Original Communication

Use of an experimental design to optimise the saponification reaction and the quantification of vitamins A_1 and A_2 in whole fish

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Abstract: In ASEAN countries, small freshwater fish species contribute to the nutritional needs of people with few livelihoods by providing them with significant amounts of protein, fat, vitamins and minerals. Some species are eaten whole (with their organs, skin, bones, head and eyes). To estimate the vitamin A content of these foods, conventional saponification has been applied but has not been able to fully release the retinol. Our objective was to optimise the conditions of vitamin A saponification in whole fish to have a reliable estimate of their contribution to intakes. The effects of temperature and saponification time on the retinol quantification of whole fish were evaluated using a two-factor experimental design. Reaction time had a significant effect on the saponification of standard retinyl palmitate and whole fish ($p \leq 0.05$). For whole fish, the best conditions for the saponification were to heat the samples to 80 °C for 43 minutes. Under these conditions, the retinol is well liberated from the matrix and protected from degradation and isomerisation reactions. The time-temperature couple used is more intense than that recommended for quantifying vitamin A in milk or enriched margarines. The protective effect of the food matrix against the release of retinol is evident. Vitamin A_2 alcohol (3,4-didehydroretinol) was detected in five species and the overall vitamin A contents ranged from 9.6 to 737.5 μg RE/100 g in species frequently consumed in Cambodia. The two species of small fish consumed whole were the ones that contained significantly more vitamin A among the ten tested ($p \leq 0.05$). *Highlights:* Vitamin A_2 alcohol was quantified in five fish species. The official saponification partially released retinol in whole fish. The optimised reaction required heating the sample to 80 °C for 43 min.

Keywords: Matrix effect, small fresh water fish, temperature, time, response surface methodology

Introduction

Promoting dietary diversification is one of the sustainable solutions to prevent micronutrient deficiencies. A balanced diet provides the necessary amounts of vitamins and minerals for the proper functioning of the body [1]. Vitamin A is essential for growth, brain development, vision and immunity [2]. Populations who do not have regular access to a balanced and healthy diet can develop vitamin A deficiency [3]. The most affected groups are located in developing countries and include infants, young children, women of childbearing age and lactating mothers [4].

Several animal products (fish, offal, red meat) contain preformed vitamin A (retinol or vitamin A_1 alcohol, 3,4-didehydroretinol or vitamin A_2 alcohol, retinyl palmitate, retinoic acid) while coloured plant products may provide provitamin A as β-carotene and α-carotene [2]. Retinyl esters are highly bioavailable [5] and they accumulate in most animal livers. The structure of 3,4-didehydroretinol is closed to retinol, exception of extra double bond on 3 and 4 position of its β-ionone ring [6]. In fish (particularly fatty fish), retinyl esters are accumulated in offal (liver, intestine, kidney and heart) and, to a lesser extent, in eyes and muscle [7]. Retinyl palmitate is the main ester identified in animal products but linoleate or retinyl stearate may also be present [5, 8]. Identifying and quantifying vitamin A_2 is important because its biological activity is high (i.e. 120% of that of vitamin A_1) and its content in small fish may be higher than that of vitamin A_1 or even exceed the latter by 100% in some species [6]. Consumption of fish containing these vitamins should be included in the dietary strategy to contribute to the fight against vitamin A deficiency.

Table 1. The distinct saponification procedures of food and biological samples

Type of samples	Conditions	References
Pork, veal, beef liver, freshwater fish	60% of KOH under nitrogen Ambient temperature for 15 min	[15–17]
Powdered milk	50% of KOH	[12]
	70 °C for 7 min	
Fortified margarine	50% of KOH	[10]
	82 °C for 25 min	
Infant formula	Ambient temperature for 18 hours	[19]
Milk	70 °C for 20 min	[21]
Liver and liver products	Ethanolic KOH	[18]
	Ambient temperature for one night	
High-fat foods	60% of KOH	[12]
	70 °C for 30 min	
Meat	10.5 M KOH	[4, 9]
Multi-ingredient sauces	70 °C for 25 min	
Mongolian gerbil liver	Ethanolic KOH 5% and 0.1% BHT	[20]
	60 °C for 10 min	
Human milk	50% of KOH	[22, 23]
	50 °C for 60 min	

KOH: potassium hydroxide; BHT: butylated hydroxytoluene.

Depending on the structure and composition of food matrices, fat-soluble vitamins are more or less well released during extraction and digestion [9–11]. In fatty samples, saponification is used to release retinyl esters and retinol bound to the complex network of proteins, lipids and carbohydrates [12, 13]. In the eyes of humans and animals, retinal is chemically bound to the photoreceptor protein of the retina and it plays an essential role in retina and eyes development [14].

However, saponification procedures introduce large variations in the quantities of vitamins because they are degraded during the reaction and their recovery is not complete. Since saponification is a chemical reaction, it can be optimized by varying the temperature, time or the concentration of alkaline agent. Potassium hydroxide (KOH) is the most commonly used alkaline agent [12, 13]. The official protocol (AOAC 992.06) recommends saponification at 70 °C for 25 min [9]. Different saponification protocols have been proposed to take into account the complexity of food matrices such as fortified margarine [10], other food and meat [4, 9, 12, 15–17] and biological samples [18–23] (Table 1). The temperature varies from room temperature to 82 °C and the reaction can last from a few minutes to 18 hours. The higher the saponification temperature, the shorter the saponification time. The temperature can be increased taking care not to exceed 80 °C as retinyl esters and retinol can isomerize above this temperature [24]. Indeed, retinoids are sensitive to various factors such as temperature, oxygen, light and traces of certain metals [5, 8]. Several types of degradation reactions can take place and they are activated by the increase in temperature. It is therefore important to optimize the vitamin A assessment by finding the ideal conditions to satisfy contradictory objectives: namely, to saponify under conditions drastic enough to release compounds bound to the food matrix while protecting them from degradation reactions activated by temperature.

In this type of situation, experimental designs are useful to optimize the output variables according to two or three factors. Response Surface Methodology (RSM) is a statistical model used to optimize analytical methods or agri-food processes [25]. It reduces the total number of experiments and, consequently, reduces time, labour and expenditure on reagents and consumables [3, 25–29]. In addition, experimental design allows to understand the impact of factors (independent variables) on responses (dependent variables) [13, 26–29]. A second-order polynomial equation describes the individual impact of each factor on responses and the synergy or antagonism of factors [3, 25, 27].

The objective of this study was to develop an optimized saponification protocol of retinoids contained in fish eaten whole by consumers. A two-factor Central Composite experimental design was tested to assess the impact of temperature and reaction time on estimated vitamin A levels. The optimized protocol was then applied to the ten most consumed fish species in the Tonle Sap Lake region (Cambodia).

Table 2. Local and scientific names, edible parts and vitamin A of the ten freshwater fish species

Local name	Scientific name	Retinol (µg RE/100 g)	3,4-didehydroretinol (µg/100 g)	Retinol equivalent (µg RE/100 g)
Big-size fish species (fillet)				
Trey chhkok	*Cyclocheilichthys enoplos*	19.3±1.1^{de}	–	19.3±1.1^{e}
Trey chhpin	*Barbodes gonionotus*	13.2±0.8^{ef}	–	13.2±0.8^{e}
Trey chrakaing	*Puntioplites proctozysron*	22.1±2.0^{de}	26.6±2.9^{cd}	54.0±3.3^{d}
Trey diep	*Channa micropeltes*	10.1±0.3^{f}	–	10.1±0.3^{e}
Trey phtouk	*Channa striata*	13.8±0.6^{ef}	–	13.8±0.6^{e}
Trey promah	*Boesemania microlepis*	9.6±1.0^{f}	–	9.6±1.0^{e}
Whole body small fish species (without organs)				
Trey kanchos	*Mystus atrifasciantus*	20.1±2.1^{de}	15.3±1.5^{d}	38.5±3.0^{d}
Trey kawmpleanh	*Trichogaster microlepis*	33.1±2.1^{c}	36.0±6.2^{c}	76.3±9.2^{c}
Whole body small fish species (with organs)				
Trey bawndol ampeou	*Clupeoides borneensis*	160.5±6.8^{b}	180.7±4.3^{b}	377.3±2.0^{b}
Trey riel	*Henicorhynchus siamensis*	422.8±1.9^{a}	262.3±11.2^{a}	737.5±15.2^{a}

Data are presented as the mean±standard deviation with three repetitions.
Different letters in the same row mean significant difference ($p<0.05$) by Tukey's test throughout the different species.
µg RE/100 g: µg retinol equivalent per 100 g of fresh weight.

Materials and methods

Chemicals

Solvents, reagents and pure chemicals (retinyl palmitate and retinol) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). 3,4-didehydroretinol was obtained from Santa Cruz biotechnology (Texas, United States) and polytetrafluoroethylene (PTFE) membranes from Sartorius (Palaiseau, France).

Fish sampling

The ten most available and consumed fish species were identified in a previous survey (unpublished data). The local and scientific names of the fish species are given in Table 2 as well as their metric scale are given in Figure 1. Six species belong to big-size ones (*Cyclocheilichthys enoplos*, *Barbodes gonionotus*, *Puntioplites proctozysron*, *Channa micropeltes*, *Channa striata* and *Boesemania microlepis*) and four belong to small-ones (*Mystus atrifasciantus*, *Trichogaster microlepis*, *Clupeoides borneensis* and *Henicorhynchus siamensis*).

Fishes were bought directly from the fishermen during the fishing hours in Kompong Chhnang near the Tonle Sap Lake (TSL) in Cambodia (12°30′55.03″ N, 104°27′28.29″ E; 12°30′37.46″ N, 104°26′54.69″ E and 12°30′44.91″ N, 104°27′10.74″ E) (see Electronic Supplementary Material 1). Fish collection was done three times during the dry season (from December 2018 to February 2019). Approximately six kilograms of each fish species were collected. The fishes were placed in a Ziploc bag to prevent any contamination and immediately stored under ice in a polystyrene box until arrival at the laboratory. Samples were prepared from edible parts of fishes as follows: only fillet for big-size species, the whole body for some small-size species and without organs for other small-size species (Table 2). The edible parts were cut into small pieces and chopped with a meat grinder (HR-12, China). Samples were stored at −20 °C for retinoid assessments.

Retinoid saponification with an official method

The saponification was realized in an amber flask heated with an electronic temperature controller Heidolph EKT 3001 temperature (Reax 2, Heidolph, Germany) connected to a magnetic stirring hotplate Bioblock Scientific 94301 (Bioblock, France). The temperature precision was ±0.1 °C. Saponifications have been carried out according to the official method [9] with retinyl palmitate (100 mg in 1 L of acetone) or whole fish (*Henicorhynchus siamensis)* as follows:

- 1mL of retinyl palmitate standard was saponified for 25 min at 70 °C with 1.25 mL of 10.5 M KOH
- 1 g of whole freshwater fish was saponified for 25 min at 70 °C with 1.25 mL of 10.5 M KOH

Afterward, the samples were cooled for 30 min and retinol was extracted by 15 mL of ethanol/hexane (4:3, v/v). After centrifugation at temperature 4 °C for 15 min at 13 000 rpm (Heraeus Multifuge X1R, Thermo Fisher Scientific, Villebon sur Yvette, France), the upper layer was

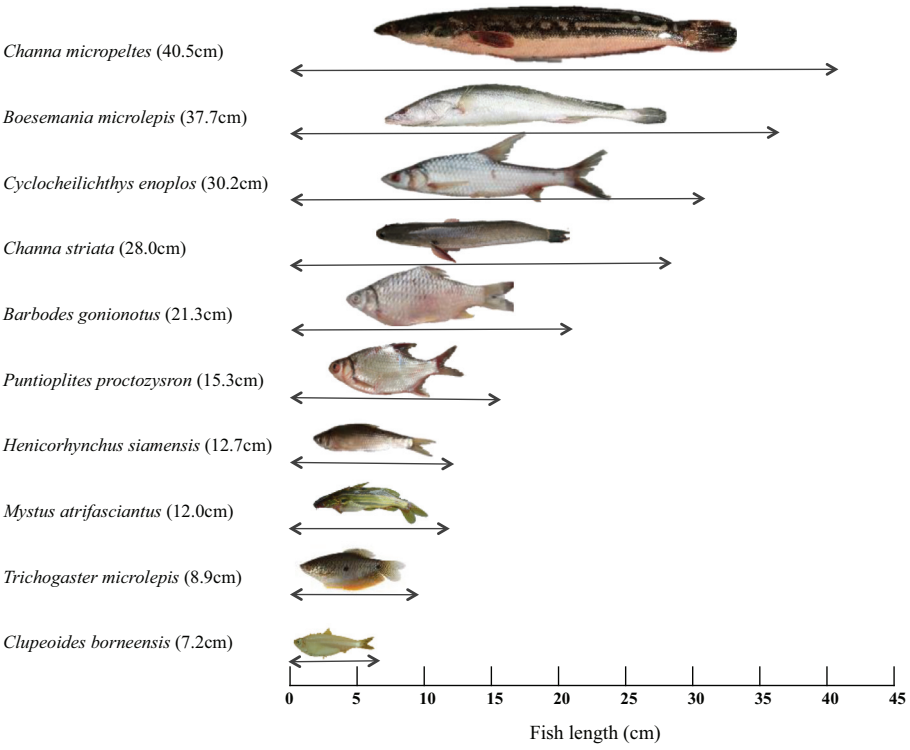

Figure 1. Picture of freshwater fish species with a metric scale.

Table 3. The experimental area in terms of temperature and heating time for saponification of retinyl palmitate standard and retinyl esters in freshwater fish

	Retinyl palmitate		Freshwater fish	
Domain	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
Minimum (−1)[a]	50.0	5.0	60.0	10.0
Central point (0)[a]	67.5	17.5	80.0	35.0
Maximum (+1)[a]	85.0	30.0	100.0	60.0

[a]Coded value.

transferred into amber glass tubes and dried under vacuum at ambient temperature for 30 min (Genevac LTD, EZ-2 series, Sp Scientific, England). The dried residues were dissolved in 1 mL of acetone and filtered with a 0.45 µm PTFE minisart SRP4 membrane.

Optimization of retinoid saponification with an experimental design

A central composite design with response surface methodology (RSM) by Box and Wilson was used to determine the effects of temperature (°C) and time (min) on the saponification reaction. This design requires an experiment number according to $N=K^2+2K+Cp$, where K is the factor number and Cp is the replicate number of the central point [3, 25, 27].

Preliminary tests have been carried out to define the boundaries of the experimental design tested (Table 3). For retinyl palmitate saponification, the temperature ranged from 50 to 85 °C and the time from 5 to 30 min. For whole freshwater fish, the temperature ranged from 60 to 100 °C over a period of 10 to 60 min.

Saponifications have been carried out with nine time-temperature pairs on retinyl palmitate or whole fish (*Henicorhynchus siamensis)* (Table 3). Three replicates were performed at the central point of the design to calculate the experimental variability.

- 1 ml of retinyl palmitate (100 mg/L) was saponified with 4 mL of KOH 50% (w/v) at different times and temperatures
- 1 g of whole freshwater fish was saponified with 4 mL of KOH 50% (w/v) at different times and temperatures

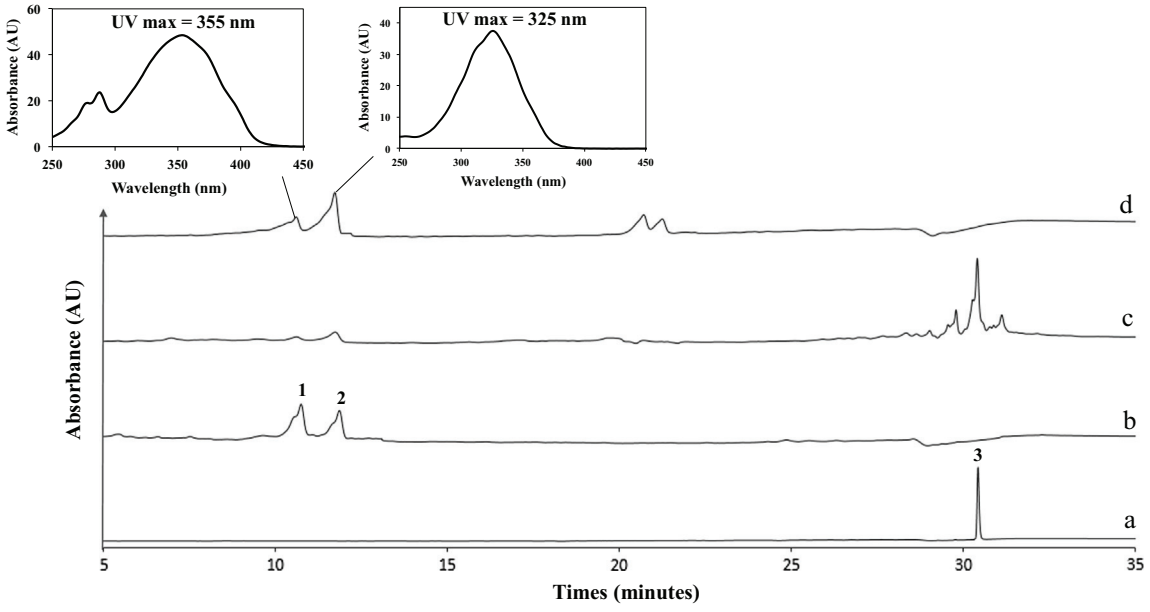

Figure 2. Chromatograms recorded at 325 nm of (a) retinyl palmitate standard, (b) 3,4-didehydroretinol and retinol standards, (c) *Henicorhynchus siamensis* sample saponified with official method and (d) *Henicorhynchus siamensis* sample saponified with the optimized method. (1) 3,4-didehydroretinol (UV max=355 nm), (2) retinol (UV max=325 nm), (3) retinyl palmitate.

To assess the effect of independent variables on the responses, RSM was applied. The basic second-order polynomial model used to fit the data was (equation 1):

$$Y = \beta_o + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{i-j} \beta_{ij} X_i X_j \quad (1)$$

where Y is a response (retinol content), X_i is the factor i [Temperature (i=1), Time (i=2)]. β_O is the constant of the model, β_i is the linear effect of X_i, β_{ii} is the quadratic effect of X_i and β_{ij} is the effect of the interaction between X_i and X_j. Contour plot and response surface plot are created from fitted polynomial equations in order to visualise the effect and interaction of factors on the response, especially to find out the optimum conditions [3]. Correlations were established with the second-order polynomial model (equation 1).

The optimised protocol was applied to the ten species of fish regularly consumed by the populations living in the floating villages of the Tonle Sap Lake and the analyses were repeated three times for each species.

High-performance liquid chromatography (HPLC)

Retinyl esters, retinol and 3,4-didehydroretinol were separated on a polymeric C_{30} (4.6 mm i.d.×250 mm, 5 µm particle size, YMC, Inc Wilmington NC) and quantified by HPLC (Agilent System 1200 series, Massy, France). The mobile phase consisted of a mix of eluent A (60:40, methanol: milli-Q water), eluent B (67.5:28.5:4, ethyl acetate:methanol:milli-Q water) and eluent C (ethyl acetate) with a flow rate 1 mL/min. Chromatograms were recorded by a UV-visible photodiode array detector at 325 nm and the distinct forms of retinyl palmitate, retinol, 3,4-didehydroretinol were clearly separated (Figure 2). External calibration was realized weekly with stock standard solutions of the pure chemical in acetone on the range of 0.5 to 20.0 mg/L. The retinol equivalent (RE) was expressed per 100 g of fresh fish as the sum of the biological activities of 100% for all-trans-retinol and 120% for 3,4-didehydroretinol [30].

Data analysis

The experiments were conducted in random order and Statgraphics plus 5.1 (STSC, Inc., USA, 1991) was used to calculate the regression coefficients based on the method of least squares (Draper and Smith 1981). The validity of the regressions and coefficients was verified by statistical tests (R^2 correlation coefficient, analysis of variance, Fisher's test). Significance was accepted at probability $p \leq 0.05$. The response surfaces and contour plots were constructed using the fitted quadratic polynomial equation obtained from the regression analysis corresponding to the fixed point and the change in the other two variables. Optimal reaction conditions result in the highest Y-response, i.e. the highest vitamin A content in whole fish.

Results

Retinoid saponification with an official method

Conventional saponification protocol applied to retinyl palmitate standard and a freshwater fish species consumed whole (i.e. with the head, skin, bones, organs such as liver and eyes) (Figure 2). The name of this specie is *Henicorhynchus siamensis* (Trey Riel) and according to its lipid content, it is a high fat fish [31, 32]. Applying standard saponification conditions (i.e. 70 °C for 25 min), retinyl palmitate was fully hydrolysed. However, when this protocol was applied to whole crushed fish, part of the retinyl palmitate was still visible on the chromatograms after saponification. The reaction only partially released the retinol because of the matrix effect, that is why we conducted this optimisation study for whole fish.

Optimisation of retinol quantification by experimental design

Retinol concentrations after saponification of retinyl palmitate and freshwater fish ranged from 7.9 to 65.9 mg/L and 1.6 to 11.9 mg/L, respectively, while 3,4-didehydroretinol concentrations after saponification of freshwater fish were lower (0.4 to 3.3 mg/L) (Table E1). The iso-responses curves clearly showed an optimum for the variables temperature and time of the saponification reaction (Figure 3). If the temperature or saponification times were too low or too high, then the vitamin A content was underestimated.

By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was established to express the retinol content according to X_i is [Temperature (i=1), Time (i=2)], and their linear and quadratic effects and interactions.

The second-order polynomial equation of retinyl palmitate standard according to the saponification parameters was:

$$Y_{[\text{retinol}]} = -8110.21 + 241.34X_1 + 135.26X_2 - 1.72X_1^2 - 0.82X_1X_2 - 1.61X_2^2 \quad (2)$$

The second-order polynomial equation of retinol and 3,4-didehydroretinol in whole body freshwater according to the saponification parameters was:

$$Y_{[\text{retinol}]} = -1088.45 + 28.44X_1 + 8.77X_2 - 0.19X_1^2 + 0.02X_1X_2 - 0.12X_2^2 \quad (3)$$

$$Y_{[3,4-\text{didehydroretinol}]} = -465.08 + 11.98X_1 + 1.53X_2 - 0.07X_1^2 + 0.01X_1X_2 - 0.03X_2^2 \quad (4)$$

The coefficient determinations (R^2) of the responses as a function of the variables (temperature and saponification time) were respectively equal to 80.57%, 84.06% and 80.00% for retinyl palmitate, retinol and 3,4-didehydroretinol in whole body freshwater fish. As the R^2 values of the models were greater than 80%, the influence of temperature and time parameters on the response can be discussed [21].

The best values of the reaction parameters for maximising the retinol content were identified for each model. Thus, the optimal temperature and time for quantifying retinol are respectively:

- 64 °C and 26 min for retinyl palmitate standard;
- 80 °C and 43 min for retinol in whole body freshwater fish;
- 82 °C and 40 min for 3,4-didehydroretinol in whole body freshwater fish.

When the p-value is ≤ 0.05, this indicates that the variable concerned has a statistically significant effect with a 95% confidence interval on the determination of retinol (Table 4). For the retinyl palmitate standard, time and temperature×temperature interaction significantly affected the retinol quantification. For whole freshwater fish, three effects (time, time×time, and temperature×temperature) significantly affected the retinol assessment, while only interaction between temperature×temperature affected on 3,4-didehydroretinol assessment. The interactions between temperature and time are well visualized in three-dimensional representations of the response surface plots, which are the graphic representations of the regression equation (Figure 3).

Vitamin A content in ten freshwater fish species

The optimized saponification protocol was applied to ten species of freshwater fish collected to assess their retinol equivalent content. Retinol was present in all fish species, while 3,4-didehydroretinol was detected in four small fish species (*Mystus atrifasciantus*, *Trichogaster microlepis*, *Clupeoides borneensis* and *Henicorhynchus siamensis*) and in a big fish species (*Puntioplites proctozysron*). Retinol and 3,4-didehydroretinol contents in ten fish species ranged from 9.6 to 422.8 µg/100 g and from 0.0 to 262.3 µg/100 g, respectively. The vitamin A content expressed as retinol equivalent varied from 9.6 to 737.5 µg RE/100 g (Table 2). The highest levels of vitamin A were found in species of small fish eaten whole (including organs and eyes) and reached values much higher than for fillets of fish eaten without their organs.

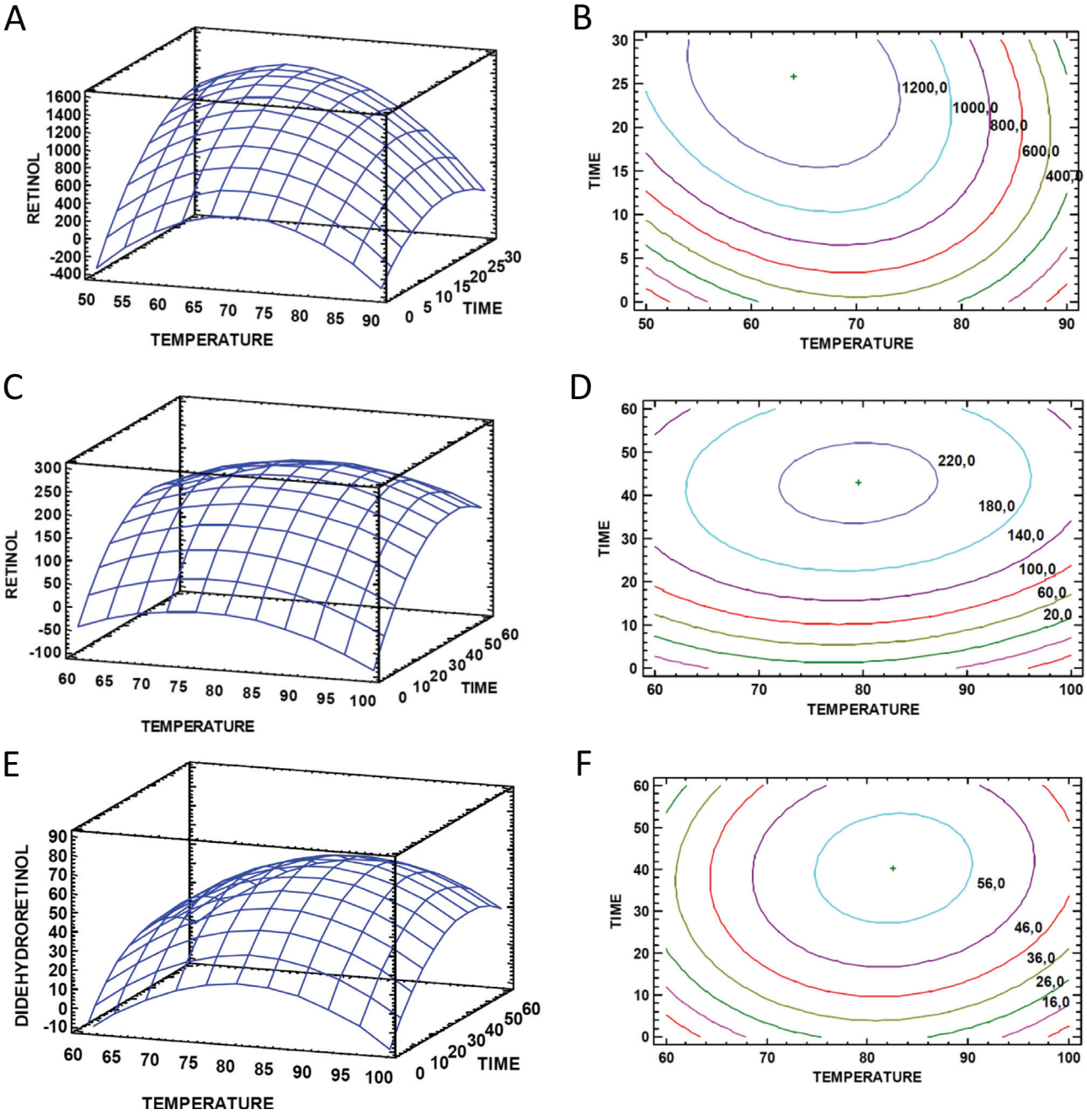

Figure 3. Iso-responses and response surfaces of polynomial regression equations generated by Statgraphics Centurion XV for retinol concentrations after saponification of retinyl palmitate alone (A, B), after saponification of *Henicorhynchus siamensis* fish (C, D) and 3,4-didehydroretinol concentrations after saponification of *Henicorhynchus siamensis* fish (E, F). The response surfaces represent the variation in retinol or 3,4-didehydroretinol concentrations modelled by the polynomial equation as a function of temperature (°C) and saponification time (min). The iso-response curves correspond to the time (min) and temperature (°C) pairs that allow a certain concentration of retinol to be obtained in the saponification medium. AU: absorbance units; min: minutes.

Discussion

Official methods have been designed for, and tested with, very specific food matrices (milk based products, muscle, etc.) [9]. In some cases, these methods may not be optimal for all food matrices, especially solid, heterogeneous foods containing bones, skin and organs. The food matrix (even when ground) can have a protective effect against hydrolysis and release reactions. It is important to have an effective method for releasing vitamin A into these matrices in order

Table 4. Regression coefficients and analysis of variance of the second polynomial

Source	Sum of squares	df	Mean square	F-ratio	p-value
Retinyl palmitate standard					
X_1	4842.20	1	4842.20	0.60	0.47
X_2	541621.00	1	541621.00	6.73	0.04*
X_1X_1	740014.00	1	740014.00	9.19	0.02*
X_1X_2	127057.00	1	127057.00	1.58	0.26
X_2X_2	168053.00	1	168053.00	2.09	0.20
Total error	483214.00	6	168053.00		
Total (correlation)	2.49	11	80535.60		
Retinol in whole freshwater fish					
X_1	276.63	1	276.63	0.14	0.73
X_2	14116.40	1	14116.40	6.89	0.05*
X_1X_1	13819.70	1	13819.70	6.75	0.05*
X_1X_2	480.93	1	480.93	0.23	0.65
X_2X_2	14893.20	1	14893.20	7.27	0.04*
Total error	10243.10	5	2048.63		
Total (correlation)	6426.30	10			
3,4-didehydroretinol in whole freshwater fish					
X_1	288.43	1	288.43	1.25	0.31
X_2	251.17	1	251.17	1.09	0.34
X_1X_1	2235.18	1	2235.18	9.72	0.03*
X_1X_2	50.06	1	50.06	0.22	0.66
X_2X_2	684.17	1	684.17	2.98	0.15
Total error	1149.8	5	229.96		
Total (correlation)	5592.31	10			

X_1: temperature; X_2: time; df: degrees of freedom; F: Fisher test; p-value: probability distribution value. *Significance was accepted at probability $p \leq 0.05$.

to estimate their potential to meet the vitamin requirements of populations. Saponification is an effective way to remove lipids, which can interfere with chromatographic separation and reduce the life of the column. It is particularly true with fatty fishes. Saponification hydrolyses the esters and this simplifies the chromatographic separation, identification and quantification. It is useful for laboratories that do not have the most efficient columns to separate compounds and the detection techniques to visualise absorption spectra for compound preliminary identification.

The retinol contents increased with temperature and time to optimal levels, and then decreased. In the experimental designs, the parameter that had a significant impact on the determination of retinol after reaction was the saponification time, which always had to be at least 26 minutes. Lower levels of temperature and time did allow releasing enough the retinol from the matrix and the higher levels of temperature and time degrade too much the retinol liberated from the matrix with chemical reactions. Elliptical contours were obtained when there is perfect interaction between the independent variables [3].

The design of the experiments makes it possible to compare the effects of the variables in isolation or in combination. A search for an optimum made it possible to identify the ideal combination of reaction to release the retinol and at the same time protect it from degradation. In practice, this means that during saponification, the temperature should not exceed 80 °C and should not last more than 43 min to maximize retinol quantification. If the thermal treatment is more severe, the released retinol and retinyl esters will probably be isomerized or cleaved and the quantification will be underestimated. The conditions necessary for the saponification of vitamin A contained in freshwater fish require more drastic conditions than for standard and enriched margarines or milk [9, 10]. The time required for saponification was shorter than in other studies for which durations reached 8 to 10 hours [33].

Many studies found that vitamin A is accumulated in the eyes and viscera of fish [16]. In our study, retinol was detected in all fish species, while 3,4-didehydroretinol was detected in only five fish species. *Henicorhynchus siamensis* is a species that contains a high concentration of retinol and 3,4-didehydroretinol and the concentration of retinol is higher than that of 3,4-didehydroretinol. However, there is no obvious biological explanation of the variation in vitamin A content between fish species [16, 17].

Henicorhynchus siamensis and *Clupeoides borneensis* were the two small indigenous fish species which contain high concentration both retinol and 3,4-didehydroretinol. Some studies revealed that the small fish species with their adult length less than 25 cm and they were consumed whole with head, bones and liver, they mostly are rich source of 3,4-didehydroretinol [6]. To promote nutrition, it is recommended to consume the head or liver of the fish as they contain many nutrients, especially vitamin A. Some studies have found that the content of retinol and 3,4-didehydroretinol is similar in fillets, while the content of the latter is higher in whole fish species [33]. This is true for some fish species in our study. The richness of 3,4-didehydroretinol may be explained by the presence and activity of enzymes that convert retinol to 3,4-didehydroretinol in some fish. [6]. There is no clear explanation for the variation in vitamin A levels between fish species. However, their diet may influence the accumulation of retinol and 3,4-didehydroretinol in their tissue. For example, green algae are known to be rich in 3,4-didehydroretinol. Fish species that prefer them as food could have their body content increased by this regular consumption [17]. The vitamin A content found is similar to the finding in previous screening of fish from Mekong River and Bangladesh [16, 17].

One of the strengths of our work is to show that it is sometimes necessary to adapt an official protocol to the complexity of a food matrix. The conditions that we propose are much faster than those recommended in other research up to 10 hours of saponification. Finally, the protocol respects the general recommendation not to exceed 80 °C in order to avoid retinol isomerisation. A limitation of this work is that the protocol developed is specific to whole fatty fish.

Electronic supplementary material

The electronic supplementary material is available with the online version of the article at https://doi.org/10.1024/0300-9831/a000729

ESM 1. Sampling location in Cambodia (Figure E1). Experimental design matrix and concentration of retinol and 3,4-didehtydroretinol after saponification of retinol palmitate (100 mg in 1 L of acetone) and *Henicorhynchus siamensis* freshwater fish (Table E1).

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History
Received May 3, 2021
Accepted August 25, 2021
Published online September 20, 2021

Conflict of interest
Authors declare that they have no conflict of interest.

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Funding
This work was supported by French scholarship program (928739F, 928756F, 954036 J and 956215 K), Institute of Technology of Cambodia, Erasmus+ (CamFoodTech) and Francophonie University Association.

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