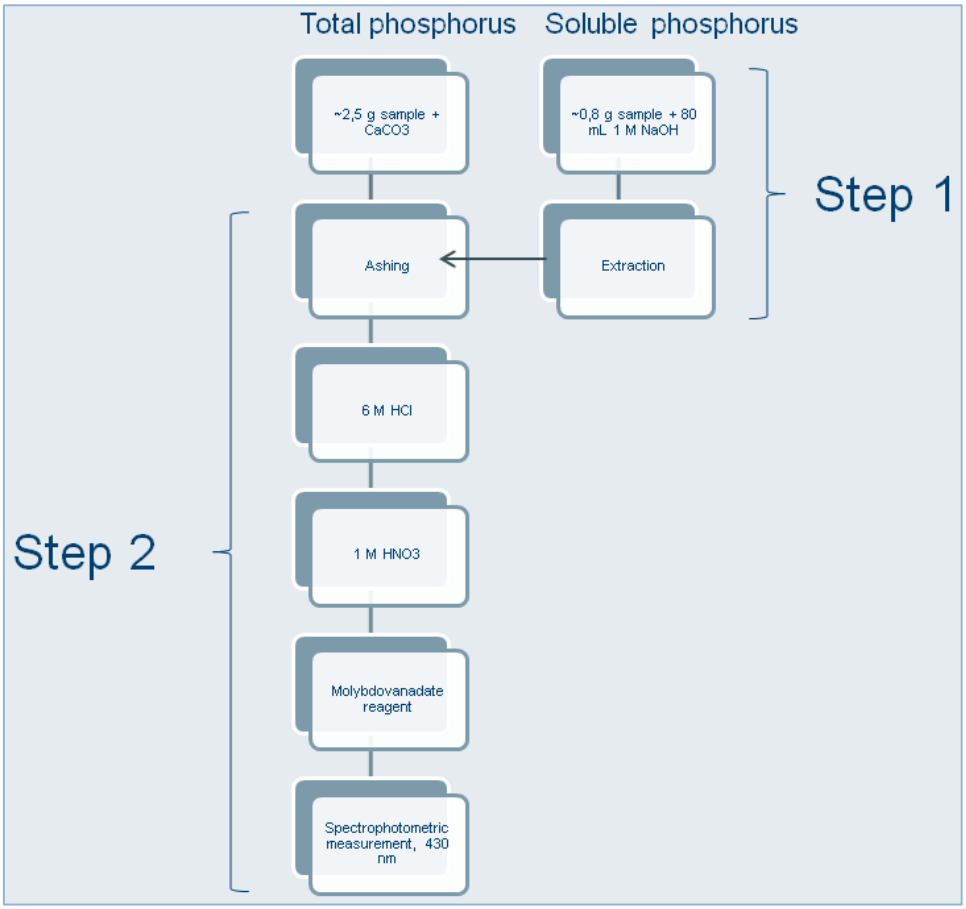

Figure 1 *The courses of the methods "total phosphorus" (ISO 6491) and "soluble phosphorus". In step 1, soluble phosphorus is extracted with an alkaline solution (sodium hydroxide) while in step 2, the exact same procedure as in "total phosphorus" is followed.*

Step 1 is not completely validated. The method has been published in scientific literature, but lacks important quality parameters. The quality parameters will be examined and determined. Step 1 demands a complete internal validation except from the method's selectivity and linearity, which is included in the standard method of total phosphorus.

Step 2 (ISO 6491) has been externally validated in a test where relevant sample materials (including fish meal) have been tested by seven different laboratories. Nofima BioLab has accredited the method “total phosphorus”. In lack of suitable certified reference material (CRM), BioLab is involved in international comparable laboratory tests several times each year to verify the trueness of the method, i.e., Association of American Feed Control Officials (AAFCO) with more than 150 participating laboratories.

2.2 Deviations from the method reference

Except from step 1, there are no deviations from the method reference ISO 6491.

2.3 Validation plan

A validation plan describes the validation factors that should be evaluated (NMKL, 2009). In this chapter the points in the validation is summarized.

2.3.1 Recovery test/spiking

Recovery (or recovery factor) is defined by IUPAC (International Union of Pure and Applied Chemistry) as, “Yield of a preconcentration or extraction stage of an analytical process for an analyte divided by amount of analyte in the original sample” (Burns et al., 2002). In an extraction step, the analyte is transferred from a complex matrix to a simpler matrix in which the instrumental detection is done. Loss of analyte can be anticipated during the extraction, and recovery gives the method’s efficiency. Recovery should, if possible, be compensated for (NMKL, 2012).

Usually the recovery is determined during a method validation by spiking, which is adding a known quantity of the analyte to the sample, extract, measure and divide by the spiked value. The spike level should be close to the concentration of the analyte in the sample (NMKL, 2012).

In this method validation the recovery test will be performed by spiking with inorganic phosphate salts and other sources of soluble phosphorus in practical diet formulations.

The recovery (*R* %) can be calculated by using equation 2.1 (NMKL, 2012).

$$R\% = \frac{Q_{extr(orig+add)} - Q_{orig}}{Q_{add}} \qquad (2.1)$$

where $Q_{extr(orig+add)}$ is the level of extracted (recovered) analyte, Q_{orig} is the original level of the analyte in the sample, and Q_{add} is the added (spiked) analyte.

The standard error of the recovery is calculated in absolute terms as the standard error of the mean (*SEM*) as shown in equation 2.2, and in relative terms as the standard uncertainty for the recovery (u_{rec}) as shown in equation 2.3 (NMKL, 2012).

$$SEM = \frac{SD}{\sqrt{n}} \qquad (2.2)$$

$$u_{rec} = \frac{\%RSD}{\sqrt{n}} \qquad (2.3)$$

where *SD* and %*RSD* are the standard deviation and the relative standard deviation of the recovery, and *n* is the number of replicates (NMKL, 2012).

It is important to not confuse recovery with bias (*b*). Incomplete recovery will lead to bias, (Linsinger, 2008) but bias is a systematic analytical error that may or may not be significant. Bias should be identified and, if possible, eliminated, but bias should usually not be corrected for (NMKL, 2012). A CRM is usually required for the determination of bias, but if no CRMs are available the recovery can be used to calculate the bias (NMKL, 2012). In both cases, bias can be calculated by equation 2.4 and relative bias (*b* %) by equation 2.5 (Linsinger, 2008; NMKL, 2012).

$$b = \frac{x_{meas}}{x_{ref}} \tag{2.4}$$

$$b\% = \left(\frac{x_{meas} - x_{ref}}{x_{ref}}\right) \times 100\% \tag{2.5}$$

where x_{meas} is the measured result while x_{ref} is the reference value, which can be a CRM, an accurately prepared sample (e.g., by spiking), well-designed intercomparisons or measurements with another method of demonstrated accuracy (Linsinger, 2008).

To see if the recovery and the bias are statistically significant, a *t*-test is performed according to equation 2.6 (NMKL, 2012).

$$t = \frac{|Q_{extr(orig+add)} - T|}{SD} \times \sqrt{n} \tag{2.6}$$

where *T* represents the calculated level of soluble phosphorus in the spiked sample, and *SD* is the standard deviation of the extracted analyte. If the bias is statistically significant, *t* is higher than t_{crit}. The value for t_{crit} (two-tailed, 95 % confidence, degrees of freedom = *n*–1) is found in a table of critical *t*-values (NMKL, 2012).

The big advantage of using recovery experiments is that the matrix is representative for real samples. The biggest limitation is that the analyte in the real sample can be strongly bound physically or chemically to the matrix, which normally will not be the case for the added analyte. This could mean that one can achieve a high recovery factor for the added analyte, without reaching a complete determination of the naturally occurring analyte (NMKL, 2012). Also, the form of the spike may present a problem as different compounds and grain sizes representing the analyte may behave differently in an analysis (Reeuwijk and Houba, 1998).

One may have four different scenarios (NMKL, 2012):

1. The native (original) analyte remains (i.e., is recovered) and the spike is partially lost, and one will achieve false bad recovery.
2. The native analyte is partially lost and the spike remains, and one will achieve false good recovery.
3. The native analyte and the spike remain, and one will achieve a true good recovery.
4. The native analyte is partially lost and the spike is proportionally lost, and one will achieve a true good recovery.

2.3.2 Precision and ruggedness

Precision and ruggedness will be measured by:

- Variation due to the differences in sample size (and the homogeneity).
- Differences between different analysts.

Precision describes the agreement among several results obtained in the same way. It is important not to confuse precision with the term accuracy, which indicates the closeness of the measurement to the true or accepted value expressed by the error (bias) (Skoog et al., 2004).

2.3.3 Limit of detection and limit of quantification

The method's limit of detection (*LOD*) and limit of quantification (*LOQ*) will be determined. Both *LOD* and *LOQ* are determined by analyzing a set of blind samples. The limit of detection is the lowest analyte concentration that can be detected with a certain degree of confidence and is commonly calculated by equation 2.7 (Armbruster et al., 1994; NMKL, 2009).

$$LOD = c \times SD_{blind} \quad (2.7)$$

where SD_{blind} is the standard deviation for the blind samples' mean value, and c is a constant which is found in a table of critical t-values (degrees of freedom = n–1 and usually $\alpha = 0.01$). For $\alpha = 0.01$ and $n = 20$, $c = 3$ is an often used approach (NKML, 2009).

The limit of quantification is the lowest analyte concentration that can be quantified with a given measurement uncertainty within a certain degree of confidence and is commonly calculated by equation 2.8 (Armbruster et al., 1994; NMKL, 2009).

$$LOQ = 10 \times SD_{blind} \quad (2.8)$$

Rigid rules for the limit of quantification cannot be given but should be evaluated in each case. $LOQ = 6 \times SD_{blind}$ is often used (NMKL, 2009). Analytical results below the limit of quantification are reported as "lower than" (using the sign <).

2.3.4 Uncertainty

Possible sources of uncertainty in the measurement procedure will be summarized, and there will be created an Ishikawa/fishbone diagram.

The method's combined measurement uncertainty will be calculated by following the procedures described in Eurachem (1995).

3 Methodology

The following methodology describes the laboratory experiments performed in connection to the validation.

3.1 Recovery test/spiking

The spiked sample and oil (50:50 rapeseed oil:fish oil) were weighed in 100 mL glass bottles in a ratio of about 83 % sample and 17 % oil. This is according to the added amount of oil in traditional fish feed (200 grams oil per 1000 grams of basal diet). The following oils were used:

Rapeseed oil: O4/11, crude degummed hot pressed from Emmelev, Denmark
Fish oil: O5/11, NorsalmOil from Norsildmel, Norway.

The total weight of the sample was about 0.8 g. The soluble phosphorus level of the mixed oil was analyzed prior to the recovery test but was found to be negligible. The analysis was then performed as described in the method description. To verify the results of the analysis, quality control measures were taken. This included analyzing four blind samples and two control samples together with each series of spiked samples. The control samples have to lie between the upper control limit (*UCL*) and lower control limit (*LCL*), defined by equation 3.1 and 3.2, respectively (Skoog et al., 2004).

$$UCL = \bar{X} + \frac{3 \times SD}{\sqrt{n}} \quad (3.1)$$

$$LCL = \bar{X} - \frac{3 \times SD}{\sqrt{n}} \quad (3.2)$$

where $\bar{X}$ is the mean for the control sample, *SD* is the standard deviation for the measurement, and *n* is the number of replicates that are obtained for each sample (Skoog et al., 2004). The mean and the standard deviation have been estimated from a set of previously analyzed control samples. The blind samples are samples going through the entire analytical process but contain only sodium hydroxide (NaOH) and no analyte.

Five spiked samples were prepared by individually adding four inorganic phosphate salts and soy lecithin to a basal diet produced at Nofima's feed technology center at Titlestad. The basal diet's approximate composition is listed in Table 1. The basal diet was analyzed for soluble and total phosphorus prior to spiking.

Table 1 The approximate composition of the basal diet used for the preparation of the spiked samples.

Basal diet	% composition	% total P in ingredient	% soluble P in ingredient
Fish meal (FM) 43/12	20	1.5-2.0[a]	0.7-1.0[a]
Soy protein concentrate (SPC) 158/10	55	0.69[b]	0.57[b]
Wheat gluten 159/10	13	0.18[b]	0.18[b]
Wheat starch 169/11	1	0.2-0.4[c]	
Wholemeal wheat 195/11	7	0.28[b]	0.25[b]
Vitamin mix T4545/10	3	0.9[c]	
Mineral mix T70/10	1	0.1[c]	

[a] *Typical values*
[b] *Analyzed values from Nofima BioLab*
[c] *From commodity tables*

The spiking materials used are listed in Table 2. "CaHPO4, new" is further described in chapter 4.2.

Table 2 Spiking materials used in the recovery test. "$CaHPO_4$, new" is further described in chapter 4.2.

Spiking material	Name	Manufacturer	Purity
NaH_2PO_4	Sodium dihydrogen phosphate	Sigma-Aldrich, LOT #BCBG0399V (purchased 2012)	>99 %
$CaHPO_4$	Calcium hydrogen phosphate	Fluka Chemika, LOT #36424911 (purchased 2003)	~99 %
$Ca(H_2PO_4)_2$	Calcium dihydrogen phosphate	Sigma-Aldrich, LOT #100M0265V (purchased 2012)	>95 %
$NH_4H_2PO_4$	Ammonium dihydrogen phosphate	Sigma-Aldrich, LOT #22702J0 (purchased 2003)	>98 %
Soy lecithin			
$CaHPO_4$, new	Calcium hydrogen phosphate	Sigma-Aldrich, LOT #BCBG6055V (purchased 2013)	>98,5 %

The spiked samples were prepared according to Table 3. The aim was an added phosphorus level of about 0.6 % and a total phosphorus level of about 1.1 % in each spike. The spikes were prepared by the assumption that all phosphorus in the spiking material is soluble in NaOH. "Spike 2, new" and "Spike 3, new" and the reason behind the preparation of these two spikes are further described in chapter 4.2.

Table 3 *The composition of the spiked samples. "Spike 2, new" and "Spike 3, new" and the reason behind the preparation of these two spikes are further described in chapter 4.2.*

Name	Spiking material	Amount of basal diet (g)	Amount of spike (g)	Soluble P in basal diet (%)	Calculated P in spike (%)	Purity of compound (%)	Calculated amount of theoretically soluble P (%)[b]
Spike 1	NaH_2PO_4	97.674	2.3240	0.56	25.81	>99	1.141
Spike 2	$CaHPO_4$	97.363	2.6360	0.56	22.76	~99	1.139
Spike 3	$Ca(H_2PO_4)_2$	97.735	2.2692	0.56	26.46	>95	1.118
Spike 4	$NH_4H_2PO_4$	97.774	2.2289	0.56	26.92	>98	1.136
Spike 5	Soy lecithin	80.457	19.5061	0.56	2.91[a]		1.018
Spike 2, new	$CaHPO_4$	97.403	2.6365	0.56	22.76	>98.5	1.137
Spike 3, new	$Ca(H_2PO_4)_2$	97.740	2.2668	0.56	26.46	>95	1.117

[a] *Found by analysis, not calculated*

[b] *Based on the assumption that all phosphorus in the spiking materials is soluble in NaOH*

The soluble phosphorus level in the basal diet was determined by following the method description. The phosphorus level in the inorganic phosphate salts were calculated based on information about the chemical composition and the purity of the compound while the soluble phosphorus level in the soy lecithin was found by analysis. The basal diet and the spike were weighed to match the desired phosphorus level of 1.1 %, but some deviation occurred due to sticky spiking material. The exact amounts of added spikes were recorded.

The sample was mixed well and to test the homogeneity of the sample, one sample of basal diet (98.5 g) and NaH_2PO_4 (1.5 g) was prepared and analyzed with 10 parallels. The sample was analyzed by the method "total phosphorus" since the aim was to the test the homogeneity and not the soluble phosphorus level.

3.2 Precision

3.2.1 Variation due to the differences in sample size

In the original method description, 40 mL of NaOH are added to about 0.4 g of sample. From April 2012 the amount of sample increased to about 0.8 g. The added volume of NaOH increased accordingly to 80 mL. The aim was to ensure less measurement uncertainty because of inhomogeneous samples. This can be measured by calculating the repeatability of the results, which indicates the precision of the method. The precision was also compared with the precision of the method "total phosphorus".

3.2.2 Differences between analysts

To see if there are differences between the analysts who perform the soluble phosphorus analysis at Nofima BioLab, five samples were analyzed by the three analysts.

4 Results and discussion

The results and discussion presented in this chapter includes both the results of the experiments performed in the laboratory and the results of the calculations.

4.1 Preliminary experiments

4.1.1 Analysis of the basal diet and the rapeseed /fish oil

The results of the analysis of the basal diet and the rapeseed/fish oil are shown in Table 4 and in Table 15, appendix 1.

Table 4 The results of the analysis of the basal diet and the rapeseed/fish oil.

	Basal diet	**Rapeseed/fish oil**	**Basal diet + rapeseed/fish oil**
Total phosphorus	0.83	Negligible	Not analyzed
Soluble phosphorus (%)	0.56	Negligible	0.56

4.1.2 Homogeneity test

The homogeneity test of a spiked sample is shown in Table 5 and in Table 16, appendix 1. The method “total phosphorus” was used for this test.

Table 5 The result of the homogeneity test of a spiked sample containing basal diet (98.5 g) and NaH_2PO_4 (1.5 g). The method “total phosphorus” was used for this test.

Spiked sample w/NaH_2PO_4	**% total phosphorus**
Result 1	1.136
Result 2	1.158
Result 3	1.180
Result 4	1.191
Result 5	1.206
Result 6	1.182
Result 7	1.223
Result 8	1.284
Result 9	1.268
Result 10	1.259
Mean value (%)	**1.21**
SD	**0.05**
%*RSD*	**4.1**

The result of the homogeneity test shows that the mixing of the spiked sample was successful; the *SD* and the %*RSD* are within acceptable limits. The difference between the highest value (1.284 %) and the lowest value (1.136 %) is 0.15 %, which is within the difference considered acceptable for the method.

4.2 Recovery test/spiking

The recovery data for the spiked samples are shown in Table 6 and in Table 17, appendix 2. The recovery factor (*R* %), the standard error of the mean (*SEM*) and the standard uncertainty for the recovery (u_{rec}) were calculated using equation 2.1, 2.2 and 2.3, respectively.

Table 6 *The recovery of phosphorus. The recovery factor (R%), the standard error of the mean (SEM) and the standard uncertainty for the recovery (u_{rec}) were calculated using equation 2.1, 2.2 and 2.3, respectively.*

Recovery of phosphorus	**Spike 1 NaH_2PO_4**	**Spike 2 $CaHPO_4$**	**Spike 3 $Ca(H_2PO_4)_2$**	**Spike 4 $NH_4H_2PO_4$**	**Spike 5 Soy lecithin**
Calculated levels in spikes (%)	**1.141**	**1.139**	**1.118**	**1.136**	**1.019**
Date of analysis	2012-12-14	2013-12-14	2012-12-20	2012-12-14+20	2012-12-20
Found mean value, $Q_{extr(orig+add)}$ (%)	1.086	0.685	0.927	1.118	1.043
Recovery, *R*%	90.8	23.5	66.6	97.0	104.4
Number of replicates, *n*	10	10	10	10	10
Standard deviation, *SD*	0.03	0.02	0.03	0.07	0.03
Relative *SD*, %*RSD*	2.9	2.6	2.9	6.4	2.7
Standard error of the mean, *SEM*	0.01	0.01	0.01	0.02	0.01
Standard uncertainty for the recovery, u_{rec}	0.9	0.8	0.9	2.0	0.9

The recovery of $NH_4H_2PO_4$ (97.0 %) was in agreement with expected recoveries (97-103 %) for this concentration range (~1 %). The recovery for NaH_2PO_4 was slightly below (90.8 %) and soy lecithin was slightly above (104.3 %) what is considered normal while for the two calcium phosphate salts the recovery is considerably lower (23.5 and 66.6 %) (WHO and FAO, 2010).

The $CaHPO_4$-salt was old (the box was opened in 2003), and although it is not described as hygroscopic it was decided to order a new box of $CaHPO_4$, which is described as “Spike 2, new” in Table 3. $Ca(H_2PO_4)_2$ was a bit lumpy, even though the container had not been opened before. The salt is described as hygroscopic in the material safety data sheet and should probably have been dried upon use. If the salt contained water, the recovery factor is false low because water molecules incorporated in the salt increase the weight of the salt. It was determined to dry the $Ca(H_2PO_4)_2$-salt in a heating cabinet at 102 °C for one hour and prepare a new spiked sample, called “Spike 3, new”. The drying reduced the weight of the $Ca(H_2PO_4)_2$-salt. The spiking experiment was then performed again for the two spikes. The new spiked samples were prepared according to Table 3. The results of the analysis are shown in Table 7 and in Table 17, appendix 2.

Table 7 *The recovery of phosphorus for the repeated analysis of spike 2 and 3. The recovery factor (R %), the standard error of the mean (SEM) and the standard uncertainty for the recovery (u_{rec}) were calculated using equation 2.1, 2.2 and 2.3, respectively.*

Recovery of phosphorus	**Spike 2, new $CaHPO_4$**	**Spike 3, new $Ca(H_2PO_4)_2$**
Calculated levels in spikes (%)	**1.137**	**1.117**
Date of analysis	2013-01-17	2013-01-17
Found mean value, $Q_{extr(orig+add)}$ (%)	0.727	0.909
Recovery, *R*%	30.7	63.5
Number of replicates, *n*	10	10
Standard deviation, *SD*	0.03	0.03
Relative *SD*, %*RSD*	3.6	2.8
Standard error of the mean, *SEM*	0.01	0.01
Standard uncertainty for the recovery, u_{rec}	1.2	0.9

The results show there were no significant difference in the recoveries for the new $CaHPO_4$-salt and for the dried $Ca(H_2PO_4)_2$-salt, and that the age of $CaHPO_4$ and the water content in $Ca(H_2PO_4)_2$ made little difference. It is, however, important to note that the added amount of salt in the spikes is low. The water content would probably have made a bigger difference if the added amount of salt was higher.

The results suggest that the calcium phosphate salts are not as soluble in NaOH as NaH_2PO_4, $NH_4H_2PO_4$ and soy lecithin. The solubility of the phosphate salts determines the bioavailability of these salts to fish. In water, most phosphates have a low solubility. The only common exceptions to this rule are the alkali metals and ammonium phosphates (Rayner-Canham and Overton, 2006). Articles published on phosphorus uptake in fish have shown that the more soluble the phosphate salt is in water, the more available it is to the fish (Nordrum et al., 1997) and that $Ca(H_2PO_4)_2$ is more digestible than $CaHPO_4$ because of its higher solubility (Hua and Bureau, 2006).

Based on the results and literature above, it was determined to check the solubility of NaH_2PO_4, $CaHPO_4$ and $Ca(H_2PO_4)_2$ in 1 M NaOH. The ammonium phosphate salt was omitted because of the good recovery. Approximately 0.19 g of NaH_2PO_4, 0.16 g of $CaHPO_4$ and 0.18 g of $Ca(H_2PO_4)_2$ was weighed in 100 mL glass bottles, added 80 mL of NaOH and analyzed as normal along with blind samples and control samples. $Ca(H_2PO_4)_2$ was dried before use. The solubility of the salts is shown in Table 8. The data from the experiments are shown in Table 18, appendix 2.

Table 8 *The solubility of NaH_2PO_4, $CaHPO_4$ and $Ca(H_2PO_4)_2$ in 1 M NaOH.*

Compound	**Calculated P in salt (%)**	**Soluble P found by analysis (%)**	**Yield/ solubility (%)**
NaH_2PO_4	25.56	23.87	93
$CaHPO_4$	22.42	7.43	33
$Ca(H_2PO_4)_2$	25.14	15.43	61

NaH_2PO_4, $CaHPO_4$ and $Ca(H_2PO_4)_2$ have solubilities of 93, 33 and 61 % in 1 M NaOH, respectively. Based on this knowledge, the results of the recovery test can be used to calculate "corrected recovery factors" where the Q_{add}-value in equation 2.1 is defined as the added amount of soluble phosphorus. It is important to note that the expression "recovery factor" cannot be used when the results are corrected for solubility since recovery is defined as the yield of the extraction. For the record the corrected value is called "corrected recovery" (*corr. R*%) in this context because this describes the value best. The results of this calculation are shown in Table 9. The recovery factors of $NH_4H_2PO_4$ and soy lecithin are not corrected.

Table 9 *The "corrected recovery" of soluble phosphorus. The recovery is corrected for the solubilities of the sodium and calcium phosphate salts. The results from the repeated analysis of spike 2 and 3 are used in the calculation. The "corrected recovery factor" (corr. R%), the standard error of the mean (SEM) and the standard uncertainty for the recovery (u_{rec}) were calculated using equation 2.1, 2.2 and 2.3, respectively. The recovery factors of $NH_4H_2PO_4$ and soy lecithin are not corrected.*

"Corrected recovery" of soluble phosphorus	**Spike 1 NaH_2PO_4**	**Spike 2, new $CaHPO_4$**	**Spike 3, new $Ca(H_2PO_4)_2$**	**Spike 4 $NH_4H_2PO_4$**	**Spike 5 Soy lecithin**
Calculated levels in spikes (%)	**1.102**[a]	**0.741**[a]	**0.897**[a]	**1.136**	**1.019**
Solubility in 1 M NaOH (%)	93	33	61	Not investigated	-
Date of analysis	2012-12-14	2013-01-17	2013-01-17	2012-12-14+20	2012-12-20
Found mean value, $Q_{extr(orig+add)}$ (%)	1.086	0.748	0.935	1.118	1.043
"Corrected recovery", *corr. R*%	97.2[a]	92.7[a]	103.4[a]	97.0	104.4
Number of replicates, *n*	10	10	10	10	10
Standard deviation, *SD*	0.03	0.03	0.03	0.07	0.03
Relative *SD*, %*RSD*	2.9	3.6	2.8	6.4	2.7
Standard error of the mean, *SEM*	0.01	0.01	0.01	0.02	0.01
Standard uncertainty for the recovery, u_{rec}	0.9	1.2	0.9	2.0	0.9

[a] *Corrected for the solubilities of the sodium and calcium phosphate salts*

As one can see there is a big difference between the recovery factors in Table 6 and Table 7 and the "corrected recovery factors" in Table 9. All the spiked samples have "corrected recovery factors" within or close to expected recoveries (97-103 %) (WHO and FAO, 2010).

The experiments show that the low recoveries of NaH_2PO_4, $CaHPO_4$ and $Ca(H_2PO_4)_2$ mainly is due to the low solubility of the salts, and that the method is fit for the intended purpose of investigating what is bioavailable to the fish, i.e., what is soluble.

4.2.1 Bias and t-test

The relative bias (*b*%) for the recovery (Table 6 and Table 7) and the *t*-values from a *t*-test was calculated by using equation 2.5 and 2.6, respectively. The calculated values are shown in Table 10.

Table 10 The bias and the t-values from the recovery test

Sample	**Spike 1 NaH_2PO_4**	**Spike 2 $CaHPO_4$**	**Spike 3 $Ca(H_2PO_4)_2$**	**Spike 4 $NH_4H_2PO_4$**	**Spike 5 Soy lecithin**	**Spike 2, new $CaHPO_4$**	**Spike 3, new $Ca(H_2PO_4)_2$**
Relative bias, *b*%	**-4.8**	**-39.9**	**-17.1**	**-1.6**	**2.4**	**-36.0**	**-18.6**
Degrees of freedom (*n*–1)	9	9	9	9	9	9	9
Table value, t_{crit}	2.26	2.26	2.26	2.26	2.26	2.26	2.26
Test value, *t*	5.42	79.80	22.35	0.76	2.71	47.96	25.32

The bias is not significant for $NH_4H_2PO_4$, but for the rest of the spiked samples the bias is significant. According to NMKL (2012) the results should be corrected for recovery for NaH_2PO_4, $CaHPO_4$ and $Ca(H_2PO_4)_2$ and soy lecithin. However, correction for recovery demands knowledge about the exact composition of the sample because the recovery factors differ. Also, in the context of finding the soluble phosphorus content in the samples, the recovery is not relevant since the soluble phosphorus is recovered as shown in Table 9. Corrected results have somewhat higher measurement uncertainty than not corrected results because correction factors have lower precision than uncorrected results (NMKL, 2012).

If the bias is calculated for the "corrected recovery" for the sodium and calcium phosphate salts in Table 9, the *t*-values are clearly lower. The results of the calculation are shown in Table 11.

Table 11 The bias and the t-values when the solubilities of the sodium and calcium phosphate salts is accounted for.

Sample	**Spike 1 NaH_2PO_4**	**Spike 2 $CaHPO_4$**	**Spike 3 $Ca(H_2PO_4)_2$**	**Spike 2, new $CaHPO_4$**	**Spike 3, new $Ca(H_2PO_4)_2$**
Relative bias, *b*%	**-1.43**	**-7.6**	**34.2**	**-1.9**	**7.4**
Degrees of freedom (*n*–1)	9	9	9	9	9
Table value, t_{crit}	2.26	2.26	2.26	2.26	2.26
Test value, *t*	1.553	9.854	3.461	1.681	1.446

The bias is not significant for "Spike 1", "Spike 2, new" and "Spike 3, new" when the results are corrected for solubility.

4.3 Precision and ruggedness

4.3.1 Variation due to the differences in sample size

The results of variation due to the differences in sample size (0.4 g and 0.8 g) compared to the method “total phosphorus”, are presented in Table 12 and in Table 19-Table 21, appendix 3. SD_r and %*RSD* are the standard deviation and the relative standard deviation for the repeatability. The repeatability limit (*r*) is an expression for the absolute difference within 95 % confidence interval between two independent single test results, obtained by using the same method on identical test material in the same laboratory by the same operator using the same equipment.

Table 12 The comparisons between the repeatability for soluble phosphorus with approximately 0.4 g and 0.8 g weighed sample and the method “total phosphorus”. SD_r and %RSD are the standard deviation and the relative standard deviation for the repeatability. The repeatability limit (r) is an expression for the precision within 95 % confidence interval.

	Soluble phosphorus		
Parameter	**Approx. 0.4 g**	**Approx. 0.8 g**	**Total phosphorus**
Number of replicates, *n*	23	52	66
Mean value	0.78	0.61	1.48
SD_r	0.042	0.041	0.038
%*RSD*	5.3	6.8	2.6
$r = 2.8 \times SD_r$	0.118	0.117	0.107

The difference between the standard deviation and the repeatability limit for 0.4 and 0.8 g of weighed sample is only 0.001. This means, the variation in weighed sample has no effect on the precision of the method, but in the future the amount of sample will continue to be approximately 0.8 g because inhomogeneous samples can occur. The repeatability limit of soluble phosphorus is close to the repeatability limit of total phosphorus.

4.3.2 Differences between analysts

The results of differences between analysts at Nofima BioLab are given in Table 13. The absolute difference between the highest and lowest value, the mean value and the standard deviation/relative standard deviation of all the results are given in the right columns.

Table 13 Results from analysis of five samples performed by three different analysts at Nofima BioLab.

Sample (journal number)	**Result ØH (% soluble phosphorus)**		**Result GH (% soluble phosphorus)**		**Result JSJ (% soluble phosphorus)**		**Abs. difference high/low value (%)**	**Mean value, all results (%)**	***SD*, all results**	**%*RSD*, all results**
2012-2034-01	Result 1: 0.567	Result 2: 0.557	Result 1: 0.567	Result 2: 0.509	Result 1: 0.493	Result 2: 0.512	0.06	0.53	0.03	6.2
	Mean value: **0.56**		Mean value: **0.54**		Mean value: **0.50**					
2012-2099-01	Result 1: 0.464	Result 2: 0.464	Result 1: 0.459	Result 2: 0.459	Result 1: 0.518	Result 2: 0.475	0.04	0.47	0.02	4.8
	Mean value: **0.46**		Mean value: **0.46**		Mean value: **0.50**					
2012-2286-01	Result 1: 0.836	Result 2: 0.771	Result 1: 0.796	Result 2: 0.827	Result 1: 0.851	Result 2: 0.864	0.06	0.82	0.04	4.2
	Mean value: **0.80**		Mean value: **0.81**		Mean value: **0.86**					
2012-2855-01	Result 1: 0.375	Result 2: 0.351	Result 1: 0.371	Result 2: 0.366	Result 1: 0.342	Result 2: 0.377	0.01	0.36	0.01	3.9
	Mean value: **0.36**		Mean value: **0.37**		Mean value: **0.36**					
2012-3948-01	Result 1: 0.753	Result 2: 0.731	Result 1: 0.710	Result 2: 0.732	Result 1: 0.696	Result 2: 0.762	0.02	0.73	0.03	3.4
	Mean value: **0.74**		Mean value: **0.72**		Mean value: **0.73**					

In general the differences in the results between the three analysts are small. The absolute difference between the highest and lowest value are between 0.01 and 0.06 % with %*RSD* between 3.4 and 6.2. This is within what can be accepted for the analysis.

4.4 Limit of detection and limit of quantification

The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were calculated by using equation 2.7 and 2.8, and based on data for 50 blind samples analyzed from 2011-09-29 to 2012-12-21. The results are shown in Table 14 and in Table 22, appendix 4.

Table 14 *The mean value of n=50 blind samples, the standard deviation (SD), the limit of detection (LOD) and the limit of quantification (LOQ). The value c=2.403 were found in a table of critical t-values.*

Parameter	mg/L	% soluble phosphorus[a]
Mean value, blind sample, *n*=50	0.3733	
SD_{blind}	0.1261	
$LOD = 2.403 \times SD_{blind}$	0.3030	-
$LOQ = 6 \times SD_{blind}$	0.7566	0.09

[a] *Assumed 0.8 g sample and blind value = 0.3733 mg/L*

The limit of detection in mg/L is lower than the average blind value. One can therefore assume that one can detect phosphorus levels down to near zero. Based on knowledge about the linearity and the previous experience with the method, one can calculate the limit of quantification using $LOQ = 6 * SD_{blind}$. This gives a *LOQ* = 0.09 % phosphorus. Values below the *LOQ* will be reported as "lower than" (<) 0.09.

4.5 Uncertainty

4.5.1 Possible sources of uncertainty

The evaluation of the possible sources of uncertainty in the measurement procedure is shown in the Ishikawa/fishbone diagram in Figure 2.

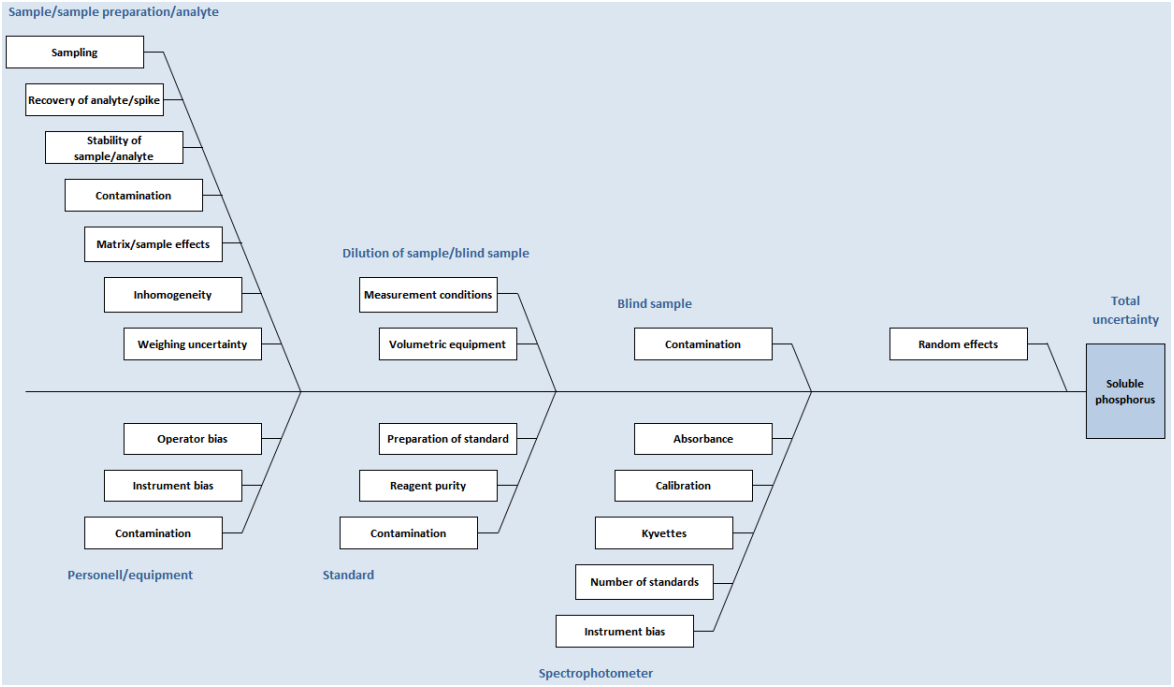

Figure 2 *Ishikawa/fishbone diagram showing the possible sources of uncertainty in the measurement procedure.*

4.5.2 Calculation of the combined measurement uncertainty

The combined measurement uncertainty of the method was calculated. The calculation was based on assumed values for the amount of weighed sample and for the soluble phosphorus level in the sample and in the blind sample.

Equation 4.1 shows the equation being used for the calculation of soluble phosphorus in the method description.

$$\%\ \text{soluble phosphorus} = \frac{A-B}{\frac{C}{V1\ (80\ \text{mL})}\times\frac{V2\ (20\ \text{mL})}{V3\ (250\ \text{mL})}\times\frac{V4\ (10\ \text{mL})}{V5\ (20\ \text{mL})}\times 10^6} \times 100\ \% \qquad (4.1)$$

where *A* is the soluble phosphorus content in mg/L, *B* is the value for the blind sample in mg/L, *C* is the weighed sample in g and 10^6 is a conversion factor from g/mL to mg/L. *V*1-*V*5 are added volumes when diluting the sample.

With an assumed value of 4.5000 mg/L for the sample (*A*) (typical value for the control sample), 0.3700 mg/L for the blind sample (*B*) (typical value for the blind sample) and an amount of weighed sample of 0.8000 g (*C*), the expression can be written as shown in equation 4.2 when the uncertainties are included.

$$\%\ \text{soluble phosphorus} = \frac{(4.5000\pm0.0022-0.3700\pm0.0015)\frac{\text{mg}}{\text{L}}}{\frac{(0.8000\pm0.0001)\ \text{g}}{(80.00\pm0.03)\ \text{mL}}\times\frac{(20.00\pm0.02)\ \text{mL}}{(250.00\pm0.09)\ \text{mL}}\times\frac{(10.00\pm0.01)\ \text{mL}}{(20.00\pm0.02)\ \text{mL}}\times 10^6} \times 100\ \% \qquad (4.2)$$

The expression account for the uncertainty in the analytical balance, the volumetric equipment and the spectrophotometer. The estimated uncertainty of the analytical balance is found in the calibration certificate, and the estimated uncertainties of the volumetric equipment are found written on the glassware or by experiments in the laboratory. The uncertainties were transformed into standard deviations using calculation rules in Eurachem (1995). The uncertainty of the spectrophotometer is theoretically calculated by using equation 4.3 (Galbán et al., 2007; Skoog et al., 2004).

$$\frac{SD_{Abs}}{Abs} = \frac{0.434}{Abs} \times \frac{SD_T}{10^{-Abs}} \qquad (4.3)$$

This expression gives the relative uncertainty in absorbance where SD_{Abs} is the standard deviation of the absorbance, *Abs* is the absorbance and SD_T is the standard deviation of the transmittance, which has a typical value of ±0.003 (Skoog et al., 2004).

The uncertainty in preparing the standard solution was calculated, but was found to be negligible (~1×10^{-7}).

The overall uncertainty from expression 4.2 was calculated to:

$$\%\ \text{soluble phosphorus} = (1.0325 \pm 0.0019)\ \% \qquad (4.4)$$

The expanded uncertainty (*U*) with a coverage factor (*k*) of 2 was calculated to ±0.0039 (%*RSD* = 0.39 %). The calculation was performed by using the spreadsheet method given in Eurachem (1995). The calculation is shown in Table 23, appendix 5.

The relative contributions to the combined standard uncertainty variance (%) are shown in Figure 3.

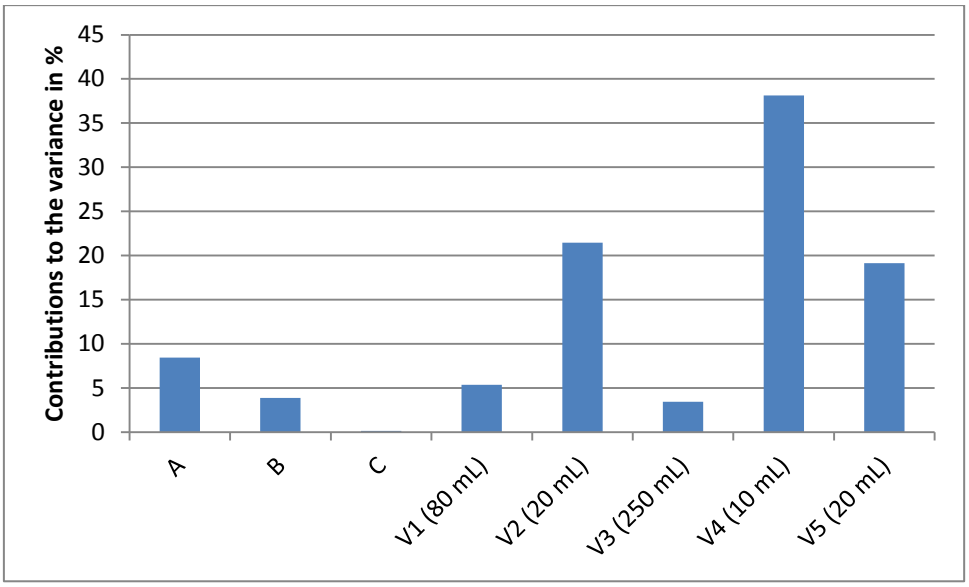

Figure 3 The relative contributions to the combined standard uncertainty variance (%).

The uncertainty contributions as standard uncertainties are shown in Figure 4. The combined measurement uncertainty is included (±0.0019).

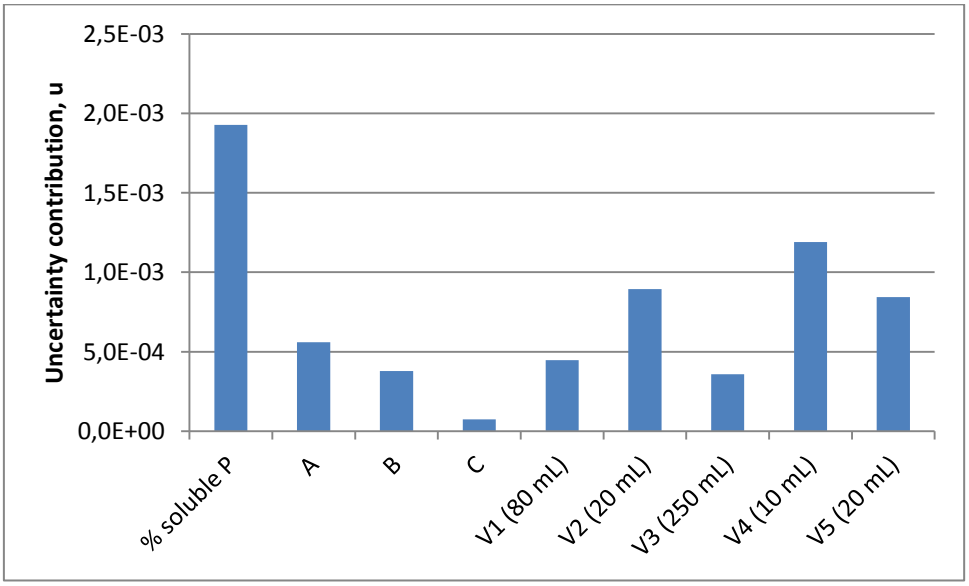

Figure 4 The uncertainty contributions as standard uncertainties.

As shown in Figure 3 and Figure 4, the largest relative contribution comes from the dilution of the sample where the smallest volume (10 mL) contributes the most.

The magnitude of the uncertainties varies depending on the value of *A*. This is shown in Figure 5.

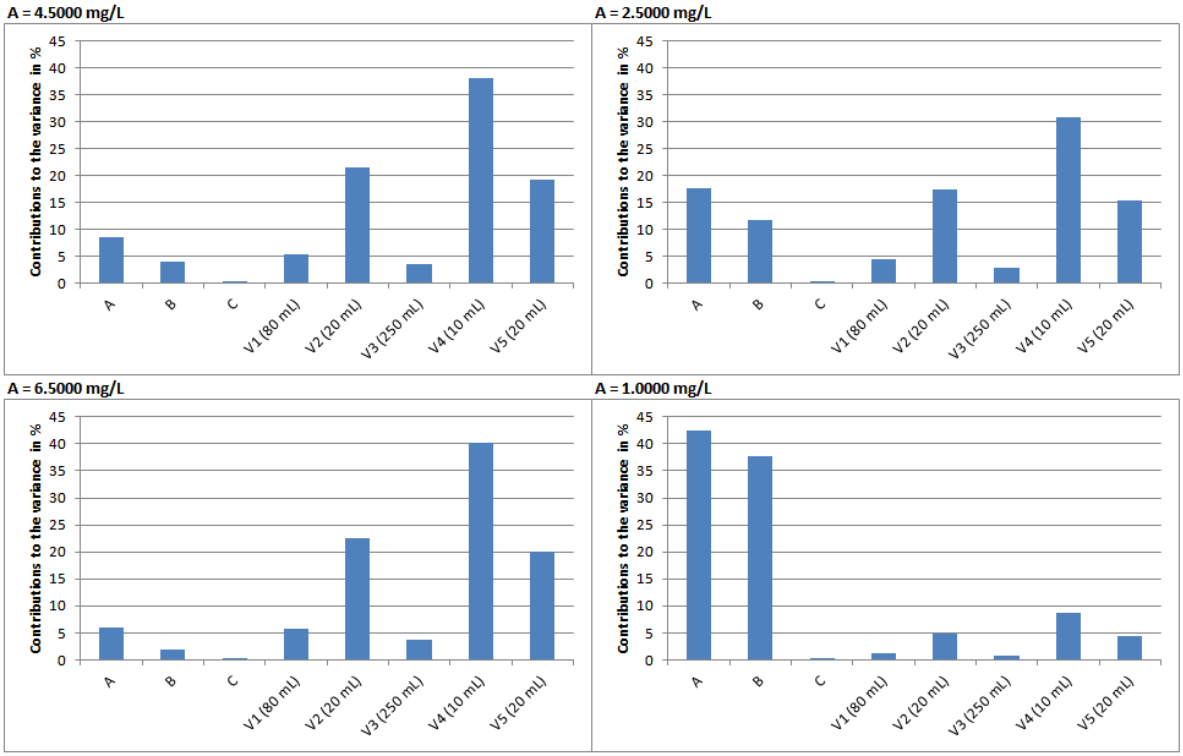

Figure 5 *The magnitude of the relative contributions to the uncertainty varies when the value of A varies.*

The uncertainty of the dilution increases when the concentration of soluble phosphorus in the sample increases (here shown for 6.5000 mg/L). When the concentration of soluble phosphorus in the sample is low (e.g., 1.0000 mg/L), the largest relative contribution comes from the uncertainty in the absorbance.

The calculation of the theoretical combined uncertainty gives a lower uncertainty than what is experienced in practice. The standard deviation found when calculating the upper and lower control limit for the control sample was ±0.068, and the repeatability (*r*) was 0.15. The number of replicates (*n*) for this calculation was 41. The calculation is shown in Table 24, appendix 6.

The difference between the theoretical and experimental uncertainty is not surprising since the theoretical calculation cannot account for uncertainties which is not quantifiable; such as random effects, inhomogeneity of the sample, sampling, matrix effects and so on.

5 Conclusion

The validation of the method for analysis of soluble phosphorus has established important method parameters, and the validation can be looked upon as complete. The validation has demonstrated that the method is fit for the intended purpose.

The recovery test/spiking showed how important it is to have knowledge about the properties of the phosphate salts, especially the solubility. If the sample being analyzed contains NaH_2PO_4, $CaHPO_4$, $Ca(H_2PO_4)_2$ or $NH_4H_2PO_4$ in known quantities, the results can be reported as “corrected for recovery”. On the other hand, values can just as well be reported as uncorrected as long as this is clearly stated. In many cases it will be more convenient to report uncorrected results because the purpose of the method is to investigate what are bioavailable to the fish. The recovery is thus not relevant since the phosphate salts that are not recovered are not bioavailable. If the exact composition of the sample is unknown or if the sample contains phosphate salts not investigated in this method validation, uncorrected values have to be reported.

Considering that the recovery test showed that the method does not give a total yield for all of the inorganic phosphate salts, the method is better referred to as “soluble phosphorus” than “not bone bound phosphorus”.

The repeatability of the method is close to the repeatability of the well-established method for analysis of total phosphorus (ISO 6491). The repeatability is similar for 0.4 and 0.8 g weighed sample. The amount of sample will for the future continue to be 0.8 g opposed to the previous 0.4 g since inhomogeneous samples can occur.

Values below 0.09 % soluble phosphorus will be reported as “lower than” (<) 0.09 %. This is the established limit of quantification for the method.

The theoretical combined uncertainty of the method was calculated to ±0.0019 at a concentration of 4.5000 mg/L soluble phosphorus. This is a typical value for the control sample. The experimental uncertainty of a similar sample is ±0.068. The difference in the results between the different analysts performing the method at Nofima BioLab was small.

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Appendixes

APPENDIX 1 – PRELIMINARY EXPERIMENTS

Table 15 Results of the preliminary analysis of the basal diet and the rapeseed/fish oil

Sample	Oil (g)	Basal diet (g)	Total amount (g)	Result (mg/L)	Result (%)	Mean value	*SD*	%*RSD*
Blind				0.3523		0.3894 mg/L	0.030	7.58
				0.4155				
				0.4106				
				0.3791				
Basal diet			0.8023	2.5880	0.548	**0.56 %**	0.026	4.60
			0.8044	2.5394	0.535			
			0.8092	2.6048	0.544			
			0.8077	2.7352	0.578			
			0.8195	2.9068	0.611			
			0.8068	2.6700	0.562			
			0.8696	2.8005	0.551			
Basal diet+oil	0.1324	0.6648	0.7972	2.3839	0.618	**0.56 %**	0.025	4.524
	0.1422	0.6662	0.8084	2.1798	0.553			
	0.1366	0.6638	0.8004	2.1530	0.547			
	0.1371	0.6656	0.8027	2.2964	0.590			
	0.1356	0.6631	0.7987	2.2041	0.563			
	0.1337	0.6650	0.7987	2.1871	0.557			
	0.1370	0.6664	0.8034	2.1263	0.537			
	0.1377	0.6673	0.8050	2.1263	0.536			
	0.1388	0.6671	0.8059	2.1603	0.546			
	0.1373	0.6658	0.8031	2.2187	0.566			

Table 16 Homogeneity test of a spiked sample w/NaH_2PO_4 (98.5 g basal diet spiked with 1.5 g NaH_2PO_4)

Sample	Amount of sample (g)	Result (mg/L)	Result (%)	Mean value	*SD*	%*RSD*
Blind		0.4899		0.3936 mg/L		
		0.2973				
Basal diet w/NaH_2PO_4	2.5226	6.1230	1.136	1.209 %	0.05	4.05
	2.5085	6.2035	1.158			
	2.5331	6.3741	1.180			
	2.5181	6.3912	1.191			
	2.5341	6.5082	1.206			
	2.5538	6.4326	1.182			
	2.5088	6.5326	1.223			
	2.4975	6.8056	1.284			
	2.5164	6.7763	1.268			
	2.5006	6.6886	1.259			

APPENDIX 2 – RECOVERY TEST

Table 17 Results from the recovery test for the spiked samples

Sample	Oil (g)	Basal diet (g)	Total amount (g)	Result (mg/L)	Result (%)	Mean value (%)	*SD*	*%RSD*	*SEM*	u_{rec}
Spike 1	0.1430	0.6698	0.8128	3.9885	1.080	1.086	0.031	2.85	0.010	0.90
	0.1391	0.6651	0.8042	3.8952	1.060					
	0.1369	0.6616	0.7985	4.0698	1.118					
	0.1371	0.6680	0.8051	4.0578	1.104					
	0.1437	0.6628	0.8065	3.8402	1.047					
	0.1341	0.6639	0.7980	4.1296	1.133					
	0.1386	0.6631	0.8017	3.9335	1.075					
	0.1324	0.6650	0.7974	4.0985	1.121					
	0.1410	0.6634	0.8044	3.8641	1.053					
	0.1365	0.6688	0.8053	3.9335	1.066					
Spike 2	0.1375	0.6654	0.8029	2.7546	0.717	0.685	0.018	2.56	0.006	0.81
	0.1458	0.6694	0.8152	2.6351	0.677					
	0.1463	0.6698	0.8161	2.6303	0.675					
	0.1453	0.6693	0.8146	2.6111	0.670					
	0.1348	0.6645	0.7993	2.7427	0.714					
	0.1363	0.6630	0.7993	2.6087	0.675					
	0.1372	0.6670	0.8042	2.6398	0.681					
	0.1342	0.6677	0.8019	2.6901	0.695					
	0.1404	0.6636	0.8040	2.5992	0.672					
	0.1395	0.6696	0.8091	2.6255	0.674					
Spike 3	0.1398	0.6625	0.8023	3.5967	0.960	0.927	0.027	2.879	0.008	0.91
	0.1396	0.6672	0.8068	3.4140	0.898					
	0.1366	0.6641	0.8007	3.4579	0.915					
	0.1323	0.6670	0.7993	3.5918	0.952					
	0.1412	0.6663	0.8075	3.3678	0.885					
	0.1337	0.6630	0.7967	3.4043	0.901					
	0.1400	0.6647	0.8047	3.5212	0.934					
	0.1698	0.6668	0.8366	3.6283	0.963					
	0.1395	0.6648	0.8043	3.5066	0.929					
	0.1380	0.6696	0.8076	3.5358	0.931					
Spike 4	0.1428	0.6635	0.8063	4.0578	1.112	1.118	0.071	6.37	0.023	2.02
	0.1436	0.6691	0.8127	4.5408	1.247					
	0.1466	0.6636	0.8102	3.6513	0.989					
	0.1403	0.6682	0.8085	4.0291	1.095					
	0.1338	0.6640	0.7978	4.0180	1.084					
	0.1528	0.6664	0.8192	4.4003	1.195					
	0.1454	0.6633	0.8087	3.9449	1.063					
	0.1437	0.6658	0.8095	4.2128	1.140					
	0.1377	0.6678	0.8055	4.1008	1.103					
	0.1533	0.6672	0.8205	4.2615	1.152					
Spike 5	0.1405	0.6698	0.8103	3.9961	1.068	1.043	0.028	2.70	0.009	0.86
	0.1312	0.6675	0.7987	3.8572	1.030					
	0.1428	0.6661	0.8089	3.8061	1.017					
	0.1366	0.6687	0.8053	3.8305	1.021					
	0.1434	0.6677	0.8111	4.0935	1.101					
	0.1395	0.6682	0.8077	3.8670	1.032					
	0.1382	0.6689	0.8071	3.9279	1.049					
	0.1407	0.6629	0.8036	3.8792	1.044					
	0.1350	0.6648	0.7998	3.9522	1.063					
	0.1457	0.6626	0.8083	3.7550	1.007					
Spike 2, new	0.1412	0.6728	0.8140	2.9800	0.782	0.727	0.026	3.64	0.008	1.15
	0.1550	0.6649	0.8199	2.7576	0.725					
	0.1369	0.6690	0.8059	2.8016	0.733					
	0.1400	0.6733	0.8133	2.8700	0.749					
	0.1321	0.6786	0.8107	2.7600	0.711					
	0.1411	0.6762	0.8173	2.7453	0.709					
	0.1475	0.6790	0.8265	2.7331	0.702					
	0.1356	0.6664	0.8020	2.8431	0.749					
	0.1557	0.6740	0.8297	2.7527	0.713					
	0.1404	0.6769	0.8173	2.7087	0.697					
Spike 3, new	0.1559	0.6795	0.8354	3.5154	0.932	0.909	0.026	2.81	0.008	0.89
	0.1476	0.6735	0.8211	3.4054	0.908					
	0.1378	0.6737	0.8115	3.4274	0.914					
	0.1453	0.6775	0.8228	3.4812	0.925					
	0.1382	0.6744	0.8126	3.4470	0.919					
	0.1422	0.6624	0.8046	3.2954	0.890					
	0.1494	0.6785	0.8279	3.3810	0.894					
	0.1510	0.6775	0.8285	3.5765	0.953					
	0.1485	0.6727	0.8212	3.2441	0.861					
	0.1505	0.6668	0.8173	3.3418	0.898					

Table 18 *Solubility of the sodium and calcium phosphate salts in 1 M NaOH, found by analysis. The reason behind the variation in number of parallels is because some of the crucibles cracked during analysis, and the samples had to be rejected*

Salt	**Weight (g)**	**Result (%)**	**Mean value (%)**	***SD***	**%*RSD***	**Calculated P in salt (%)**	**% yield/ solubility**
NaH_2PO_4	0.1936	24.137	23.87	0.256	1.09	25.56	93.4
	0.1874	23.620					
	0.1906	23.857					
$CaHPO_4$	0.1671	7.618	7.430	0.467	6.28	22.42	33.1
	0.1636	7.383					
	0.1636	7.575					
	0.1627	7.471					
	0.1639	7.965					
	0.1644	6.568					
$Ca(H_2PO_4)_2$	0.1831	15.517	15.43	0.929	6.02	25.14	61.4
	0.1877	16.249					
	0.1856	14.109					
	0.1896	15.836					

APPENDIX 3 – PRECISION

Table 19 Precision calculation for soluble phosphorus with approximately 0.4 g weighed sample

Sample type	Sample ID	Result 1	Result 2	Diff.	Diff^2	Mean value	n
Hydrolysate	2011-0115-09	0.216	0.176	0.04	0.0016	0.20	1
Hydrolysate	2011-0115-10	0.252	0.212	0.04	0.0016	0.23	2
Oppk.LV	2011-0124-01	2.540	2.583	-0.04	0.0018	2.56	3
T1 råst.kol.dry	2011-0197-05	0.803	0.733	0.07	0.0049	0.77	4
T1 nøytr.hydr	2011-0197-06	0.663	0.659	0.00	0.0000	0.66	5
Fish feed	2011-0462-01	0.478	0.497	-0.02	0.0004	0.49	6
Fish feed	2011-0462-02	0.889	0.754	0.14	0.0182	0.82	7
Fish feed	2011-0462-03	0.962	0.991	-0.03	0.0008	0.98	8
Fish feed	2011-0462-04	1.166	1.136	0.03	0.0009	1.15	9
Fish feed	2011-0462-05	0.927	1.107	-0.18	0.0324	1.02	10
Fish feed	2011-0462-06	0.882	0.859	0.02	0.0005	0.87	11
Fish feed	2011-0462-07	0.915	0.906	0.01	0.0001	0.91	12
Fish feed	2011-0463-01	0.512	0.589	-0.08	0.0059	0.55	13
Freeze dried fish	2011-1530-01	0.657	0.656	0.00	0.0000	0.66	14
Freeze dried fish	2011-1530-02	0.743	0.793	-0.05	0.0025	0.77	15
Freeze dried fish	2011-1530-03	0.683	0.661	0.02	0.0005	0.67	16
Freeze dried fish	2011-1530-04	0.675	0.661	0.01	0.0002	0.67	17
Freeze dried fish	2011-1530-05	0.640	0.715	-0.08	0.0056	0.68	18
Freeze dried fish	2011-1530-06	0.644	0.669	-0.03	0.0006	0.66	19
Freeze dried fish	2011-1530-07	0.695	0.719	-0.02	0.0006	0.71	20
Freeze dried fish	2011-1530-08	0.692	0.718	-0.03	0.0007	0.71	21
Freeze dried fish	2011-1530-09	0.661	0.659	0.00	0.0000	0.66	22
Freeze dried fish	2011-1530-10	0.647	0.656	-0.01	0.0001	0.65	23

***n*=**	23	**SUM D^2=**	**0.080**	**Mean value=**	**0.78**

Repeatability

SD_r = SQR(SUM(D*D)/2K)	0.042
%*RSD*	5.3
$r = 2.8 \times SD_r$	0.118

Table 20 Precision calculation for soluble phosphorus with approximately 0.8 g weighed sample

Sample type	Sample ID	Result 1	Result 2	Diff.	Diff^2	Mean value	*n*
SKRUK-A	2012-1720-01	0.440	0.449	-0.01	0.0001	0.44	1
SKRUK-B	2012-1720-02	0.662	0.834	-0.17	0.0296	0.75	2
SKRUK-B	2012-1720-02	0.607	0.615	-0.01	0.0001	0.61	3
SKRUK-C	2012-1720-03	0.429	0.584	-0.16	0.0240	0.51	4
SKRUK-C	2012-1720-03	0.556	0.583	-0.03	0.0007	0.57	5
SKRUK-D	2012-1720-04	0.397	0.375	0.02	0.0005	0.39	6
SKRUK-E	2012-1720-05	0.564	0.649	-0.09	0.0072	0.61	7
SKRUK-F	2012-1720-06	0.425	0.396	0.03	0.0008	0.41	8
77/12	2012-1847-01	0.623	0.653	-0.03	0.0009	0.64	9
Diet 1 0,8mm	2012-2034-01	0.567	0.557	0.01	0.0001	0.56	10
Diet 2 0,8mm	2012-2034-02	0.707	0.716	-0.01	0.0001	0.71	11
Diet 3 0,8mm	2012-2034-03	0.793	0.825	-0.03	0.0010	0.81	12
Diet 4 0,8mm	2012-2034-04	0.627	0.635	-0.01	0.0001	0.63	13
Diet 5 0,8mm	2012-2034-05	0.771	0.826	-0.05	0.0030	0.80	14
Diet1:1,6-2,3mm	2012-2035-01	0.500	0.575	-0.08	0.0056	0.54	15
Diet2:1,6-2,3mm	2012-2035-02	0.663	0.704	-0.04	0.0017	0.68	16
Diet3:1,6-2,3mm	2012-2035-03	0.792	0.839	-0.05	0.0022	0.82	17
Diet4:1,6-2,3mm	2012-2035-04	0.636	0.645	-0.01	0.0001	0.64	18
Diet5:1,6-2,3mm	2012-2035-05	0.745	0.796	-0.05	0.0026	0.77	19
Diet A	2012-2099-01	0.464	0.464	0.00	0.0000	0.46	20
Diet B	2012-2099-02	0.784	0.736	0.05	0.0023	0.76	21
Diet C	2012-2099-03	0.943	0.999	-0.06	0.0031	0.97	22
Diet D	2012-2099-04	0.444	0.434	0.01	0.0001	0.44	23
Diet E	2012-2099-05	0.706	0.790	-0.08	0.0071	0.75	24
Diet F	2012-2099-06	0.963	0.925	0.04	0.0014	0.94	25
Diet G	2012-2099-07	0.383	0.397	-0.01	0.0002	0.39	26
Diet G	2012-2099-07	0.366	0.328	0.04	0.0014	0.35	27
Diet H	2012-2099-08	0.656	0.520	0.14	0.0185	0.59	28
Diet H	2012-2099-08	0.483	0.520	-0.04	0.0014	0.50	29
Diet H	2012-2099-08	0.462	0.594	-0.13	0.0174	0.53	30
Diet I	2012-2099-09	0.637	0.67	-0.03	0.0011	0.65	31
Diet I	2012-2099-09	0.688	0.597	0.09	0.0083	0.64	32
NTC 12030 FM	2012-2099-10	0.944	0.923	0.02	0.0004	0.93	33
86/12	2012-2152-01	0.559	0.571	-0.01	0.0001	0.57	34
88/12	2012-2286-01	0.836	0.771	0.06	0.0042	0.80	35
05 SSC 50	2012-2855-01	0.375	0.351	0.02	0.0006	0.36	36
12 SSC 200	2012-2855-02	0.566	0.535	0.03	0.0010	0.55	37
13 SSC 200	2012-2855-03	0.492	0.394	0.10	0.0096	0.44	38
15 SSC 200	2012-2855-04	0.438	0.474	-0.04	0.0013	0.46	39
20 Transfer 25	2012-2855-05	0.652	0.583	0.07	0.0048	0.62	40
26 Transf.Sm.75	2012-2855-06	0.464	0.444	0.02	0.0004	0.45	41
40 Opal 250	2012-2855-07	0.473	0.490	-0.02	0.0003	0.48	42
Beinrest	2012-2876-01	0.129	0.081	0.05	0.0023	0.11	43
Beinrest	2012-2880-01	0.238	0.182	0.06	0.0031	0.21	44
>4,6mm	2012-2961-01	0.046	0.017	0.03	0.0008	0.03	45
T2	2012-2980-02	0.021	0.012	0.01	0.0001	0.02	46
T3	2012-2980-03	0.007	0.057	-0.05	0.0025	0.03	47
T4	2012-2980-04	0.043	0.045	0.00	0.0000	0.04	48
3:T1 Bunnfall K	2012-3002-01	1.535	1.532	0.00	0.0000	1.53	49
4:T2 Bunnfall K	2012-3003-01	2.458	2.477	-0.02	0.0004	2.47	50
5:Bunnsediment	2012-3004-01	0.774	0.793	-0.02	0.0004	0.78	51
6:Krystaller v.	2012-3005-01	0.926	0.975	-0.05	0.0024	0.95	52

***n*=**	52	**SUM D^2=**	**0.177**	**Mean value=**	**0.61**

Repeatability

SD_r = SQR(SUM(D*D)/2K)	0.041
%*RSD*	6.8
$r = 2.8 \times SD_r$	0.117

Table 21 Precision calculation for total phosphorus

Sample type	Sample ID	Result 1	Result 2	Diff.	Diff^2	Mean value	n
Kontroll	2012-2706-01	0.034	0.078	-0.04	0.0020	0.06	1
1% - 2t	2012-2706-02	0.258	0.279	-0.02	0.0004	0.27	2
2,5% - 0,5t	2012-2706-03	0.744	0.814	-0.07	0.0048	0.78	3
2,5% - 1t	2012-2706-04	0.847	0.854	-0.01	0.0001	0.85	4
2,5% - 2t	2012-2706-05	0.899	0.854	0.05	0.0021	0.88	5
5% - 0,5t	2012-2706-06	0.967	0.887	0.08	0.0063	0.93	6
5% - 1t	2012-2706-07	1.125	1.067	0.06	0.0034	1.10	7
5% - 27	2012-2706-08	1.125	1.140	-0.01	0.0002	1.13	8
10% - 2t	2012-2706-09	1.108	1.127	-0.02	0.0004	1.12	9
1% - 2t	2012-2707-02	0.233	0.244	-0.01	0.0001	0.24	10
2,5% - 0,5t	2012-2707-03	0.786	0.866	-0.08	0.0064	0.83	11
2,5% - 1t	2012-2707-04	0.831	0.862	-0.03	0.0010	0.85	12
2,5% - 2t	2012-2707-05	0.847	0.835	0.01	0.0002	0.84	13
5% - 0,5t	2012-2707-06	1.062	1.053	0.01	0.0001	1.06	14
5% - 1t	2012-2707-07	1.109	1.147	-0.04	0.0014	1.13	15
5% - 2t	2012-2707-08	1.114	1.151	-0.04	0.0013	1.13	16
10% - 2t	2012-2707-09	1.152	1.162	-0.01	0.0001	1.16	17
>4,6mm	2012-2708-01	7.209	7.261	-0.05	0.0027	7.24	18
4,6-2,3	2012-2709-01	6.686	6.741	-0.06	0.0030	6.71	19
002 12/012	2012-3213-01	2.084	2.204	-0.12	0.0144	2.14	20
3435005/8	2012-2726-01	2.366	2.194	0.17	0.0296	2.28	21
106265	2012-2727-01	1.717	1.731	-0.01	0.0002	1.72	22
106091	2012-2728-01	1.699	1.709	-0.01	0.0001	1.70	23
3434544/9	2012-2729-01	2.095	2.093	0.00	0.0000	2.09	24
05 SSC 50	2012-2855-01	1.151	1.122	0.03	0.0008	1.14	25
12 SSC 200	2012-2855-02	1.036	1.022	0.01	0.0002	1.03	26
13 SSC 200	2012-2855-03	1.078	1.039	0.04	0.0015	1.06	27
15 SSC 200	2012-2855-04	1.046	1.060	-0.01	0.0002	1.05	28
20 Transfer 25	2012-2855-05	1.666	1.651	0.01	0.0002	1.66	29
26 Transf.Sm.75	2012-2855-06	1.453	1.451	0.00	0.0000	1.45	30
40 Opal 250	2012-2855-07	1.283	1.290	-0.01	0.0000	1.29	31
Beinrest	2012-2876-01	1.699	1.653	0.05	0.0021	1.68	32
Bein	2012-2880-01	4.321	4.368	-0.05	0.0022	4.34	33
0% 2t	2012-2881-01	0.019	0.010	0.01	0.0001	0.01	34
1% 2t	2012-2881-02	0.225	0.225	0.00	0.0000	0.23	35
2,5% 0,5t	2012-2881-03	0.142	0.131	0.01	0.0001	0.14	36
2,5% 1t	2012-2881-04	0.158	0.274	-0.12	0.0135	0.22	37
2,5% 2t	2012-2881-05	0.215	0.217	0.00	0.0000	0.22	38
5% 2t	2012-2881-06	0.196	0.178	0.02	0.0003	0.19	39
0% 2t	2012-2882-01	0.007	0.032	-0.03	0.0007	0.02	40
1% 2t	2012-2882-02	0.406	0.351	0.06	0.0030	0.38	41
2,5% 0,5t	2012-2882-03	0.382	0.408	-0.03	0.0007	0.40	42
2,5% 1t	2012-2882-04	0.468	0.453	0.02	0.0002	0.46	43
2,5% 2t	2012-2882-05	0.562	0.684	-0.12	0.0149	0.62	44
5% 2t	2012-2882-06	0.540	0.511	0.03	0.0008	0.53	45
2,5% 0,5t	2012-2883-01	0.163	0.179	-0.02	0.0003	0.17	46
2,5% 1t	2012-2883-02	0.237	0.248	-0.01	0.0001	0.24	47
2,5% 2t	2012-2883-03	0.347	0.308	0.04	0.0015	0.33	48
mai 2012	2012-2936-01	12.633	12.468	0.16	0.0272	12.55	49
>4,6mm	2012-2961-01	0.633	0.614	0.02	0.0004	0.62	50
2,3-4,6	2012-2962-01	0.546	0.566	-0.02	0.0004	0.56	51
T1	2012-2980-01	0.373	0.380	-0.01	0.0000	0.38	52
T2	2012-2980-02	0.306	0.312	-0.01	0.0000	0.31	53
T3	2012-2980-03	0.347	0.352	-0.01	0.0000	0.35	54
T4	2012-2980-04	0.352	0.363	-0.01	0.0001	0.36	55
1Gips fra trik.	2012-3000-01	0.634	0.614	0.02	0.0004	0.62	56
3:T1 Bunnfall K	2012-3002-01	2.338	2.437	-0.10	0.0098	2.39	57
4:T2 Bunnfall K	2012-3003-01	2.923	2.999	-0.08	0.0058	2.96	58
5:Bunnsediment	2012-3004-01	2.836	2.785	0.05	0.0026	2.81	59
6:Krystaller v.	2012-3005-01	1.423	1.300	0.12	0.0151	1.36	60
7 FBH1	2012-3006-01	6.964	6.971	-0.01	0.0000	6.97	61
3628907-01-171	2012-3092-01	1.499	1.506	-0.01	0.0000	1.50	62
3629050-01	2012-3093-01	1.520	1.557	-0.04	0.0014	1.54	63
Intro svev 40	2012-3140-01	1.795	1.795	0.00	0.0000	1.80	64
00191	2012-3418-01	1.366	1.409	-0.04	0.0018	1.39	65
00201	2012-3422-01	2.397	2.412	-0.02	0.0002	2.40	66

n=	66	SUM D^2=	0.189	Mean value=	1.48

Repeatability

SD_r = SQR(SUM(D*D)/2K)	0.038
%RSD	2.6
$r = 2.8 \times SD_r$	0.107

APPENDIX 4 – LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

Table 22 Calculation of the limit of detection (LOD) and limit of quantification (LOQ)

Date	Result 1 (mg/L)	Result 2 (mg/L)	Result 3 (mg/L)	Result 4 (mg/L)	Mean value (mg/L)
2011-09-29	0.2197	0.1269			0.1733
2011-12-30	0.5144	0.3111			0.4128
2012-02-03	0.6412	0.4011			0.5212
2012-02-11	0.6091	0.2374			0.4233
2012-02-24	0.1899	0.4471			0.3185
2012-02-27	0.2251	0.1912			0.2082
2012-04-13	0.2259	0.2842			0.2551
2012-04-20	0.2608	0.2101			0.2355
2012-04-24	0.3497	0.3181			0.3339
2012-05-04	0.5510	0.6641			0.6076
2012-05-09	0.2339	0.5958			0.4149
2012-05-14	0.3823	0.3198			0.3511
2012-05-30	0.3372	0.4143			0.3758
2012-10-01	0.2884	0.3006	0.3471	0.2053	0.2854
2012-10-15	0.4169	0.5139	0.4896	0.4630	0.4709
2012-11-07	0.5763	0.3134			0.4449
2012-11-09	0.4312	0.3464			0.3888
2012-11-30	0.3523	0.4155	0.4106	0.3791	0.3894
2012-12-14	0.4041	0.3562	0.4351	0.2845	0.3700
2012-12-21	0.3652	0.3944	0.4894	0.4237	0.4182

n	50
Mean value	0.3733
SD	0.126
%RSD	33.8

	mg/L	%
LOD	0.3030	-
LOQ	0.7566	0.09

APPENDIX 5 – CALCULATION OF UNCERTAINTY

Table 23 Numerical calculation of the combined uncertainty of the method. The calculation was performed by using the spreadsheet method given in Eurachem (1995)

	Symbol	*A*	*B*	*C*	*V1* (80 mL)	*V2* (20 mL)	*V3* (250 mL)	*V4* (10 mL)	*V5* (20 mL)	$f=10^6$	%
	Value	4.5000	0.3700	0.8000	80	20	250	10	20	1E6	100
	Standard u(x_i)	0.002239	0.0015163	0.0001	0.06	0.03	0.15	0.02	0.0282843	0	0
	***SD*, u(x_i)**	0.002239	0.0015163	0.0000577	0.0346410	0.0173205	0.0866025	0.0115470	0.0163299	0	0
A	4.5000	4.5022	4.5000	4.5000	4.5000	4.5000	4.5000	4.5000	4.5000	4.5000	4.5000
B	0.3700	0.3700	0.3715	0.3700	0.3700	0.3700	0.3700	0.3700	0.3700	0.3700	0.3700
C	0.8000	0.8000	0.8000	0.8001	0.8000	0.8000	0.8000	0.8000	0.8000	0.8000	0.8000
***V1* (80 mL)**	80	80	80	80	80.03	80	80	80	80	80	80
***V2* (20 mL)**	20	20	20	20	20	20.02	20	20	20	20	20
***V3* (250 mL)**	250	250	250	250	250	250	250.09	250	250	250	250
***V4* (10 mL)**	10	10	10	10	10	10	10	10.01	10	10	10
***V5* (20 mL)**	20	20	20	20	20	20	20	20	20.02	20	20
$f=10^6$	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E6
%	100	100	100	100	100	100	100	100	100	100	100
% soluble P	**1.0325**	**1.0331**	**1.0321**	**1.0324**	**1.0329**	**1.0316**	**1.0329**	**1.0313**	**1.0333**	**1.0325**	**1.0325**
u(y,x_i)		0.000560	-0.000379	-0.000075	0.000447	-0.000893	0.000358	-0.001191	0.000843	0	0
u(y)^2, u(y,x_i)^2	3.717E-06	3.134E-07	1.437E-07	5.552E-09	1.999E-07	7.982E-07	1.279E-07	1.418E-06	7.107E-07	0	0
Sum r_i, u(y,x_i)^2/u(y)^2	1	0.084306	0.038654	0.001493	0.053769	0.214706	0.034412	0.381479	0.191180	0	0
100 % Sum r_i, u(y,x_i)^2/u(y)^2	100	8.430555	3.865415	0.149338	5.376945	21.470575	3.441245	38.147902	19.118026	0	0
u_c(y)	**0.0019**										
u(y,x_i)/u(x_i)		0.250000	-0.250000	-1.290532	0.012906	-0.051580	0.004130	-0.103131	0.051625	0	0
	% soluble P	***A***	***B***	***C***	***V1* (80 mL)**	***V2* (20 mL)**	***V3* (250 mL)**	***V4* (10 mL)**	***V5* (20 mL)**	**$f=10^6$**	**%**
ABS(u(y,xi))	0.001928	0.000560	0.000379	0.000075	0.000447	0.000893	0.000358	0.001191	0.000843	0	0

Expanded uncertainty, *k*=2	**0.0039**
%*RSD*, *k*=2	**0.39**

APPENDIX 6 – CONTROL SAMPLE

Table 24 The reproducibility and repeatability for the control sample, calculated when creating a control chart for the method

Date	Result 1	Result 2	Diff.	Diff^2	Mean value	*n*
2011-05-12	1.135	1.101	0.03	0.0012	1.12	1
2011-05-20	1.308	1.273	0.04	0.0012	1.29	2
2011-05-26	0.996	0.978	0.02	0.0003	0.99	3
2011-06-17	1.172	1.086	0.09	0.0074	1.13	4
2011-06-20	1.004	1.010	-0.01	0.0000	1.01	5
2011-07-01	0.990	1.000	-0.01	0.0001	1.00	6
2011-07-08	1.028	1.018	0.01	0.0001	1.02	7
2011-08-31	1.011	1.023	-0.01	0.0001	1.02	8
2011-09-20	1.034	1.035	0.00	0.0000	1.03	9
2011-09-26	1.031	1.033	0.00	0.0000	1.03	10
2011-09-29	1.055	1.040	0.01	0.0002	1.05	11
2011-12-30	1.052	1.007	0.05	0.0020	1.03	12
2012-01-20	0.987	1.011	-0.02	0.0006	1.00	13
2012-01-27	0.943	0.941	0.00	0.0000	0.94	14
2012-02-03	1.071	1.060	0.01	0.0001	1.07	15
2012-02-24	1.034	1.047	-0.01	0.0002	1.04	16
2012-03-02	1.032	1.091	-0.06	0.0035	1.06	17
2012-03-09	0.987	0.965	0.02	0.0005	0.98	18
2012-03-28	1.172	1.049	0.12	0.0151	1.11	19
2012-04-13	1.056	1.034	0.02	0.0005	1.05	20
2012-04-20	1.024	1.060	-0.04	0.0013	1.04	21
2012-04-30	0.997	1.007	-0.01	0.0001	1.00	22
2012-05-04	0.938	0.981	-0.04	0.0018	0.96	23
2012-05-09	0.985	0.969	0.02	0.0003	0.98	24
2012-05-21	1.007	0.999	0.01	0.0001	1.00	25
2012-05-30	1.027	1.065	-0.04	0.0014	1.05	26
2012-06-15	0.978	0.980	0.00	0.0000	0.98	27
2012-06-28	1.059	1.026	0.03	0.0011	1.04	28
2012-07-04	1.009	1.045	-0.04	0.0013	1.03	29
2012-08-16	1.442	1.032	0.41	0.1681	1.24	30
2012-08-31	0.995	1.018	-0.02	0.0005	1.01	31
2012-09-13	1.036	1.042	-0.01	0.0000	1.04	32
2012-09-18	1.160	1.175	-0.02	0.0002	1.17	33
2012-09-28	1.020	1.034	-0.01	0.0002	1.03	34
2012-10-09	1.043	1.052	-0.01	0.0001	1.05	35
2012-10-15	1.031	1.112	-0.08	0.0066	1.07	36
2012-11-05	0.999	1.000	0.00	0.0000	1.00	37
2012-11-09	1.029	1.023	0.01	0.0000	1.03	38
2012-11-13	0.997	1.035	-0.04	0.0014	1.02	39
2012-11-30	0.999	1.000	0.00	0.0000	1.00	40
2012-12-14	1.011	1.006	0.00	0.0000	1.01	41

***n*=**	**41**	**SUM D^2=**	**0.218**	**Mean value**	**1.04**

Reproducibility

Mean value	1.04
SD	0.068
Upper control limit (*UCL*)	1.185
Lower control limit (*LCL*)	0.897

Repeatability

SD_r = SQR(SUM(D*D)/2K)	0.052
$r = 2.8 \times SD_r$	0.146

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