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Vitamin B₂ concentration in cow milk: Quantification by a new UHPLC method and prediction by visible and near-infrared spectral analysis

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ABSTRACT

Until now, there are few information on vitamin B₂ concentration variability in milk. In this study, a novel analytical method to quantify total vitamin B₂ in milk was developed and applied on 676 samples. In parallel, spectral analysis (colorimetry and near infrared spectroscopy) were performed to develop prediction models of vitamin B₂ concentration in milk. The analytical method includes an acid and enzymatic extraction followed by vitamin B₂ quantification by Ultra High Performance Liquid Chromatography coupled with fluorimetry. Samples analysis showed a wide range of concentration from 0.78 to 4.58 mg/L with a mean of 2.09 ± 0.48 mg/L. Two prediction models based on colorimetric analysis allow estimation of vitamin B₂ concentration in milk. Thus, this work shows an analytical method and, for the first time, a prediction method to enable enhancement of researches on vitamin B₂ content of milk and its variation factors.

1. Introduction

B-vitamins are essential micronutrients in human diet. Among them, vitamin B₂ and its principal component, riboflavin, play an important role in energy metabolism. Vitamin B₂ main food contributors in human nutrition are dairy products, offal and eggs and recommended nutrient intakes for riboflavin are 1.0–1.6 mg/day for adults and 0.4–1.4 mg/day for children (depending to age and gender) (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2017). However, an important proportion of the population of western countries suffer from insufficient vitamin B₂ consumption to cover the dietary recommendations. Riboflavin deficiency would affect 5 to 75% of the population in some European countries (Kennedy, 2016; Troesch, Hoef, McBurney, Eggersdorfer, & Weber, 2012).

Bovine milk and dairy products contribute significantly to the vitamin B₂ reference intakes in many countries, however very few research efforts have been directed on studying factors affecting its concentration in milk. Indeed, since 1950's, it was considered that B-vitamins provision to ruminants through diet and rumen bacterial synthesis was sufficient to cover animal requirements, and that concentration of these vitamins in cow milk was almost constant. However, during the last decade, a few studies showed variations of vitamin B₂ concentration in cow milk according to season and management practices (Laverroux, Vallet, Chassaing, Girard, Agabriel, Martin, & Graulet,

2014; Poulsen, Rybicka, Larsen, Buitenhuis, & Larsen, 2015; Poulsen et al., 2015; Shingfield et al., 2005). Considering the major role of dairy products in vitamin B₂ provision in human diet whereas a significant part of the population does not cover its dietary recommendations (Troesch et al., 2012), it led to a renewed interest in milk vitamin B₂ quantification and identification of main drivers of its concentration variations. Indeed, a greater characterization and comprehension of variation factors is necessary to have a better estimation of vitamin B₂ content in dairy products.

Historically, vitamin B₂ was quantified with microbiologic or global fluorometric methods (Hampel, Dror, & Allen, 2018). In 90's, novel methods using High Performance Liquid Chromatography (HPLC) with spectrophotometric or fluorometric detection were developed and showed more reproducibility and less dependency on matrix content (Kanno, Shirahuji, & Hoshi, 1991; Russell & Vanderslice, 1992). They also conduced to highlight the vitamin B₂ composition in milk. Vitamin B₂ in bovine milk includes riboflavin as the prevalent form but also a non-negligible proportion (5–25%) of some other flavins as 5'-phosphate-riboflavin (FMN) and flavin-adenine-dinucleotide (FAD) (Kanno et al., 1991). In the last few years, HPLC methods with mass spectrometric detection have emerged and permitted greater sensitivity (Hampel, York, & Allen, 2012; Schmidt, Schreiner, & Mayer, 2017). Currently, the common technique for vitamin B₂ analysis is HPLC separation followed by fluorescence detection. However this technique is

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skewed for flavins other than riboflavin (Hampel et al., 2018). In order to increase the intensity of research on vitamin B₂ content of milk, our laboratory chose to develop a method of quantification for the sum of all forms of vitamin B₂ by flavins conversion into riboflavin.

However, since the knowledge level is poor, an additional rapid method useful for research purposes as well as for in-farm or in dairy plant determination, could allow increase data acquisition in less time and for a lower price. As vitamin B₂ is a pigment that affects the yellow color of milk (Nozriere et al., 2006), a spectrophotometric method could be an interesting approach for a quick estimation of its concentration. However, β -carotene also impacts the milk yellowness (Calderon et al., 2007), so it could interfere in the vitamin B₂ estimation by spectrophotometry. A more complete spectral analysis (including the infrared segment) could also be proposed to take account not only the pigment property of vitamin B₂ but rather its complete spectral signature, both in the visible and the near-infrared ranges.

The aim of this work was in a first step to set the quantitative method developed in our laboratory for vitamin B₂ analysis in milk by fluorometry after Ultra-High Performance Liquid Chromatography (UHPLC) and to validate its use to study variation factors of vitamin B₂ concentration in milk, and in a second step to evaluate spectral analysis as spectrophotometry (color) and near-infrared spectroscopy (NIRS) in their abilities to estimate vitamin B₂ content of milk. For this, raw cow milks were analyzed for vitamin B₂ concentration, color and NIRS. Additionally, some raw cow milk samples were also analyzed for β -carotene concentration to estimate influence of this component on vitamin B₂ prediction by spectral analysis.

2. Material and methods

2.1. Sample collection

A total of 676 cow raw milk samples were collected. They were obtained from single cows (356 samples from 67 cows, INRA Experimental Unit Herbipole, UE1414, Saint-Genès-Champanelle, France) or farm tanks (320 samples from 68 commercial farms). Samplings were conducted on milk from one milking (morning or evening, 31%), milk corresponding to day milk (morning plus evening, 22%) and milk from tanks which had grouped 2 days of milkings (47%). All seasons were represented with 59%, 16%, 15% and 10% of samplings in winter, spring, summer and autumn, respectively. Samples came from several geographical origins (all single samples from Auvergne region of France; 36% from Bretagne lowland region of France, 42% from Auvergne-Rhône-Alpes mid-mountain region of France and 22% from Po valley of Italy for tank samples). Samples also differed gradually by the breeding and feeding system, from highly intensive systems rich in concentrate diets ($n = 72$) to highly extensive systems exclusively based on grazing ($n = 133$). All these differences were supposed to let get a wide range of milk composition. All milk samples were kept at 4 °C until the evening then frozen and brought to the laboratory for storage at -20 °C until analysis.

2.2. Analytical method

2.2.1. Standards and reagents

Riboflavin standard was purchased from Sigma-Aldrich (St Louis, USA, purity = 98%). Solvents were of "gradient grade for HPLC" and other chemical products were of "analytical grade" and all were purchased from Sigma-Aldrich (St Louis, USA) or VWR (Radnor, USA). For enzymatic hydrolysis, papain from *Papaya latex* and acid phosphatase from potato were purchased from Sigma-Aldrich. Ultrapure water from Simplicity® system (Merck Millipore, Darmstadt, Germany) was used for sample preparation and chromatographic analysis. Certified reference material (milk-based infant/adult nutritional formula powder, SRM 1849a, NIST®) was purchased from LGC Standards (Molsheim, France).

2.2.2. Vitamin B₂ analysis

As vitamin B₂ is sensitive to natural light, especially ultraviolet radiations, all preparation steps were performed in a dark room with a yellow light. Milk samples were unfrozen at room temperature during 30 min on a balancer. For each analytical series, a control cow milk was analyzed in triplicate either with or without a known added quantity of riboflavin to calculate a mean recovery for the series. A blank (where water replaced milk sample) was also analyzed to quantify vitamin B₂ potentially present in enzymes preparation. Experimental samples were analyzed in duplicate.

The first step of extraction consisted to an acid hydrolysis for milk protein breakdown and most of vitamin B₂ release (Jakobsen, 2008). In a glass tube with screw cap, 2 mL of milk were mixed with 2 mL of 0.1 M HCl. Tubes were incubated for 30 min at 100 °C in a dry block heater. After cooling at room temperature, pH was adjusted to 4.5 with 120 μ L of 2 M sodium acetate. The second step of extraction was an enzymatic hydrolysis for complete release of vitamin B₂ bound to proteins and conversion of phosphorylated forms to riboflavin simplest form (Jakobsen, 2008; Santschi, Berthiaume, Matte, Mustafa, & Girard, 2005). For this, 200 μ L of 1% reduced glutathione and 400 μ L of a mixture of papain (60 U) / acid phosphatase (14 U) were added to tubes. Then, they were vortexed and incubated under constant agitation 85 min at 45 °C in a water bath. The activity of enzymes was stopped by 10 min incubation in an ice water bath, and enzymes were inactivated by 10 min incubation at 100 °C in a dry block heater. After cooling at room temperature, tubes were centrifuged at 2000 g for 10 min at room temperature. Supernatants were filtered through a 0.2 μ m GHP filter (Pall, Port Washington, USA) before UHPLC analysis.

Chromatographic analysis was performed on a Waters Acquity UPLC® system (Milford, USA) equipped with a fluorescence detector. Injection volume was set at 15 μ L. Separation of riboflavin was performed using an Acquity UPLC® HSS T3 column (150 \times 2.1 mm, 1.8 μ m particle size, Waters) following a Waters application note (Doneanu, Chen, & Mazzeo, 2011) with some modifications to improve the separation of interfering compounds of the biological matrix. Column temperature was set at 30 °C and gradient elution was operated at a flow rate of 0.3 mL/min with two mobile phases. Mobile phase A was 10 mM ammonium formate, 0.1% formic acid in ultrapure water, and mobile phase B was 10 mM ammonium formate, 0.1% formic acid in methanol. The gradient of mobile phase B was as follows: 0% for 2 min, exponential increase to 20% until 8 min, linear increase to 35% until 12 min, linear increase to 90% until 18 min, stay at 90% for 1 min, decrease to 0% until 20 min and stand-by for 2 min. In these conditions, riboflavin was eluted after 14.3 min and was quantified fluorometrically with 450/520 nm as excitation and emission wavelengths, respectively. Calibration curves corresponded to peak areas of standard solutions injected at five different concentrations. Vitamin B₂ concentration in a sample milk resulted from the analytical concentration corrected for extraction dilution (calculated with final extract weight and initial milk weight), blank concentration and series recovery.

2.2.3. Method validation

The analytical method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, precision and accuracy in accordance with ICH guidelines and Eurachem guide (Harmonised Tripartite Guideline, 2005; Magnusson & Örnemark, 2014).

Range chosen to study performances of the analytical method was from 0.6 to 3.5 mg/L in milk that corresponded respectively to half and twice concentrations traced in food composition databases.

Linearity, LOD and LOQ were evaluated on calibration curves. Standards solutions with five different concentrations of riboflavin (0.3, 0.6, 0.9, 1.2 and 1.5 mg/L) were injected at 16 different days. These concentrations corresponded to milk extract concentration (due to extraction dilution). Calibration curves were obtained by plotting peak areas against concentration, and linear regression model was performed on these. Linearity was evaluated by p-value and correlation coefficient.

LOD and LOQ were determined as $3\sigma/S$ and $10\sigma/S$, respectively, where σ was standard error of intercept and S was slope of linear regression.

Recovery was calculated by spiking a control cow milk with a known amount of riboflavin (in triplicate at 16 different days, known amount = 1.7 mg/L of sample). Recovery was also studied on the five levels of standard solutions to compare to milk samples and evaluate matrix effect.

Repeatability and reproducibility were estimated by triplicate extraction / injection of a control cow milk sample, at 16 different days.

Accuracy was validated by 23 extractions / injections of a certified milk powder NIST® 1849a dispatched on 10 days. This certified reference material (CRM) was dissolved in ultra-pure water prior to injection (approximately exactly 0.2 g in 2 mL) to reflect vitamin B₂ concentration of test samples.

2.3. Spectral analysis

Before scanning, milk samples were unfrozen at laboratory temperature for 30 min on a balancer.

Color analysis was performed with a spectrophotometer (CM-2600d, Minolta, Tokyo, Japan). 20 mL of milk sample were placed in a glass cell (CR A502, Minolta) and analyzed thrice for percentage of light reflectance (10 nm step between 400 and 700 nm). Due to the large volume used, only 352 milk samples were analyzed for color. Color data were used as follows: 1) data were expressed using the CIELAB color space: L* as lightness (from 0 for black to 100 for white), a* as color under green–red axis and b* as color under blue–yellow axis (both from –120 to +120), C* as chromaticity and h* as hue (both calculated from a* and b* to have another visualization of color); 2) reflectance (R) values were converted into absorbance (A) using the formula $A = \log(1/R)$ and color spectrum was built for each milk sample.

A Foss NIRSystems model 6500 scanning visible-near-infrared spectrometer equipped with an auto cup module (Foss NIRSystems, Silver Spring, MD, USA) produced the near-infrared spectra. 500 μ L of milk were placed on a GF/A glass microfibre filter (Whatman, Maidstone, UK), oven-dried at 30 °C during 24 h and placed in a 50 mm diameter ring cup according to the methodology proposed by [Thyholt and Isaksson \(1997\)](#). Then they were scanned in reflectance mode at 2 nm intervals from 400 to 2498 nm. Spectra were recorded using ISIScan (version 2.21 Infracsoft International, South Atherton St. State College, PA, USA). Each spectrum was time averaged from 32 scans. Reflectance values were converted into A using the formula $A = \log(1/R)$ to obtain NIRS absorbance spectra.

2.4. Development of predictive models of vitamin B₂ concentration using spectral data

A total of 88 milk samples analyzed for vitamin B₂ concentration, color and NIRS spectra together, were randomly chosen from the initial population samples. These samples were used for comparison purposes. This number of samples corresponded to 30% of samples containing these three determinations. Predictive models were developed using all other data. They were validated using the cross-validation method (4-fold).

Models from CIELAB parameters were developed using multiple linear regression models of R software (version 3.5.1 assisted by RStudio 1.1.463, “lm” function). Samples with vitamin B₂ concentration not included in ± 2 standard error area were considered as outliers and were not used for models development. Cross-validation of these models was performed with “train” function of “caret” package.

Models from color or NIRS spectra were developed using the modified partial least squares regression (MPLS) of WinISI 4 (version 4.12.0.15440, Foss Analytical A/S, Hilleroed, Denmark). Spectral similarity was assessed with the standardized Mahalanobis distance (H). Predicted samples showing an H-distance to the center of model population higher than 3 were considered as outliers and were removed

for model development ([Shenk, Westerhaus, & Fahey, 1994](#)). NIRS data were subjected to standard normal variate and detrending ([Barnes, Dhanoa, & Lister, 1989](#)) and first-derivative treatments (1,4,4), where the first digit is the number of the derivative, the second is the gap over which the derivative is calculated, and the third is the number of data points in the first smoothing. As Foss NIRSystems spectrometer allowed analyzing spectrum from visible to near-infrared area (400–2498 nm each 2 nm), three models were performed: using data of the visible part of spectrum (400–1098 nm), the near-infrared part of spectrum (1100–2498 nm) and the whole spectrum (400–2498 nm). Models were validated by cross-validation using WinISI4 program (version 4.12.0.15440).

All calibration models were evaluated by the coefficient of determination of calibration (R^2C) and of cross-validation (R^2CV) and by the standard error of calibration (SEC) and of cross-validation (SECV).

Obtained models were then applied to the external dataset. Prediction results were evaluated by the coefficient of determination of prediction (R^2P), standard error of prediction (SEP), bias and SEP corrected by the bias (SEPC).

2.5. Influence of β -carotene on vitamin B₂ prediction

A total of 115 samples of the population analyzed for vitamin B₂ were also quantified for β -carotene concentration as described by [Chauveau-Duriot, Doreau, Noziere, and Graulet \(2010\)](#). Extraction consisted to an alcoholic deproteinization followed by a purification using organic solvents (ethanol, diethyl ether and ethyl acetate) and then a potassium hydroxide saponification. Chromatographic analysis was performed on a Waters Acquity UPLC® System (Milford, USA) equipped with a PhotoDiodeArray detector and β -carotenes were quantified at 450 nm by comparison to peak areas of standard solution. The total concentration of β -carotene was the sum of the concentrations of *all-trans*- β -carotene, *9-cis*- β -carotene and *13-cis*- β -carotene isoforms.

In a first step, the “cor.test” function of R software calculated the correlation between β -carotene and vitamin B₂ concentrations in milk. In a second step, models developed for predicting vitamin B₂ in section 2.4 and showing better performances were applied to the dataset composed by the 115 samples analyzed for vitamin B₂ and β -carotene contents. Then, residuals (difference between predicted and observed values) were calculated for each individual sample. Finally the “lm” function of R software (version 3.5.1 assisted by RStudio 1.1.463) permitted to evaluate effect of β -carotene concentration on residuals obtained for each method.

3. Results

3.1. Vitamin B₂ concentration in cow milk

3.1.1. Vitamin B₂ analytical method performances

Chromatograms ([Fig. 1](#)) obtained for milk extracts showed a single peak with a retention time and a UV–visible spectrum corresponding to riboflavin. [Table 1](#) shows performances of the method developed for vitamin B₂ quantification as chromatographic analysis or complete procedure (extraction and chromatographic analysis). Calibration curves were performed with standard solutions and showed a linearity range between 0.304 and 1.520 mg/L ($p < 0.001$, $r^2 = 0.98$), a LOD of 0.049 mg/L and a LOQ of 0.167 mg/L.

The mean recovery was 96% with variations between 87 and 107%. Variations were both intra-assay and inter-assays. Recoveries of standard solutions and milk samples were similar within a same assay. Therefore, we decided to include a recovery rate on a control cow milk in triplicate in all extraction series.

To validate analytical method on milk samples, it was necessary to calculate linearity range, LOD and LOQ in samples. The extraction procedure lead to a sample dilution (about 2.3). It also bring a small amount of riboflavin (concentrations in blank samples, with enzymes

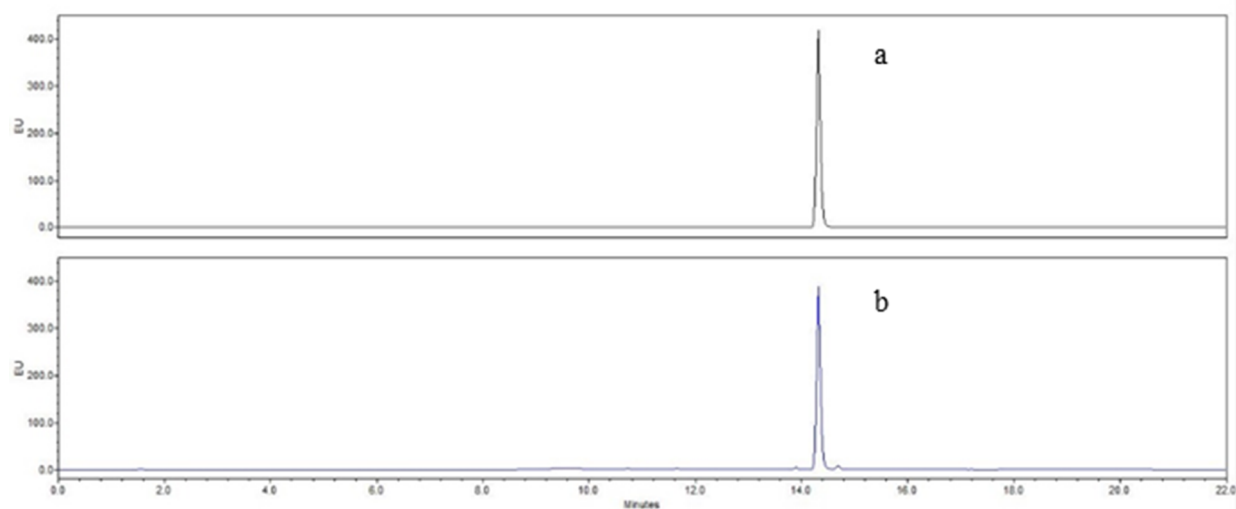


Fig. 1. Chromatograms of a standard solution (a) and a control cow milk extract (b).

but without milk sample substituted by water, ranged from 0.012 to 0.133 mg/L). Thus, values obtained with standard solutions analysis were corrected for dilution, blank result and recovery rate. Therefore, analytical method allow quantification of vitamin B₂ in milk from 0.656 to 3.548 mg/L with LOD of 0.048 mg/L and LOQ of 0.330 mg/L.

Repeatability and reproducibility, calculated on a control cow milk are 2.07% (0.053 mg/L) and 5.90% (0.152 mg/L) respectively for an average concentration of 2.581 mg/L.

Analysis of the CRM milk powder NIST® 1849a allowed evaluating the accuracy of the method at 99.2%. Indeed, the mean vitamin B₂ concentration obtained was 20.20 mg/kg (\pm 1.20 mg/kg, n = 23 determinations) for a certified concentration of 20.37 \pm 0.52 mg/kg.

3.1.2. Vitamin B₂ concentration in cow milk

This new method was used to quantify vitamin B₂ in 676 milk samples. According to the method, mean vitamin B₂ concentration in raw cow milk was 2.09 \pm 0.47 mg/L with a range from 0.78 to 4.58 mg/L. Five samples had a concentration greater than the application area of the method. According to sample type, average vitamin B₂ concentration was 1.98 \pm 0.35 mg/L [from 1.33 to 3.07, n = 320] in tank milk, and 2.20 \pm 0.54 mg/L [from 0.78 to 4.58, n = 356] in individual milk. According to season, mean vitamin B₂ concentration was 2.06 mg/L in autumn and winter, 2.13 mg/L in spring and 2.18 mg/L in summer, with equivalent variations inside seasons. According to geographical origin of tank milk, samples from French lowland region contained 2.05 \pm 0.33 mg/L, samples from French mid-mountain region contained 2.06 \pm 0.38 mg/L and samples from Italian lowland region contained 1.70 \pm 0.14 mg/L of vitamin B₂. Milk samples from highly intensive systems rich in concentrate diets contained 1.70 \pm 0.14 mg/L and samples from highly extensive systems based on grazing contained 2.51 \pm 0.59 mg/L of vitamin B₂.

Table 1

Performances of the analytical method of vitamin B₂ quantification in milk developed in our laboratory.

	Vitamin B ₂ as standard = chromatographic analysis	Vitamin B ₂ in milk = complete procedure
Application area (mg/L)	0.304 – 1.520	0.656 – 3.548
LOD (mg/L)	0.049	0.048
LOQ (mg/L)	0.167	0.330
Repeatability		2.07% (i.e. 0.053 mg/L)
Reproducibility		5.90% (i.e. 0.152 mg/L)

LOD: limit of detection.

LOQ: limit of quantification.

3.2. Predictive models of vitamin B₂ content

Means, standard deviations and minimal and maximal values of calibration and external datasets are presented in Table 2. Calibration and external datasets had equivalent mean and standard deviation values as the whole database. Calibration and validation statistics of prediction models for vitamin B₂ content in milk are given in Table 3. On the basis of the CIELAB parameters, multiple linear regression model using all the three L*, a* and b* indexes (called Lab* thereafter) provided the best prediction potency (per comparison to models using one or two of these indexes only, data not shown) and was characterized by R²CV of 0.73 and SECV of 0.23 mg/L (10.4% of the mean). MPLS model developed with color spectral data was characterized by R²CV of 0.88 and SECV of 0.16 mg/L (7.3% of the mean). MPLS models developed with NIRS spectral data were characterized by R²CV of 0.51 and SECV of 0.29 mg/L (14.3% of the mean) for the visible segment, 0.23 and 0.34 mg/L (17.0% of the mean) for the near-infrared segment, and 0.52 and 0.29 mg/L (14.3% of the mean) for the whole spectrum.

Application of these models on an external dataset showed R²P of 0.82 for Lab* model, 0.87 for model with Minolta visible spectrum, 0.59 for model with Foss visible spectrum, 0.44 for model with Foss near-infrared spectrum and 0.51 for model with Foss total spectrum. SEP were 0.17 (8.0% of the mean) for Lab* model, 0.16 (7.2% of the mean) for model with Minolta visible spectrum, 0.26 (12.7% of the mean) for model with Foss visible spectrum, 0.31 (15.4% of the mean) for model with Foss near-infrared spectrum and 0.29 mg/L (14.0% of the mean) for model with Foss total spectrum. Fig. 2 illustrates predicted vs. observed values of external dataset for the best models. Model developed with Minolta visible spectrum shows the best adequacy on the largest range of concentrations.

Table 2
Characteristics of vitamin B₂ concentrations in milk sample datasets used for prediction models.

Dataset	Analysis	Parameter used for prediction model	n	Mean	Sd	Min	Max
				mg/L			
Whole database	Vitamin B ₂ content		676	2.09	0.47	0.78	4.58
Calibration datasets	Vitamin B ₂ content and color	CIELAB parameters	241	2.19	0.43	1.33	3.24
		Visible spectrum: 400–700 nm (10 nm)	208	2.24	0.47	1.33	3.59
	Vitamin B ₂ content and NIRS	Visible spectrum: 400–1098 nm (2 nm)	504	2.06	0.42	1.22	3.49
		Near-infrared spectrum: 1100–2498 nm (2 nm)	510	2.02	0.39	1.22	3.17
		Total spectrum: 400–2498 nm (2 nm)	519	2.05	0.42	1.17	3.49
External dataset			88	2.12	0.41	1.44	3.16

The whole database, calibration datasets and the external dataset are characterized by number of samples (n), mean, standard deviation (Sd), minimal (Min) and maximal (Max) values.

3.3. Influence of β -carotene on prediction of vitamin B₂ concentration in milk

One hundred and fifteen milks were analyzed both for vitamin B₂ and β -carotene concentrations. In this sub-population, vitamin B₂ average concentration was 1.99 ± 0.35 mg/L [from 1.33 to 2.88 mg/L] and β -carotene average concentration was 0.092 ± 0.050 mg/L [from 0.024 to 0.294 mg/L]. Fig. 3a illustrates relationships between vitamin B₂ and β -carotene concentrations. Correlation coefficient between observed vitamin B₂ and β -carotene concentrations is 0.327. Fig. 3b and c show that β -carotene concentration have no significant effect on residues of predicted vitamin B₂ concentration ($p = 0.78$ for Lab* model, and $p = 0.64$ for model based on Minolta visible spectrum).

4. Discussion

4.1. Reliability of the analytical method for milk vitamin B₂ analysis

The analytical method developed for vitamin B₂ quantification in milk shows an analytical range from 0.65 to 3.55 mg/L that includes mean riboflavin concentration in whole cow milk traced in food composition databases (1.70 mg/L for Ciquial-Anses (France) in pasteurized whole cow milk, 1.80 mg/L for EFSA (Europe), 1.69 mg/L for USDA (USA) and 1.88 mg/L for Health Canada in whole cow milk). LOQ of 0.167 mg/L as a standard and of 0.321 mg/L as a milk sample are similar to previous chromatographic methods proposed in the literature and allow analysis of samples with low vitamin B₂ content (Guneser & Yuceer, 2012; Sunaric, Denic, & Kocic, 2012). A more recent analytical method achieved to a lower limit of quantification (0.019 mg/L;

Schmidt et al., 2017), however we didn't try to improve this parameter to evaluate lower concentration than 0.3 mg/L as no publication showed it in milk. Instead, we prefer to focus on the accuracy of the method. This objective was reached since the mean accuracy value obtained is 99.2% of a CRM (NIST® 1849a) certified value. Reproducibility for extraction and chromatographic analysis is 5.90% and is in accordance with method of Schmidt et al. (2017). The method developed in our laboratory to quantify vitamin B₂ in milk also allows quantification of the three vitamin B₆ forms (pyridoxamine, pyridoxal and pyridoxine) with good performances (data not shown).

The mean vitamin B₂ concentration of the 676 analyzed samples is 2.09 mg/L with a wide range from 0.78 to 4.58 mg/L. To compare these results with food composition databases and literature, it's important to separate samples of tank milk to individual milk. Actually, tank milk, and even more milk from dairy industry, were averaged. Concentrations of constituents should be thus less variable in tank milk samples than in individual milk samples. Range of vitamin B₂ concentration in analyzed tank milk samples was from 1.33 to 3.07 mg/L. Food composition databases concentrations (1.69–1.88 mg/L) are in this range, but in the lower moiety. Literature studying tank milk reported mean concentration close to 1.65 mg/L with a range from 1.30 to 2.12 mg/L (Amador-Espejo, Gallardo-Chacon, Nykanen, Juan, & Trujillo, 2015; Kim, Kim, Choi, Min, & Kwak, 2008; Poulsen et al., 2015b; Sunaric et al., 2012), that corresponds to our observations. Range of vitamin B₂ concentration in individual milk samples of our study was from 0.78 to 4.58 mg/L. Poulsen et al. (2015a) have studied individual variations in riboflavin content and found concentrations from 0.73 to 2.84 mg/L. Therefore, range of vitamin B₂ concentrations analyzed with our method is in accordance with variability known for dairy milk as well as individual milk.

Table 3
Calibration and validation statistics and comparison of prediction models of vitamin B₂ content of milk.

Analysis / Material	Data used for models	Calibration		Cross-validation		Comparison with external dataset			
		SEC mg/L(%)	R ² C	SECV mg/L(%)	R ² CV	SEP mg/L(%)	SEPC mg/L	R ² P	Bias mg/L
Color / Minolta spectrophotometer	Lab* parameters	0.22 (10.2%)	0.74	0.23 (10.4%)	0.73	0.17 (8.0%)	0.17	0.82	-0.04
	Visible spectrum	0.15 (6.7%)	0.90	0.16 (7.3%)	0.88	0.16 (7.2%)	0.16	0.87	-0.03
NIRS / Foss NIRSystems spectrometer	Visible spectrum	0.28 (13.4%)	0.57	0.29 (14.3%)	0.51	0.26 (12.7%)	0.26	0.59	0.00
	Near-infrared spectrum	0.32 (15.7%)	0.33	0.34 (17.0%)	0.23	0.31 (15.4%)	0.31	0.44	0.00
	Total spectrum	0.26 (12.9%)	0.60	0.29 (14.3%)	0.52	0.29 (14.0%)	0.29	0.51	-0.03

SEC: standard error of calibration, mg/L (percent of the mean).

R²C: coefficient of determination of calibration.

SECV: standard error of cross-validation, mg/L (percent of the mean).

R²CV: coefficient of determination of cross-validation.

SEP: standard error of prediction, mg/L (percent of the mean).

SEPC: standard error of prediction corrected for the bias, mg/L.

R²P: coefficient of determination of prediction.

Five prediction models were developed and cross-validated. There performances were compared by application of these models to an external dataset.

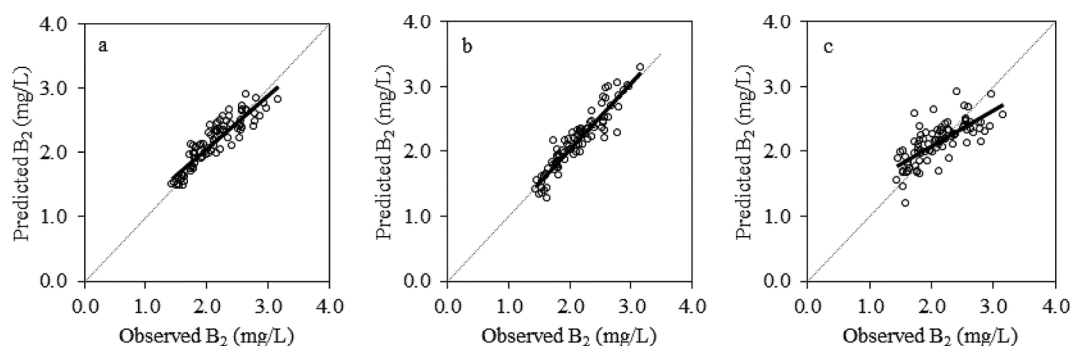


Fig. 2. Performances of prediction models of vitamin B₂ concentration in milk evaluated using an external dataset. Predicted vs. observed values of vitamin B₂ concentration when Lab* linear regression model (a), MPLS model with Minolta visible spectra (b), and MPLS model with Foss total spectra (c) were applied on an external dataset.

On average, food composition databases and literature include slightly lesser concentrations (1.77 and 1.65 mg/L, respectively) than our analyzed samples (2.09 mg/L). However, it should be highlighted that vitamin B₂ in milk includes several forms, free or bound to proteins, phosphorylated or not (Kanno et al., 1991; Koop, Monschein, Macheroux, Knaus, & Macheroux, 2014). Nevertheless, food composition databases and much of the literature only analyzed riboflavin and not others flavins such as FMN and FAD. However, proportions of FMN and FAD were estimated to 5–25% of total flavins in milk (Kanno et al., 1991; Russell & Vanderslice, 1992). Therefore we developed an extraction method based on an acid hydrolysis followed by a protease / phosphatase hydrolysis, first to improve the release of vitamin B₂ linked to milk proteins and second to convert phosphorylated forms into riboflavin. Consequently, this method allowed to analyze the sum of all vitamin B₂ forms. This point was confirmed by chromatograms of milk extracts that showed only one peak corresponding to riboflavin. Thus, mean riboflavin concentration of 1.70 mg/L reported by databases and literature and mean vitamin B₂ concentration of 2.09 mg/L obtained with our analytical method were in accordance, as our method allowed to include the 20% of complex flavins forms not analyzed with previous methods. So our method permits to study the sum of vitamin B₂ forms present in milk.

4.2. Vitamin B₂ concentration in cow milk

We observe that vitamin B₂ concentration is lower and less variable in tank milks than in individual milks (1.98 ± 0.35 vs. 2.20 ± 0.54 mg/L, respectively, $p < 0.001$). No articles in the literature compared these two types of milk samples. However, Poulsen et al. (2015a) analyzed 892 individual milk samples grouped in 42 milk

samples of herds and found riboflavin concentration ranging from 1.20 to 2.20 mg/L in herd milks while it varied from 0.73 to 2.84 mg/L in individual milk samples. Therefore, variability of vitamin B₂ concentration is more important in individual milk samples and analysis of this type of milks should allow advances in studies of variation factors.

The database of analyzed milk samples used for the present work was not built to compare vitamin B₂ concentrations between milk samples but rather to reach a large variability in milk production conditions. However, observation of the whole results would suggest that vitamin B₂ concentration varies according to season, geographical origin, breeding and feeding system. It would tend to increase during spring and summer and with more extensive systems. These observations consolidate first studies on variations of vitamin B₂ content in milk that mentioned a seasonal variation with some management practices and some variations with breed and lactation stage (Poulsen et al., 2015a, 2015b).

Therefore, the level of variability in our sample population is highly interesting to evaluate the application field of the method as well as to develop a prediction method based on spectral analysis of milk samples.

4.3. Prediction of vitamin B₂ concentration in cow milk

Color data allow to develop models for prediction of vitamin B₂ content in milk ($R^2_{CV} = 0.73$ and 0.88 , Table 3). However the MPLS model based on spectral data is a bit more adjusted than the multiple regression Lab* model. Actually, SEP obtained on an external dataset is lower for spectral model than for Lab* model (0.16 vs. 0.17 mg/L, respectively) and R^2_P is higher (0.87 vs. 0.82, respectively) (Table 3). Fig. 2 shows that prediction is better in low and high concentrations for MPLS model, as predicted values are closer to observed values. The

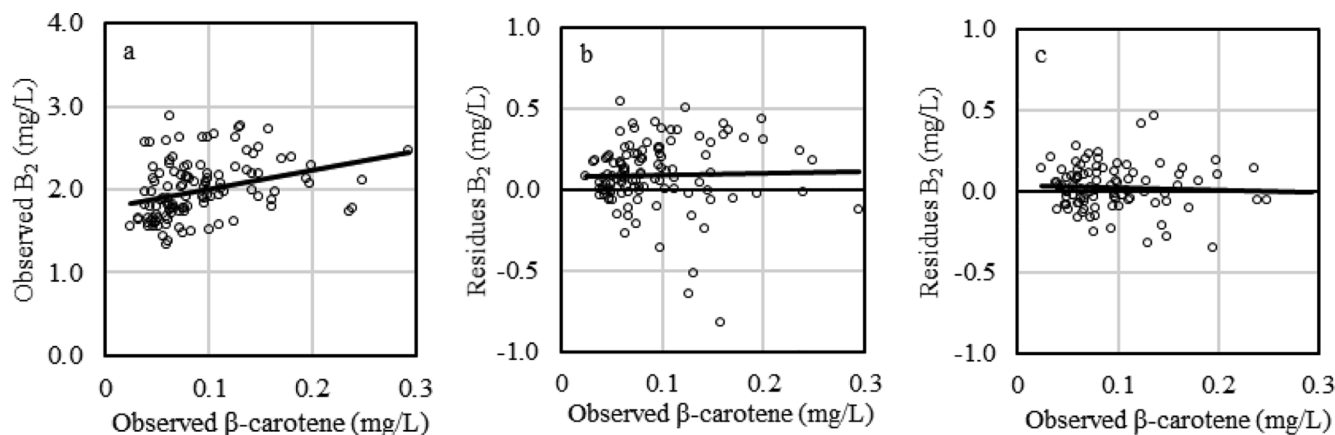


Fig. 3. β -carotene concentration vs. vitamin B₂ concentration (a), and β -carotene concentration vs. vitamin B₂ residues (predicted – observed values) from Lab* model (b) and MPLS model based on Minolta visible spectra (c).

success in the ability to get predicting models of milk vitamin B₂ concentration is not really a surprise considering both its concentration level as well as the spectral activity of its molecule (orange-yellow pigment).

Models developed with spectra obtained with NIRS are not enough accurate, either with visible part and/or near-infrared part of spectra ($R^2CV < 0.55$, Table 3). Difference of results between Minolta visible spectrum and Foss visible spectrum raises questioning. The analysis with the Minolta spectrophotometer was performed using 20 mL of milk corresponding to around 40 µg of vitamin B₂ (considering a mean concentration of 2.0 mg/L) whereas only 500 µL of milk were used for analysis with the Foss spectrometer, corresponding to 1 µg of vitamin B₂ spotted on to the filter (40-fold less). Moreover, it should be noticed that milk preparation is different before the acquisition of spectra by both instruments. To avoid interfering noise signal due to milk water, milk samples were heat dried prior analyzing with Foss spectrometer, which was not the case with Minolta spectrophotometer. Some tests in our laboratory, with vitamin B₂ additions in milk (data not shown), showed differences between milk Foss spectra obtained with liquid sample or with warm dried sample. But it remains to be explored in the specific case of vitamin B₂ quantification. To date, estimation of vitamin B₂ content with NIRS analysis cannot be discarded but remains to be performed with another type of preparation.

4.4. Influence of β -carotene concentration on vitamin B₂ content prediction

CIELAB parameters, especially b* index, are known to predict concentrations of yellow pigments, like carotenoids, in different feed (Kljak, Grbesa, & Karolyi, 2014; Melendez-Martinez, Vicario, & Heredia, 2003). In milk, 75–85% of carotenoids are β -carotene (Graulet, Cirie, & Martin, 2019) and some authors showed correlation between b* index and β -carotene in milk (Agabriel et al., 2007; Calderon et al., 2007; Noziere et al., 2006). Therefore, it is important to ensure that no interaction and/or interference due to variation of β -carotene content occurs on milk vitamin B₂ prediction by color/spectral analysis.

For this, 115 samples among our milk total population were analyzed both for vitamin B₂ and β -carotene content. Vitamin B₂ concentrations were in agreement with the total population concentration, and β -carotene concentrations were in the variation range of the literature, still in the lowest part (Agabriel et al., 2007; Noziere et al., 2006). First, evolution of both components contents was studied with a correlation test. Coefficient of correlation between the two components was 0.327 ($p < 0.001$; Fig. 3a). The significance indicated that the two parameters generally evolved in the same way. Data distribution illustrates a large variability, but generally, a milk rich in vitamin B₂ is also rich in β -carotene. In a second step, we studied if prediction errors of vitamin B₂ content were explained by β -carotene concentration. Thus, we observed that β -carotene concentration has no link with residuals of models (Fig. 3b and c). These two results showed that there seems to be no influence of β -carotene concentration on vitamin B₂ prediction models. However, these tests must be made on milk samples containing more β -carotene to validate these results on the complete variation range of β -carotene concentration.

Several points could explain these results. In milk, mean concentrations are 10-fold higher for vitamin B₂ than for β -carotene (2.0 mg/L i.e. 5 µM and 0.2 mg/L i.e. 0.4 µM, respectively). On the other hand, their molecular extinction coefficients are 13 cm⁻¹.mM⁻¹ and 140 cm⁻¹.mM⁻¹ at 450 nm, respectively. So milk contains 10 more times of vitamin B₂ than β -carotene, but coloring strength is 10 times more important for β -carotene than for vitamin B₂ (at least for the maximal absorption at 450 nm). Therefore, the two components could perform simultaneously on intrinsic spectral parameters. However, environmental elements could interfere in their respective spectral response since vitamin B₂ is mainly present as free form or non-covalently bound (Kanno et al., 1991; Ndaw, Bergaentzle, Aoude-Werner, &

Hasselmann, 2000) in the aqueous part of milk, while β -carotene is mainly retained in the core of fat globules because of its strong hydrophobicity (Bernard et al., 2018). So impact on color could not be the same for these two components.

5. Conclusion

The analytical method developed for quantification of vitamin B₂ in milk was evaluated and its accuracy was validated according to reference procedure. Analysis of 676 milks allowed to validate that range, limit of quantification and reproducibility of this method are consistent with variations potentially obtained with milk from various origins (breed, diet, season, geographical area). Spectrophotometer analysis allowed to estimate vitamin B₂ concentration in milk with models based on CIELAB parameters or spectral data. Using our dataset, we observed that there is no interaction of β -carotene on prediction.

These results are promising to study the effects of husbandry practices (like ruminant diet), characteristics of animals (species, breeds, physiological stages) as well as technological treatments on milk vitamin B₂ concentration. Moreover, the spectral approach provides a quick-and-easy solution to predict the vitamin B₂ concentration (especially as an indicator of the nutritional quality of milk) in very large experimental designs, in commercial farms or dairy plants.

CRediT authorship contribution statement

Sophie Laverroux: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Writing - original draft, Visualization. **Fabienne Picard:** Methodology, Formal analysis, Resources, Validation. **Donato Andueza:** Validation, Writing - review & editing, Supervision. **Benoît Graulet:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.128310>.

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