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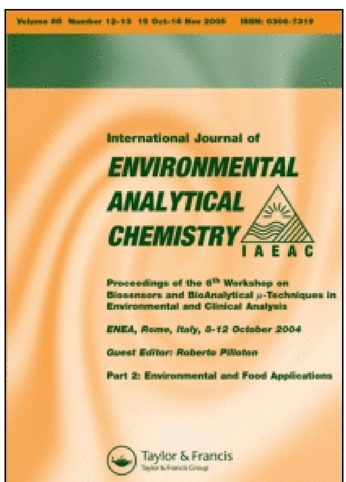

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Improved spectrophotometric vitellogenin determination via alkali-labile phosphate in fish plasma – a cost effective approach for assessment of endocrine disruption

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Vitellogenin (VTG) is a well known protein biomarker for exposure to environmental estrogens and possible endocrine disruption in fish. VTG is very dominant in plasma after the onset of vitellogenesis and the protein is heavily phosphorylated. This enables indirect quantification through measurement of alkali-labile protein bound phosphate (ALP) as an alternative to the more expensive enzyme linked immunosorbent assay. Good correlation has previously been shown between ALP and actual VTG levels but little effort has been made to investigate the method in an analytical way e.g., to assure the origin of the measured phosphate. During this method development care has been taken to rule out non-VTG sources of phosphate such as phospholipids and free phosphate in the blood plasma. Sample preparation has been simplified and unnecessary steps have been omitted. The common spectrophotometric measurement for ALP involves measurement at two wavelengths and calculation of corrected absorbance values. With a quick phase separation step the spectrophotometric phosphate determination using molybdic acid and ascorbic acid has been improved and all matrix interference has been eliminated. The final ALP method presented here has a detection limit of 3.2 μg PO_4^{3-}/ml plasma which is six times lower than similar methods and it also has less variability. A high sample throughput in comparison to previous ALP methods is possible after scaling down sample and reagent volumes to fit in a 96 well microtiter plate. The cost for buying all chemicals and plastic consumer goods for setting up the indirect protocol for the analysis of 1000 samples is only circa 350 euro. This is only 1% of the material cost for buying commercially available test kit for direct quantification of VTG in the same number of samples. The ALP method should thus be of interest also for applied scientists outside advanced research laboratories.

Keywords: vitellogenin; alkali-labile phosphate; endocrine disruptors; *Carassius carassius*; biomarker

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1. Introduction

An increasing number of man-made chemicals have been identified as potent endocrine disrupting chemicals (EDCs) with the ability to work as agonists or antagonists to endogenous hormones. The European Commission has prioritised 146 EDCs that are of

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concern for human and wildlife exposure due to their persistence or high volume use [1]. Most attention and research on endocrine disruption has been directed towards organic chemicals in the aquatic environment and especially those interfering with the female sex hormones. These xenoestrogens can interfere with the vertebrate hypothalamo-hypophysis-gonadal axis which is the predominant system for regulating vitellogenesis in female fish. Vitellogenesis is the hormonally controlled process whereby protein and lipids essential for the growing eggs are formed in liver cells, transported in the blood, taken up as vesicles in the ovary and finally split into the yolk components that are stored in yolk platelets inside the evolving oocytes. During the blood transport these components are embedded in a macromolecule called vitellogenin (VTG). Xenoestrogens have the ability to mimic the estrogen-induced production of VTG in liver cells and therefore increasing blood levels of VTG in male and immature fish has been established as a biomarker for endocrine disruption [2,3].

There are different methods for quantification of VTG and in a review of different methods for measuring (anti-) estrogenic effects it was concluded that indirect quantification through alkali-labile phosphoprotein (ALP) is an inexpensive and rapid alternative [4]. Throughout this text ALP is defined as the amount of phosphate that can be obtained by alkaline treatment of isolated plasma proteins.

Phosphate quantification may appear as a non-discriminating and blunt tool to quantify a biomarker protein but is feasible due to the unusual features of this molecule. In vertebrates VTG is a macromolecule consisting of lipovitellin and phosvitin subunits where the latter has an amino acid backbone unusually rich in serine which makes it the most phosphorylated amino acid sequence in nature [5].

VTG is stated to be the only phosphorous containing protein in the blood of oviparous vertebrates [6], and this together with the high degree of phosphorylation enables the indirect quantification of VTG via ALP. The method has been used in at least 27 publications for quantification in fish e.g. [7–10] but also for measurement in mussel [11–13], clam [14–17], crustacean [18] and in lizard [19]. Validations have correlated measured ALP and direct measurement of VTG in *Centropomus undecimalis* [20], *Salmo gairdneri* [21], *Scyliorphinus canicula L.* [22], *Oncorhynchus mykiss* [6] and *Sceloporus occidentalis* [23] resulting in correlation coefficients, r, between 0.73 and 0.99. Although widely used there is certainly room for improvement of the ALP methodology and all VTG quantification methods need to be standardised before they can be used in regulatory tests [24]. To achieve maximum correlation between the indirect ALP method and the actual level of VTG, the origin of the quantified phosphate must be secured.

When measuring ALP it is important to ensure that phosphate from non-VTG sources are excluded in the quantification. Estrogens induce VTG production but also elevate levels of serum phospholipids in vertebrates [19]. Lipids occur both in the plasma and are embedded in VTG where they constitute around 18% of the mass of the macromolecule [22]. Half [22] or two thirds [25] of these embedded lipids are phospholipids. Phospholipids should preferably not be present in the isolated protein fraction when measuring ALP but small amounts may not be a problem since they are not always alkali-labile [26]. It has been shown that in an ether extract containing both VTG and phospholipids the latter did not interfere with the ALP measurement [17].

It has been suggested but not shown in experiments that VTG can be dephosphorylated during handling and storing [20]. The sample treatment needs to be gentle enough to keep all protein bound phosphate intact while free phosphates are removed from the plasma, before an alkaline hydrolysis of the bound phosphate is performed.

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Additionally, a good sample treatment is also needed to make sure that the final spectrophotometric quantification of phosphate in the ALP methodology can be performed without matrix interference.

The aim of this paper was to improve and assess the indirect method based on ALP and establish a procedure that is simple, accurate and cheap. How ALP is affected by different procedures for collection of blood samples, separation of plasma fraction and the necessity of blood additives has not been discussed elsewhere. The method would be more straightforward if all plasma proteins where precipitated instead of extracting VTG from plasma. The number of washing steps should be minimised and the use of hazardous organic solvents avoided. Another aim was to improve the final spectrophotometric determination so that the procedure of Stanton [28] involving two readings and calculation of corrected absorbance values could be abolished. Different critical steps in the analytical procedure were identified during a thorough literature revision and thereafter the whole procedure was evaluated from blood sampling to the final spectrophotometric determination by experiments on blood from Crucian carp (*Carassius carassius*) or human plasma.

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2. Experimental

2.1 *Equipment and chemicals*

The spectrophotometric determination was performed with an ELx 808IU plate reader from BIO-TEK INSTRUMENTS inc. with absorbance measured at 630 nm. The instrumentation was set to hold a constant temperature of 40°C during colour development, since colour development was supposed to be faster at this temperature than at room temperature [27]. The *Tris*-buffer used to wash protein pellet was 0.05 M *Tris*(hydroxymethyl)aminomethane Base (pro analysi, Merck), with pH adjusted to 7.6 with HCl and the TBS-buffer was identical but with 0.9% NaCl. Standard solution of 127 μg PO_4^{3-}/ml was prepared in 1M NaOH from oven dried H_2KPO_4. Molybdic acid reagent solution was 1% $(NH_4)6Mo_7O_{24}$ X $4H_2O$ (pro analysi, Merck) prepared at different acidity during method development and ultimately in 2M sulphuric acid (pro analysi, Merck). This reagent is stable for storage [28]. Ascorbic acid (pro analysi, Merck) solutions were prepared at different concentrations in pure water and stored in dark flasks at 4°C where they are stable for about 7 weeks [27]. The finally chosen concentration of ascorbic acid was 5%. 1-butanol (pro analysi, Merck), acetone (AnalaR®, BDH) and aprotinin (Sigma-Aldrich) was used during sample preparation.

2.2 *Blood samples from wild Crucian carp*

All experiments involving live fish were approved by an ethical board. Wild Crucian carp (20–35 g, 10–15 cm) were captured in early July 2006, kept in 200 L aquaria containing aerated, filtrated and circulated tap water with 0.1% of iodine-free table salt and fed with pellet (Koi foder, Simontorp Säteri AB, Sweden). Fish were anaesthetised with benzocaine (ethyl-p-aminobensoat, Sigma no. E-1501). Benzocaine is recommended for the use on freshwater fish and is in contrast to the much used MS222 not carcinogenic [29]. A solution with 4% benzocaine in methanol was diluted in water to a concentration of 50–500 mg benzocaine/L which was enough to immobilise fish within 30 seconds. Length and weight were noted before blood was collected from the caudal vein of the immobilised

fish using one or several 75 µl heparinised glass hematocrit tubes (Vitrex) which were emptied into ice-cold centrifuge tubes. By working swiftly it was possible to collect a maximum of 600 µl of blood from the larger fishes. Blood was kept on ice and centrifuged within 2 hours and plasma that was not analysed on the same day was stored at −80°C. The anaesthetised fish were sacrificed by decapitation.

2.3 *Development of a procedure*

The methodology described below is based on a protocol previously used for indirect measurement of VTG via ALP in clams and mussels [30] adapted for measurement on smaller carp fish. This original procedure involved blood sampling, separation of plasma, separation of plasma proteins, alkaline treatment of plasma proteins, and finally a spectrophotometric determination of phosphate. After initial rough testing with this method and a thorough literature review we identified several crucial steps in the procedure that needed to be validated and also steps that should be added for improvement. These new steps are included as steps 4 and 6 in Figure 1, where the other steps emanate from the original procedure.

The individual analytical steps illustrated in Figure 1 are further discussed in the text below and were examined by experiments steps 1–7.

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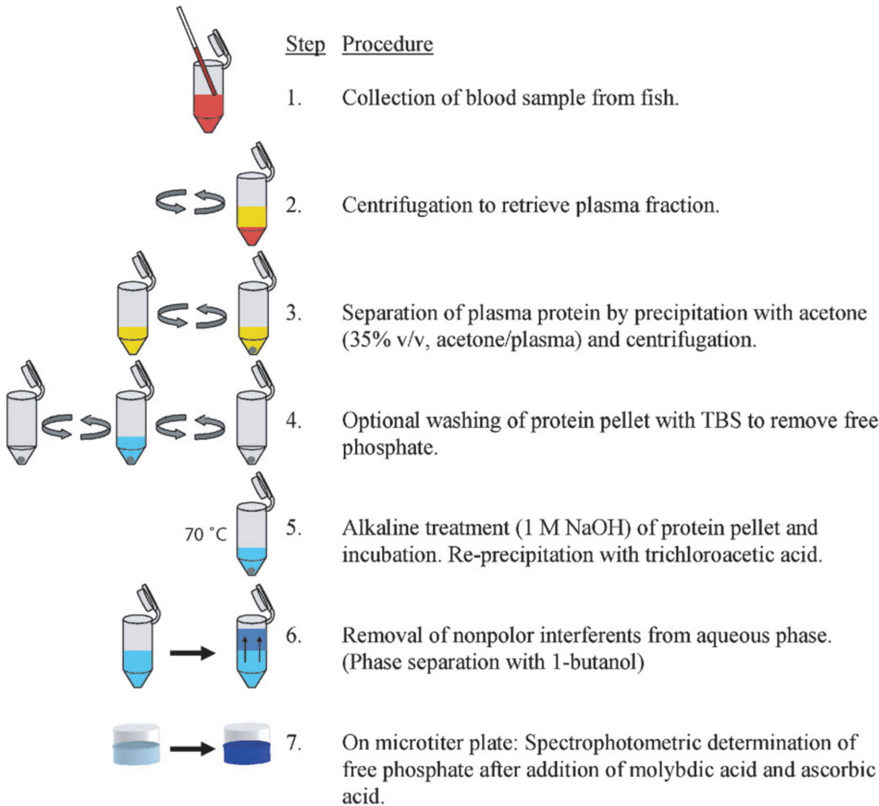

Figure 1. Scheme of ALP-analysis in samples of fish plasma.

Already at the point of blood sampling the methods reported in earlier publications are very diverse. Antiproteolytic agents has in some cases been added to the collected fish blood (25 µl aprotinin/ml blood in [10] and $10\,\mu l\,ml^{-1}$ in [6]), injected in fish prior to sampling (5 TIU aprotinin/~130 g individual in [31]) or in many cases not used at all. The blood samples have been stored on ice [6,8,32], shaken [33], left for clotting on ice [21], left for clotting in refrigerator [34] or left for clotting at room temperature [35].

There is no stringency in the centrifugation speed used to collect plasma. The lowest gravity reported was 1300 g [8] and the highest 10,000 g [33]. Tough centrifugation could lead to haemolysis and contamination of plasma with intracellular phosphorous from erythrocytes [7]. Spinning at gravity above 2000 g is not used in clinical laboratories to avoid haemolysis of human blood samples, since this could lead to overestimation of phosphate and potassium in plasma [36]. On the other hand, it might be possible that haemolysis leads to underestimation of ALP since blood contains iron. Ferric salts are said to retard the reduction of phosphomolybdic acid [37].

A percentage of 30–40% of acetone is claimed to precipitate VTG from eggs of rainbow trout [14]. An old but often cited procedure involves precipitation directly from blood with trichloroacetic acid (TCA) followed by washing in several steps with different solvents and solvent combinations [19]. Extraction of nonpolar lipoproteins with methyl-*t*-butyl-ether has been used as an alternative to precipitation of all proteins predominantly when working with hemolymph or tissue extracts from mussels and clams [30,38].

Washing of the protein pellet might serve as a mean to ensure that the removal of plasma is complete. This is important, since fish plasma itself contains from 40 to 256 µg/ml of free phosphate [39,40]. Repeated washing with different non-polar solvents will remove plasma lipids and lipids associated with the protein and if so wanted the combined solvent extract can be treated with strong acid for estimation of lipid phosphorous in fish plasma [22,41]. A washing procedure with a minimum number of washes and liquids is preferable in a straightforward ALP method.

Protein bound phosphate is released by dissolving the pellet in 1 M NaOH. Most protein bound phosphate is released already at 0.1 M NaOH and 30°C [42] and the process of alkali-induced elimination of phosphate from phosphoserine peptides is thought to be through β-elimination. Phospholipids have variable stability to alkaline treatment [26] and even if deacylated the liberation of free orthophosphate might still be minimal. As discussed below, the reduction of phosphomolybdate is very dependent on pH. If TCA is present in the final colorimetric reaction, standards will have to be compensated to the same acidity.

Fatty acids, originating from the plasma or the VTG macromolecule itself, can cause problematic emulsions or a finely dispersed precipitate during the spectrophotometric measurements. It has been suggested that calculation of corrected optical densities will compensate for the influence of non-Raylight scattering caused by these emulsions [28]. This mathematical procedure has been adopted for ALP measurement in some cases [13,16,17] and demands for additional reading at lower wavelength. Selective extraction of lipoproteins with ether, as mentioned in Section 3 above, may give less interference during spectrophotometric measurement in comparison to a methodology based on precipitation of all plasma proteins. However, the extraction approach may also give problems, since some phospholipids are probably co-extracted giving an extra addition of phosphate during the alkaline treatment. If lipids cause a problem during spectrophotometry these could be removed by introducing phase separation with an organic solvent when phosphate has been released into the aqueous solution.

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Colorimetric determination of phosphate by complex binding with molybdate and reduction of the phosphomolybdate complex is a well known method and gives a blue colour with absorbance maximum around 815–860 nm [28,43,44]. Ascorbic acid gives a selective reduction of the phosphomolybdate complex while leaving the molybdate substrate unreacted and colourless. However, selectivity is only achieved if working at certain pH intervals and pH between 0.9 and 2.8 must be avoided to rule out the reduction of molybdic acid [43]. For accurate quantification acidity must be identical in samples and standards. Besides pH, the reaction speed is influenced by the concentration of ascorbic acid, concentration of molybdic acid and temperature [44]. Altogether it is obvious that for a fast and reproducible colour development the colorimetric determination should be carefully standardised.

2.3.1 *Addition of antiproteolytic agent (step 1)*

The necessity of adding antiproteolytic agent (aprotinin) to blood samples was tested by pouring blood from 2 full hepatocrit tubes (75 µl) into an empty ice-cold centrifugation tube and blood from 2 hepatocrit tubes from the same fish into an ice-cold centrifugation tube containing 50 µl aprotinin (5–10 TIU/ml, Sigma-Aldrich, Sweden). Samples were centrifuged at 1200 g and 4°C for 10 minutes and a duplicate with 50 µl of supernatant was transferred to new centrifugation tubes from the aprotinin diluted plasma and a single volume of 50 µl from the undiluted plasma. ALP results from pure plasma and aprotinin diluted plasma were compared after compensating for dilution with aprotinin, using blood from three fishes.

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2.3.2 *Isolation of plasma through centrifugation (step 2)*

To investigate the influence of centrifugation speed on the measured ALP, blood from 4 fishes of various sex with aprotinin added was pooled, divided on 6 tubes and then spun at three different speeds at 4°C: 800 g 10 min, 3500 g 10 min, 10,000 g 1 min.

2.3.3 *Isolation of plasma proteins (step 3)*

Precipitating proteins directly from blood did not seem as an attractive alternative since plasma is more suitable for freeze storage than blood. Accordingly frozen and thawed plasma was used for all determinations.

To 100 µl plasma 54 µl acetone was added to generate a 35% (v/v) mixture. The tube was mixed by vortex, centrifuged at 10,000 g for 5 min and the supernatant discarded. In cases of further washing the pellet was centrifuged only at 5000 g to facilitate easier resuspension in washing buffer.

2.3.4 *Washing of plasma proteins (step 4)*

Plasma fluid contains free phosphate and it is therefore important to make sure that the precipitation with acetone and a subsequent removal of plasma supernatant will not leave traces of phosphate from the plasma. ALP was measured in triplicates of washed and unwashed pellet of human plasma to mimic a sample containing low levels of protein bound phosphate but yet high levels of free phosphate. For protein pellets originating from human plasma the influence of 0–3 washes with TBS or *Tris* was investigated. Washing with *Tris* and ethanol was also tested.

The washing procedure must be gentle to avoid loss of protein bound phosphate. To mimic a protein sample with high levels of bound phosphate bovine casein was used, since it has a high content of phosphoserine and phopshothreonine. ALP was measured in quadruplicates of 15–30 mg of unwashed protein powder and in protein that had been washed up to three times with TBS or *Tris*.

The washing procedure involved adding 300 µl of washing liquid and the pellet was dissolved by stirring and by vortex. The protein was again precipitated and isolated by addition of 162 µl of acetone, brief mixing by vortex, centrifugation and removal of supernatant. Last centrifugation before alkaline treatment was always at 10,000 g and otherwise at 5000 g.

2.3.5 *Alkaline treatment to release protein bound phosphate (step 5)*

100 µl of 1 M NaOH was added to tubes containing the pellets and the tubes were incubated at 70°C for 90 min and shaken intermittently. After incubation the proteins were again precipitated, this time with 40 µl of 100% trichloroacetic acid. The tubes were mixed by vortex and centrifuged at 20,000 g for 5 min and 105 µl of the supernatant was transferred to a new tube and diluted to 435 µl with distilled water. Duplicates of 145 µl were either directly added to two wells in the microtiter plate or the sample was first phase-separated according to step 6 below. To test the alkaline-stability of the most common phospholipid in fish [31] 500 µg of phosphatidylcholine (Sigma) dissolved in ethanol was transferred to a tube and evaporated to dryness. The phospholipids were then treated in the same way as a protein pellet.

2.3.6 *Phase separation with 1-butanol (step 6)*

During initial measurements based on the original method traces of a milk-like emulsion appeared in plasma samples, when molybdic acid was added. However, replacing the molybdic acid with pure H_2SO_4 at equal acidity did not generate emulsion. This indicates that the emulsion could be fatty acids binding to the molybdate. Centrifugation just before colorimetric reading was tested for removing the emulsion. However, this resulted in loss of phosphomolybdate in accordance with previous findings [28]. The disturbance was too big for calculation of scattering correction after measuring absorbance at two wavelengths as described by the same author. The introduced washing step improved the situation. Phase separation with 1-butanol was tested as a way to achieve totally clean samples for spectrophotometry. The procedure was evaluated for samples of human plasma containing exceptionally amounts of lipids as could be visually observed. To 435 µl of aqueous sample containing the released phosphate, an equal amount of 1-butanol was added. The centrifugation tube was mixed by vortex and the phases were separated by centrifugation at 20,000 g for 5 min. From the lower water phase two replicates of 145 µl were added to two wells in a microtiter plate. Since ascorbic acid is a selective reducer the background absorbance could be estimated by replacing ascorbic acid with water in one of the well duplicates.

1-butanol has a water solubility of 7.7% and this solubility is facilitated by TCA in the aqueous phase. By comparing standards, submitted to imitated sample treatment, with untreated standards mixed directly on the microtiter plate, it was investigated weather traces of butanol and TCA in the aqueous phase could influence the spectrophotometric measurement.

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2.3.7 *Optimisation of colorimetric determination (step 7)*

Since pH and concentration of molybdic acid are critical the influence of these parameters was investigated. As mentioned earlier pH must be below 0.9 or above 2.8 for a selective reduction of only phosphomolybdate. High ascorbic acid concentration at pH 2.8–3.5 accelerates the reaction rate but will give a continuous colour development [43], which may have an impact on the reproducibility. Hence, the lower window was chosen and the influence of acidity was further investigated at pH 0.25–1.0. This was accomplished by mixing 25 µl of standard or 1 M NaOH with different ratios of water and sulphuric acid in a series of plate wells, with subsequent addition of 50 µl of molybdate in slightly acidic solution and 50 µl of ascorbic acid. The pH in the different mixtures was estimated with CurTiPot software [45] suitable for simulations at high ionic strength. The final concentration of molybdic acid in the plate well was around 0.2%. Between 0.1 and 0.2% has been recommended elsewhere [43]. Different concentrations of ascorbic acid were tested.

When the conditions for colorimetric determination have been optimised, ascorbic acid will act as a highly selective reducer. It was tested whether one of the sample duplicates might serve as a measurement for non-phosphor related background absorbance by intentionally excluding the reducer in this well.

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3. Results and discussion

3.1 *Development of a procedure*

3.1.1 *Addition of antiproteolytic agent and stability of the protein*

Before comparing ALP analysis of a pure blood sample with one containing aprotinin the latter had to be compensated for dilution. By centrifugation of 5 different blood samples it was determined that the plasma fraction constitutes $52 \pm 1\%$ of a collected volume of blood. Hence, it was assumed that 150 µl blood contains 75 µl plasma and that 50 µl aprotinin will dilute plasma 5/3 times. For three fishes ALP was determined in pure fish blood and in blood with antiproteolytic agent added and the results are presented in Figure 2.

There was no dramatic effect of excluding aprotinin in the process of collecting fish blood prior to ALP analysis at least when blood was swiftly transferred to ice-chilled tubes. Aprotinin did not seem to increase phosphoproteins stability but instead it was noticed that adding aprotinin more often resulted in pink plasma samples after centrifugation, while plasma from pure blood were apparently more non-disturbed and displayed a yellow colour. Aprotinin has been used in ALP analysis as well as for immunological quantification of VTG to prevent the tryptic cleavage of VTG at arginine and lysine amino acids. It could be argued, that as long as no phosphatase enzymes are active and e.g., the phosphoserines are intact it is of minor importance if the protein is cleaved enzymatically. If any antiproteolytic agent should be used in combination with ALP, it is more tempting to suggest PMSF which is a serine protease inhibitor. Immunological methods depending on an intact binding site for an antibody are perhaps more sensitive to degradation/fragmentation of the VTG molecule than an indirect spectrophotometric method based on ALP. It has for example been shown that when using ELISA, VTG concentration appears to be lower when aprotinin has been added to samples [46]. Separation by anion-exchange chromatography also depends on the stability of the protein as secured by protease inhibitors and there are species differences in the

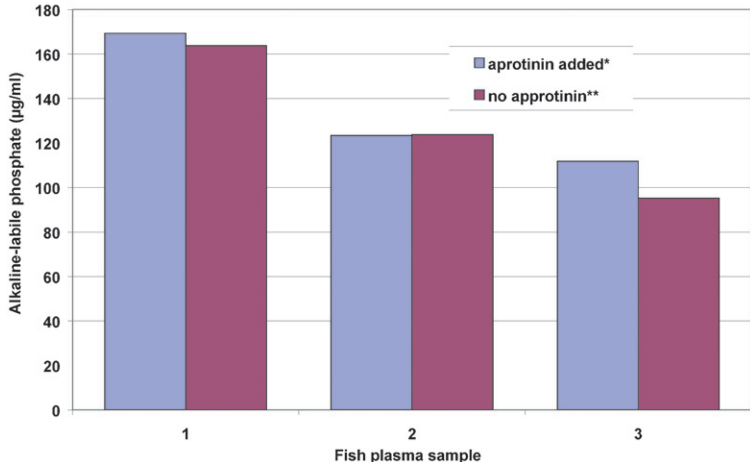

Figure 2. Influence of adding aprotinin before ALP determination.

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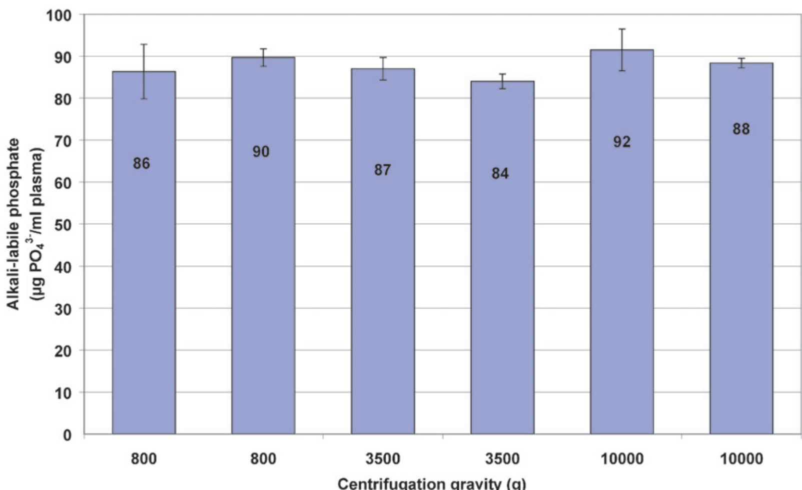

Figure 3. Influence of blood centrifugation speed before ALP determination.

stability of VTG [47]. In this context we think that ALP is a more robust method and we suggest a fast transfer of blood from sedated fish into ice-cold tubes without aprotinin and centrifugation within 2 hours.

3.1.2 *Isolation of plasma through centrifugation*

The influence of centrifugation speed, when separating plasma, on the determined ALP concentration in fish blood is shown in Figure 3.

To our surprise, centrifugation speed seemed to have no influence on the measured ALP (Figure 3). The fastest spin only lasted for 1 minute and spinning for longer time might have resulted in elevated ALP levels as a result of haemolysis. As mentioned above, centrifugation of blood at 1200 g will more often result in pink plasma when aprotinin has

been added and this could mean that pure blood is more tolerant for high centrifugation speed. Anyhow there is nothing to be gained from heavy centrifugation so we suggest that 800–1200 g for 10 minutes is sufficient.

3.1.3 *Isolation of plasma proteins*

Adding acetone to generate a 35% acetone/plasma solution was an effective way to precipitate plasma proteins. After centrifugation at 5000 g a relatively clear supernatant could be removed without disturbing the precipitated pellet. A complete precipitation without discriminating between proteins is feasible as long as the background level of protein phosphorylation is negligible as in fish plasma. For complete precipitation acetone is known to be a good alternative [14]. In cases, where high selectivity is searched for, extraction solvents like *t*-butyl methyl ether could be better. For example it can facilitate data interpretation by reducing the background when VTG is analysed by HPLC. It has been shown that 99% of VTG can be extracted from homogenised gonad samples of invertebrates in *Tris*-HCl buffer [48]. Whether this might be advantageous for determination of VTG in plasma from oviparous vertebrates remains to be investigated. One complication is that the recovery of fish VTG with *t*-butyl methyl ether might be less, since the presence of the phosvitin moiety gives the molecule more water soluble properties [49].

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3.1.4 *Washing of plasma protein*

Human plasma was used to mimic a sample with low levels of protein bound phosphate and relatively high level of free phosphate. There was no difference in using TBS or *Tris* as washing buffer (data not displayed). The effect of washing a pellet with plasma proteins with ethanol and/or *Tris* is displayed in Figure 4.

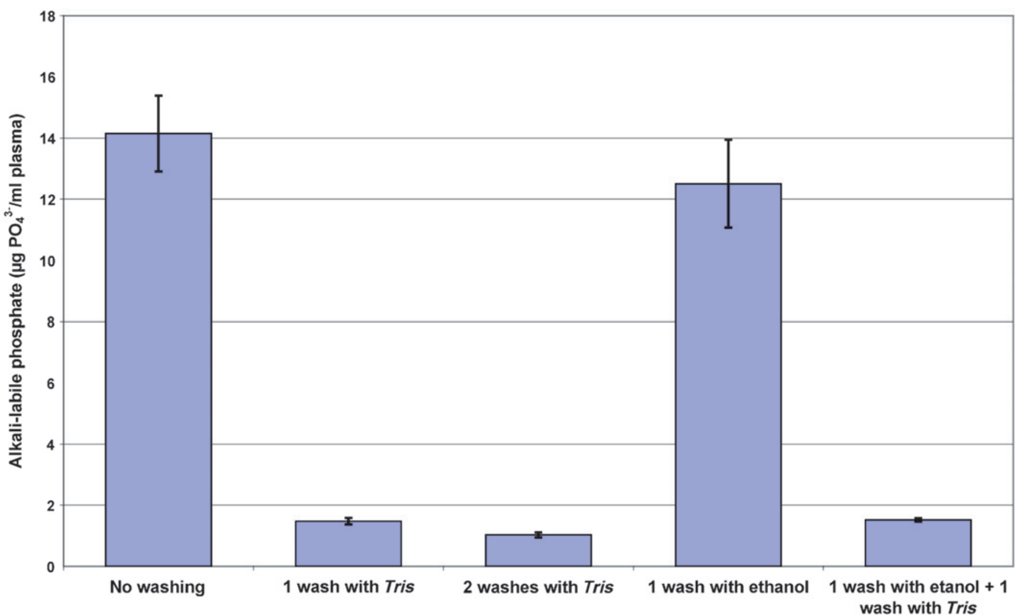

Figure 4. Influence of washing plasma protein pellet before ALP determination.

Higher standard deviation in the first column in Figure 4 indicates varying success in removal of plasma liquid. After one wash with *Tris* the measured ALP decreased with 90%. This illustrates that traces of plasma with substantially higher phosphate content than the protein pellet will disturb the measurement, if washing is not included in the protocol. After one or two washes with *Tris*, ALP was below detection limit ($<3.2\,\mu g$). When using a second washing with ethanol the protein pellet becomes pale and loses the yellowish colour while the ALP remains unchanged. The ethanol wash serves the purpose of removing phospholipids. Casein was used to mimic a protein sample rich in bound phosphate. The influence of washing on ALP measurement of casein is displayed in Figure 5.

ALP associated with bovine casein powder decreased with 10–20% after one wash and with 30% after two or three washes. The choice of TBS or *Tris* as washing buffer had no significant influence. The protein powder used was not of pure quality and thus might very well contain unbound phosphate. Phosphorylated serine and threonine, which are very abundant in casein, remain intact during the washing procedure. Figures 4 and 5 together indicate that two washes with *Tris* and ethanol is efficient to remove unwanted free phosphate while preserving the protein bound fraction until the alkaline treatment.

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3.1.5 *Alkaline treatment to release protein bound phosphate*

The alkaline-stability of 500 µg phosphatidylcholine was tested by subjecting it to the same treatment as the isolated plasma proteins. No phosphate was detected. The average molar weight for the standard was $M = 768$ which mean that total liberation of all phosphate would have generated 61 µg phosphate. Deacylation of the phospholipids are likely to occur but the phosphate ester bonds are perhaps protected by the polar solvent [26]. It has

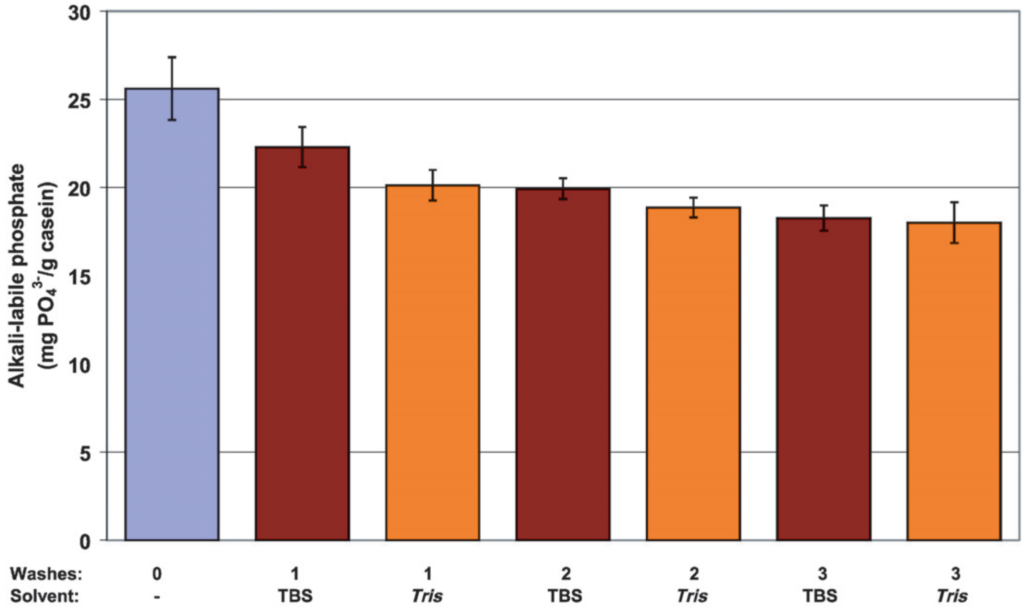

Figure 5. Influence of washing a pellet of casein protein before ALP determination.

also been shown elsewhere that when ALP is determined by alkaline treatment of an ether extract containing both VTG and phospholipids the protocol is not affected by the latter compound [17].

3.1.6 *Phase separation with 1-butanol*

The effect of introducing a phase separation step after the alkaline treatment of the protein pellet before spectrophotometry is illustrated in Figure 6.

In non phase-separated and phase-separated samples absorbance after 60 min was 0.172 ± 0.028 and 0.184 ± 0.10, respectively. The two values do not differ significantly on the 95% probability level. The lower average in non-treated samples was caused by a single lower value in one replicate. Treating with butanol markedly reduced the variability. The reducer, ascorbic acid, seemed to be very selective and if no reducer was added to phase-separated samples the absorbance was practically identical to reagent blanks (0.041 ± 0.001 vs. 0.037 ± 0.002). Excluding ascorbic acid in samples that had not been phase-separated again gave a higher variability and also higher background absorbance (0.076 ± 0.014). Traces of 1-butanol or TCA in the aqueous phase after phase separation do not influence the reduction of the phosphomolybdate complex or absorbance reading. Calibration curves prepared by standards in centrifugation tubes with subsequent simulated sample pre-treatment and phase separation were identical to calibration curves prepared directly on the microtiter plate.

In conclusion, these results suggest that phase separation with butanol eliminates interference and enables quantification without risking overestimation of phosphate. The separation step might be excluded from the protocol, if one corrects for non phosphate related absorbance by adding duplicate samples to the plate and excluding the reducer

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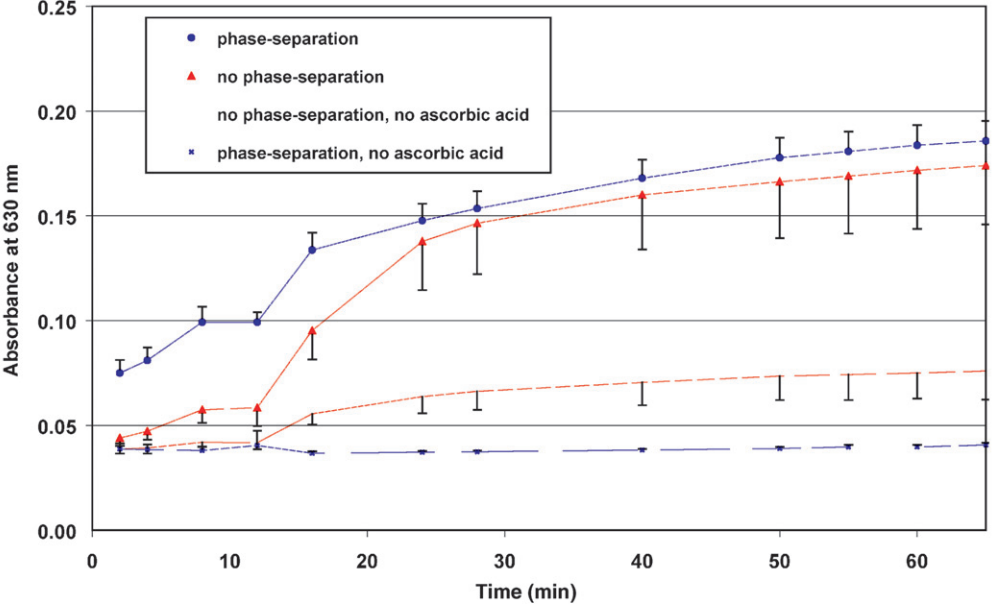

Figure 6. Spectrophotometric phosphate determination after phase separation.

in one of the wells. The correction will then be made by subtracting the absorbance value obtained with no reducer from the absorbance value of the sample. These slightly corrected absorbance values will avoid a small systematic overestimation of phosphate, since it seems that washing with *Tris* removes most but not all of the interfering compounds. Using phase separation or corrected absorbance values there is no need for measuring at two wavelengths as suggested elsewhere [28].

3.1.7 *Optimisation of the colorimetric determination*

3.1.7.1 *Acidity*. The final step in the ALP process is the reduction of phosphomolybdate complex by the addition of ascorbic acid. The influence of pH at this stage is shown in Figure 7 where the acidity in plate wells were varied while keeping other parameters fixed e.g. time for development, temperature, concentration of molybdate reagent and concentration of ascorbic acid.

Above pH 0.8 the reagent blank showed elevated absorbance which is in agreement with what has been reported earlier [43]. Between pH 0.53 and 0.89 absorbance was stable for a standard solution. Adding 50 µl of a molybdic acid solution prepared in 2 M H_2SO_4 will give pH 0.6 for the colour reaction in the plate well and this was chosen for all other experiments.

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3.1.7.2 *Reagent concentration*. The influence of ascorbic acid on the reaction rate for the phosphomolybdate complex in standard wells at pH 0.6 is shown in Figure 8.

The selectivity of the reducer was obvious as there were no difference between a standard well without ascorbic acid and a reagent blank. Reaction rate for the reduction of phosphomolybdate increased with increasing concentration of ascorbic acid. A higher concentration of ascorbic acid than used here could speed up the reaction even more but then there is a risk that colour development would continue over time [43]. We have chosen a concentration of ascorbic acid of 1%, incubation at 40°C and a development time of 60 min for the final method. These parameters will ensure good reproducibility in the spectrophotometric measurements.

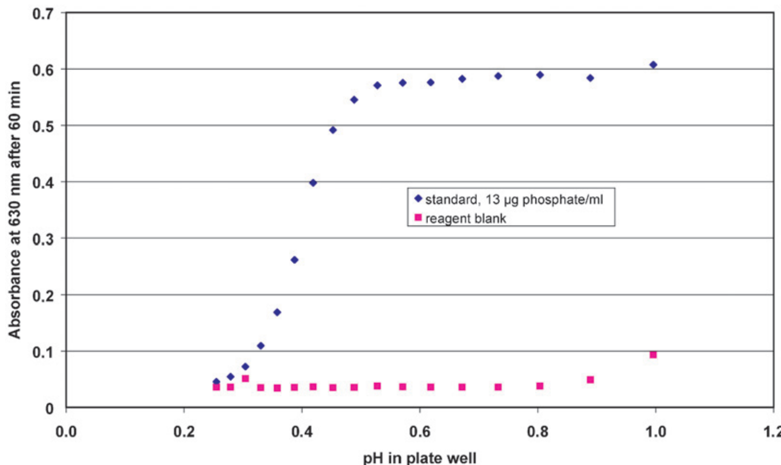

Figure 7. pH dependency for the reduction of phosphomolybdate.

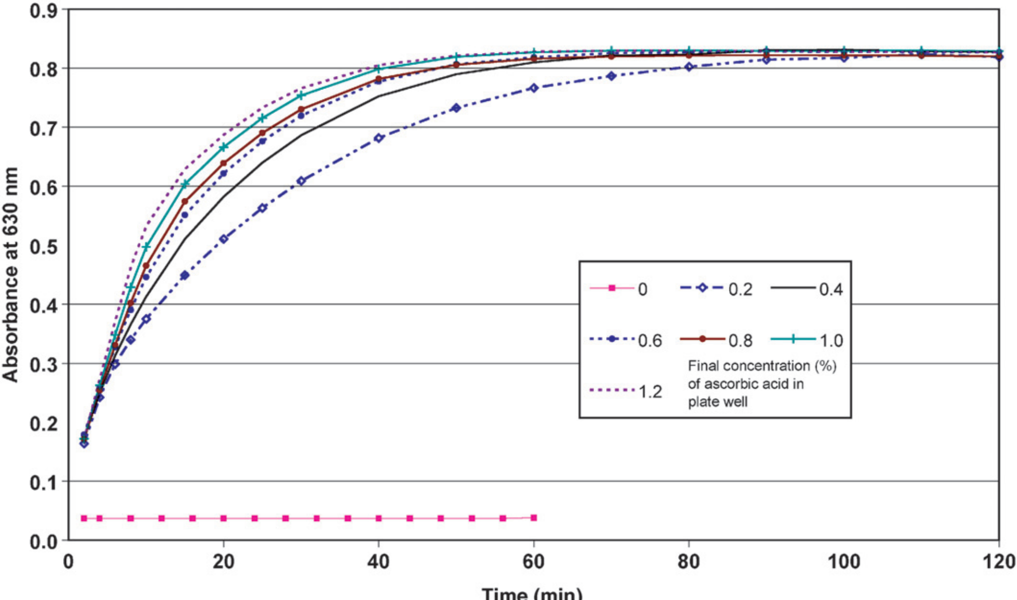

Figure 8. Influence of regent concentration on spectrophotometric phosphate determination.

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3.2 *Suggested final method*

3.2.1 *Sample treatment*

Collect blood and transfer swiftly to the bottom of ice-chilled tubes and centrifuge for 10 minutes at 800–1200 g within 2 hours. To 100 µl of plasma add 54 µl of acetone, mix by vortex, and centrifuge at 5000 g for 5 minutes. Carefully discard the supernatant to leave an intact pellet. Wash and dissolve the pellet by adding 300 µl of *Tris* and mixing by vortex, add 162 µl acetone, mix quickly by vortex, centrifuge for 5 min at 5000 g and discard the supernatant. Repeat the washing a second time with ethanol and finish by centrifuging at 10,000 g and discarding the supernatant.

For alkaline treatment add 100 µl of 1 M NaOH and mix by vortex. Incubate at 70°C for 90 min with interruption for mixing by vortex after 30 and 60 min.

Add 40 µl of 100% trichloroacetic acid and mix by vortex (In order to avoid the hazardous formation of chloroform the alkaline solution should be left to cool down to room temperature before adding trichloroacetic acid). Centrifuge at 20,000 g for 5 min and transfer 105 µl of the supernatant to a new tube and dilute to 435 µl with distilled water. Add approximately 435 µl of 1-butanol and mix by vortex. Centrifuge at 20,000 g for 5 min and carefully transfer two volumes of 145 µl from the lower aqueous phase to two plate wells. These two wells serve as either duplicates for the spectrophotometric measurement or with one well for measuring the background absorbance by not adding the reducer. The PO_4^{3-} concentration in the sample well as calculated with the aid of the calibration curve has to be multiplied by four to give the concentration in 100 µl of plasma, given that the exact volumes in the protocol above are used. The results should generally be presented as µg of PO_4^{3-} per ml of plasma. However, in cases where the levels of VTG do not exceed a few % of the total protein concentration normalisation against the protein

content can be recommended to compensate for fluctuations in biomass, as previously discussed by Matozzo *et al.* [49]. However, with high levels of VTG concentration in plasma big changes in blood concentration are masked by the normalisation procedure, which then makes this procedure less appropriate.

3.2.2 *Preparation of calibration curve*

For a standard calibration curve duplicate wells at each concentration were prepared by adding 50 µl of 127 µg/ml PO_4^{3-} standard in 1 M NaOH and 240 µl water to the first plate well in each of two rows. Add 25 µl 1 M NaOH and 120 µl water to the remaining wells in these rows. Dilute stepwise by mixing and transferring 145 µl from the first well to the next and so forth giving a series of 7 solutions with a dilution factor of two. Two wells containing only 25 µl of 1 M NaOH and 120 µl water serve as reagent blanks.

3.2.3 *Colour development*

For colour development add 50 µl of 1% molybdate reagent in 2 M H_2SO_4 to all wells. Add 50 µl of 5% ascorbic acid to all standards. Add ascorbic acid to either both of the sample duplicates or only to one while adding 50 µl of water to the other duplicate for measurement of the background absorbance. Shake plate for 60 seconds and incubate plate at 40°C for 60 min and read absorbance at 630 nm.

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3.3 *Method validation*

The intra-assay variation was determined by repeated analysis of pooled plasma. In four replicates of 100 µl of plasma, that had been treated with phase separation before the spectrophotometric measurement, ALP was 24.4 ± 1.6 µg PO_4^{3-}/ml plasma. For the alternative quantification method, without phase separation and with correction for background absorbance, ALP was 23.8 ± 1.6 µg PO_4^{3-}/ml plasma. Thus, the variation was very similar independently on what strategy that was used. Standard deviation was lower than in a study with hormone injected male Crucian carp where ALP was 68.8 ± 4.2 µg PO_4^{3-}/ml [33]. (The variation was 6.1% for repeated analysis of 50 µl of pooled plasma and 68.8 µg was the mean individual concentration in non-pooled plasma from the same group.) In our method the limit of detection, calculated as two times the standard deviation was 3.2 µg PO_4^{3-}/ml plasma. This is six times better than earlier published limit of detection [8] and limit of quantification [21]. The instrumental limit of detection for the spectrophotometric measurement, calculated as three times the standard deviation of the reagent blanks, was 0.4 µg PO_4^{3-}/ml.

4. General discussion

Indirect quantification of a biomarker protein by measuring protein bound phosphate is depending on that there are no other non-VTG phosphoproteins present at significant concentrations in plasma. In the African clawed toad *Xenopus laevis* VTG is said to account for all ALP in serum [19] and it was boldly stated that 'VTG is the only phosphorous containing protein in the blood of oviparous vertebrates' [6]. It has also been shown that the fraction containing VTG is the only eluent fraction giving measurable concentrations of ALP, when serum samples have been separated by gel filtration [7,31,50,51].

For ALP to be a useful biomarker for endocrine disruption it should be sufficient with a method that can detect a significant deviation from the normal background level of protein bound phosphate. We have lowered the detection limit six times and background levels are easy to measure if they are as high as 46.3 or 340 µg PO_4^{3-}/ml as was reported for male specimens of Crucian carp [33] and fathead minnow [8], respectively. When the background concentration of ALP in 19 species of supposedly unexposed fish was measured ALP was never below our detection limit of 3.2 µg PO_4^{3-}/ml plasma [41]. Access to instrumentation capable of absorbance reading at longer wavelength, then in this study, would slightly improve the sensitivity. Further improvement is likely, if malachite green is added to the molybdic acid solution [52,53]. This is an approach which will be further investigated. Favourably, the same pH window between 0.4 and 0.9, used in the present study, is suitable when using molybdic acid in combination with malachite green [54]. We have promising results indicating that by doing this we will be able to additionally lower the detection limit by one order of magnitude.

A fluorescence method for quantification of ALP was recently presented with an extraordinary low detection limit of 5 ng/ml, which was 2000 times more sensitive than the lowest result presented for fish plasma in the same study [55]. However, no studies on matrix influences at these low concentrations were presented but only an instrumental detection limit for the final quantification of inorganic phosphate. However, a method with very low detection limit is not an advantage if it only detects more of the non-VTG protein bound phosphate. From the study cited above it could be concluded that in 17β-estradiol injected males and females the ALP always constituted $2 \pm 1\%$ (w/w) of the VTG quantified with ELISA [55]. This ratio should be consistent also when measuring low concentrations of VTG. However, in blood from control male and female fish ALP constituted 293% and 64%, respectively.

The key to reliable results at low ALP concentrations seems to be the separation of free and bound phosphate. In adult rainbow trout the amount of protein bound phosphate in plasma has been reported to be 9–18 µg PO_4^{3-}/ml [41] while the level of free phosphate in plasma in the same species is 256 µg/ml [40]. Here the washing of the protein pellet surely is of importance. Generally, this problem has not been discussed in earlier publications. An increase of ALP as a result of higher VTG levels is of course harder to detect if the used method has an ever present high interference from non-VTG phosphate. The influence of washing procedures has not been studied previously as detailed as in this study. An old and often cited sample treatment involves washing with several different solvents and solvent mixtures including the hazardous chloroform [19]. In our method washing with *Tris* and ethanol generates a protein pellet free of co-extracted phosphate. Phosphatidylcholine and similar phospholipids are soluble in ethanol and hence removed by the second washing step. If small amounts remain in the protein fraction this is not a problem since they were shown not to be alkali-labile in the sense of not generating free phosphate.

When measuring ALP it is important to prevent premature release of the protein-bound phosphate until the plasma proteins have been isolated by precipitation and the alkaline solution added. The effect of freezing and thawing of samples was not investigated but it can be expected that spectrophotometric determination of ALP is less affected as compared to VTG quantification with anti-bodies. ELISA gave higher results when VTG was quantified after freezing [46].

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In this study we have not investigated the precise correlation between ALP and VTG, as measured with e.g. ELISA, in the model fish Crucian carp. These correlations, some of which are referred to in the introduction, demonstrate the biological relevance of the ALP approximation. However, because of the between species variation in protein phosphorylation the extrapolation of ALP levels to those of VTG is problematic. This does not diminish the value of ALP as a biomarker but is a remainder that the extrapolation is uncertain, if studies based upon different endpoints are compared. The matter may be even more complicated since it has been reported that pollution exposure may lead to increased phosphorylation of egg yolk proteins. In a study with clam (*Mya arenaria*), exposure to municipal effluent resulted in increased levels of ALP in isolated egg yolk vitellin from gonads but this increase was not associated with an increase in VTG concentration [15]. Increased phosphorylation of VTG has not been shown for fish. This may depend on that in oviparous vertebrates the VTG production mainly takes place in the liver in contrast to the situation in clam. Detailed studies of the post-translational modifications have shown that phosphorylation is completed before VTG is excreted into the bloodstream [56]. The liver cells of fish are both producers of VTG and target cells for contaminants and it would be interesting to study if post-translational modifications of VTG in fish might be altered in the presence of contaminants.

However, even when immunoassay based methods for quantification of VTG are used problems arise. Species variability in affinity causes a problem when anti-bodies for one species and VTG-standard from the same species are used for estimation of VTG concentration in another species. This is due to the fact that the anti-bodies are species-specific and cross-reactivity is somewhat unpredictable [57,58]. Not all laboratories have the possibility to develop their own species-specific antibodies or standards to be used for a certain fish of interest. There are commercial kits, with good reproducibility even at low concentrations, available even though often expensive and with a limited storage time. As stated elsewhere, when choosing the best method to measure VTG levels one needs to take into account feasibility, sensitivity, reproducibility and cost-effectiveness [2]. Simple, universal and robust laboratory procedures are preferable to advanced and specific methods, if VTG is to be used as a cost efficient environmental biomarker for endocrine disruption in a number of different species. The indirect ALP method offers a big cost reduction. A commercial anti-body based test kit approximated for 50 samples is circa 1800 euro (35 euro/sample). During our method development we spent circa 350 euro on chemicals and plastic consumer goods that would cover the need for the analysis of 1000 samples at a cost of circa 0.35 euro/sample.

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5. Conclusions

Cheapness and versatility has drawn attention to the indirect quantification of VTG via ALP but there has been less interest in improvement and validation of the ALP methodology. The method has previously been correlated to direct immunological measurements of VTG but there has been no critical assessment of the pre-treatment procedures used for plasma samples. The spectrophotometric determination of inorganic phosphate is an old, generally accepted and simple technique. However, said so one should not overlook the 90 years of accumulative knowledge of its limitations such as sensitivity for pH and reagent concentrations.

Here we present a validated method for ALP determination with the following improvements:

- Lower detection limit and less variability.
- A simple and straightforward way of isolating the plasma proteins by precipitation with acetone and two washing steps. Older methods involve precipitation with diluted trichloroacetic acid followed by numerous washing steps with (hazardous-) organic solvents.
- The addition of antiproteolytic agent can be omitted.
- All matrix interference has been completely removed by the introduction of a phase separation step and there is no need for reading at two wavelengths and calculating corrected absorbance values.
- (The selectivity of the reducer ascorbic acid has been utilised in an inventive approach to enable quantification of background interference in the spectrophotometry as an alternative to phase separation or to be used as an extra precaution if so wanted.)
- A high sample throughput is now possible after scaling down sample and reagent volumes from the original method to fit in a 96 well microtiter plate.

Immunological methods for quantification of low levels of VTG will probably still be asked for in research related to endocrinology and fish physiology and it is not the author's intention to discredit them. When trying to assess the ecological impact of endocrine disruption knowledge is limited by the number of samples that can be afforded. Indirect quantification of VTG through ALP is a strong alternative that should facilitate large scale environmental monitoring at many locations and in many fish species. A cost effective method might also encourage small and less equipped laboratories to incorporate measurement of the well known biomarker into their environmental programmes.

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