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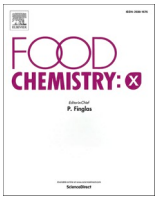

Protein binding assays for an accurate differentiation of vitamin B12 from its inactive analogue. A study on edible cricket powder

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ABSTRACT

Inactive analogues of vitamin B12 (cobalamin, Cbl) can mimic the active Cbl in food if using the traditional microbiological measurements. Thus, overestimated Cbl was recently revealed in edible insects employing immunoaffinity adsorption, HPLC-separation and mass spectrometry (https://doi.org/10.1016/j.foodchem.2021.129048). Here we demonstrate the utility of a convenient binding assay to evaluate Cbl in edible cricket powders. The assay employed the Cbl-specific protein intrinsic factor (IF) and the analogue-detecting protein haptocorrin. The excessive analogues had a weak affinity for IF, resulting in a modest overestimate of Cbl. This overestimate was corrected by a novel mathematical procedure, based on the ratio of analogue/Cbl in the sample and their relative affinities for IF. We found that 100 g of cricket powders contained 40–60 μg of analogues and 0.75–2.2 μg of Cbl. This result was confirmed by HPLC. A correct approach to Cbl-measurements is essential for nutritional assessment of any analogue-containing food.

Introduction

Cobalamin (Cbl, vitamin B12) is a crucial coenzyme produced by a number of microorganisms (Martens, Barg, Warren & Jahn, 2012). Herbivores obtain Cbl from their gastrointestinal bacteria, which ferment plant material and produce Cbl plus other corrinoids (Cbx), also called Cbl-analogues (Girard, Santschi, Stabler & Allen, 2009; Fedosov, 2012; Okamoto et al., 2021). Only Cbl is active in animal cells, and a system of specific proteins facilitates absorption and cellular uptake of the "active" Cbl to the exclusion of its analogues (Fedosov, 2012). The most important component of this system is a gastric protein intrinsic factor (IF) that strongly binds Cbl but has a low affinity for most Cbx (Stupperich & Nexø, 1991; Fedosov, Fedosova, Kräutler, Nexø & Petersen, 2007; Fedosov, 2012). The formed IF-Cbl complex is absorbed via a receptor-mediated endocytosis in the small intestine, while the majority of analogues bind to another gastric protein haptocorrin (HC), which hardly discriminates between Cbx and Cbl (Stupperich and Nexø, 1991; Fedosov et al., 2007). Preferential binding of Cbl to IF apparently prevents entrance of analogues into the body even when they are present in excess, such as that found in the digestive system of ruminants (Ortigues-Marty, Micol, Prache, Dozias & Girard, 2005; Girard et al., 2009).

Humans and other carnivores/omnivores lack the plant-fermenting rumen, which contains Cbl-producing microbiome and precedes the small intestine (where the uptake takes place). These species obtain Cbl from food of animal or bacterial origin, with a daily requirement of B12 = 2–5 μg for humans (Obeid et al., 2015; Green et al., 2017). The typical Cbl-containing food (e.g. meat, milk, fish, eggs) usually provides sufficient quantities of the vitamin with an insignificant contamination by the inactive analogues (Watanabe, 2007; Watanabe et al., 2022). Therefore, the specificity of Cbl absorption by the human intestinal system is not particularly burdened by excessive quantities of Cbx. This situation is currently changing due to the introduction of novel insect-based foods, which are environmentally friendly and which possess

Abbreviations: BSA, bovine serum albumin; Cbl, cobalamin/vitamin B_{12}; Cbi, cobinamide; Cbx, unspecified corrinoid unequal Cbl; CN/HO/Me/AdoCbl, cyano-/aquo-/methyl-/5′-deoxyadenosyl-cobalamin; $CN^{57}Cbl$ or ^{57}Cbl, radioactive $CN[^{57}Co]Cbl$ (the same for a 58-cobalt isotope); CPM, counts per minute; DMB, 5,6-dimethylbenzimidazole; fA, factor A; fS, factor S; HC, haptocorrin; HPLC, high performance liquid chromatography; IF, intrinsic factor; pB, pseudo B12; ΣCor, total content of corrinoids (Cbx + Cbl).

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generally promising nutritional profiles (Van Huis & Oonincx, 2019; Kårlund et al., 2020), but which lack an accurate characterization of Cbl-content.

Several recent investigations challenge the accuracy of the microbiological measurements, which continue to be frequently employed in food industry. It is increasingly recognized that many unconventional foods contain large pools of the inactive Cbx, where Cbl represents just a small fraction of total corrinoids (Watanabe, Takenaka, Abe, Tamura & Nakano, 1998; Edelmann, Aalto, Chamlagain, Kariluoto & Piironen, 2019; Schmidt, Call, Macheiner, & Mayer, 2019; Okamoto et al., 2021; Watanabe et al., 2022). The excessive levels of Cbx can compromise the accuracy of food declarations, if employing the microbiological Cbl-assay with *Lactobacillus delbrueckii* (Davis, Moulton & Kelly, 1973). For example, pseudo B12 (pB), factor A (fA) or factor S (fS) (inactive in humans and animals) can substitute for Cbl during incubation with *L. delbrueckii* (Watanabe et al., 1998), which results in an inaccurate, overestimated quantitation of Cbl (Watanabe et al., 2022).

The major difference between Cbl (Fig. 1) and its potential mimics concerns the nucleotide base (Fig. 1, blue solid box) situated below the corrin ring (Fig. 1, red dashed box). In Cbl this is 5,6-dimethylbenzimidazole (DMB), while in pB, fA and fS the adenine derivatives substitute for DMB. Many bacteria either have a low specificity for such change or can replace the unsuitable nucleotide by DMB, e.g. *L. delbrueckii*, *Euglena gracilis*, *Chlamydomonas reinhardtii*, *Pavlova lutheri*, etc (Helliwell et al., 2016; Watanabe et al., 2022). In both cases, the analogues promote the growth of microorganisms nearly as efficiently as Cbl.

To bypass the inadequate Cbl-specificity of microbiological methods, other more accurate approaches have been introduced, involving partial purification and concentration of Cbl (present in minute quantities in the original samples), followed by HPLC and mass-spectrometry (Watanabe et al., 2022). The evaluation of Cbl is precise, but the method is rather elaborate and requires pure standards of the analogues (expected to be present in the analyzed extract). In addition, some Cbx-molecules deviate considerably from Cbl in their hydrophobicity index, and thereby might disappear during the purification procedure As a result, the assessment of Cbx/Cbl proportion would appear incorrect.

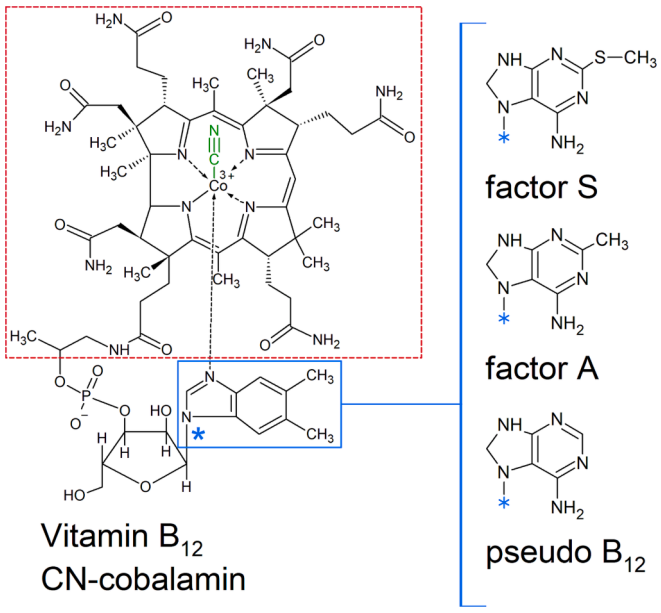

Fig. 1. Chemical structures of Cbl and selected Cbx. Cyanocobalamin (CNCbl, $M_r = 1355$) is shown to the left, while modifications in its nucleotide (blue solid frame) are shown to the right (blue bracket) for several relevant Cbl-analogues. The "upper" surface of Cbl contains [Co^{3+}]-coordinated cyanide group (green). The upper ligand in enzymatically active forms of Cbl is either a methyl- or 5'-deoxyadenosyl-group. Both decompose upon exposure to light, giving HO/H_2OCbl. Red frame outlines the structure of an "incomplete" analogue cobinamide (Cbi), lacking the whole nucleotide loop. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

An easier procedure of Cbl-measurement, and one bypassing the need for expensive HPLC and mass spectrometry platforms, employs a competitive binding assay with a Cbl-specific protein IF (Lau, Gottlieb, Wasserman & Herbert, 1965; Sobczyńska-Malefora et al., 2021). This method has a high sensitivity and does not require any purification of Cbl. Therefore, it is widely used in clinical medicine and biomedical research when measuring Cbl in blood or animal tissues, where the content of Cbx is low (Fedosov, 2012). Nevertheless, this approach is largely unused by the food industry, possibly because there is a lack of systematic studies on samples containing Cbl-analogues in great excess. Indeed, it has been established that the competitive binding assay with IF gives lower values of Cbl than the microbiological method, when both were applied to items with high levels of Cbx (Watanabe et al., 1998). Yet, the error introduced by Cbx into the Cbl-measurements by IF is of genuine concern but has never before been examined in a quantitative way, making applicability of this otherwise convenient method uncertain, at least for sources with a high content of Cbx.

We sought to correct this knowledge gap in the methodology of using a competitive binding assay for Cbl measurement by examining the impact of analogues on determination of Cbl in two preparations of edible cricket powder, which reportedly contain a high Cbx/Cbl ratio (Schmidt et al., 2019; Okamoto et al., 2021). By doing so, we aim to provide an accurate, convenient, and relatively low-cost quantitative assay for Cbl in complex substrates containing high levels of Cbx, such as those the food industry is increasingly presented with. A manual setup of the competitive binding assay employed two different Cbl-binding proteins (HC and IF). The HC-based measurements assessed the content of total corrinoids (ΣCor = Cbl + Cbx), while the IF-based measurements assessed primarily Cbl. Competitive binding curves of Cbl *vs* a pure Cbx-sample were constructed for each sample to be analyzed. Importantly, a novel mathematical correction was incorporated into the analytical scheme that allowed us to calculate the "true" Cbl on the background of highly excessive Cbx. In addition, we used the HPLC analysis to confirm the composition of samples and verify the results of IF-based measurements.

Materials and methods

Materials

All salts and standard reagents were purchased from Sigma-Aldrich (USA, MO). Radioactive tracer CN[$^{57}Co^{3+}$]Cbl was obtained from MP Biomedicals LLS (USA, OH). The scintillation cocktail OptiPhase HiSafe 3 was supplied by Perkin Elmer (USA, MA). The Cbl-specific binding protein IF was obtained from Xeragenx (USA, MO). The Cbx-specific binding protein HC was purified from buffalo milk (HC) as described elsewhere (Fedosov et al., 2019). CNBr-activated Sepharose was from Merck (Germany, Darmstadt). Two samples of cricket protein powder were a generous gift from Entomo Farms (Canada, Norwood, Ontario) and included (i) a regular cricket protein powder (Lot: C3211029) with the listed content of vitamin B12 = 7.65 μg per 20 g; and (ii) an organic cricket protein powder (Lot: O3210910m) with the listed B12 = 11.85 μg per 20 g. For production details of the powders see Section Insect housing and processing.

Insect housing and processing

The crickets were raised in 20,000 square foot retrofit chicken barns. Each barn housed approximately 35 million crickets. Ingredients of conventional cricket chow included corn, corn gluten meal, soy, flax cake, vitamin pack and mineral pack. Organic chow contained corn, soy, fish meal, vitamin pack and mineral pack. The harvesting method involved shaking the cardboard housing units over a large rubber bin

when no remaining feed was present. This procedure dislodged the crickets, which fell into the bin. Any unexpected/accidental carry over of feed would be insignificant in terms of the biomass and the B12-content. The harvested insects were powdered and processed in commercial ovens for at least 100 min at 125 °C. During processing, no parts of the animal were abstracted. There was no isolation of components (protein, fats etc.), or exposure to sterilizing radiation.

Extraction procedure

In a typical experiment, 0.5 g of cricked powder was suspended in 15 mL of 0.32 M NH_4-Acetate buffer, pH 4.6. The sample was homogenized for 2 min with help of a blade disperser, whereupon the solution was placed in several 2 mL screw-top vials. Then KCN was added to the final concentration of 0.1 mM, and the homogenates were heated for 10 min at 95 °C. The vials were cooled under water flow and left – with periodical agitation – for approximately 1 h under the light from two standard bulbs (2×450 lm) to accomplish conversion of all Cbl-forms to CNCbl (Pratt, 1964; Pratt, 1972). The samples were centrifuged on a benchtop centrifuge (20 000 g, 10 min) and stored frozen at −20 °C. This procedure is based on a previously established efficient method for extraction of total Cbl tested in an artificial mixture with a very sturdy Cbl-binder HC from buffalo milk (Fedosov, Nexo & Heegaard, 2023).

In a variant of the extraction procedure, the homogenization was performed in 0.15 M NaCl containing 10 mM Na-phosphate buffer, pH 6.8. The obtained extract (+KCN) was heated and illuminated before measurement of Cbl.

In another variant, the NH_4-Acetate extract (pH 4.6) was neutralized by adding Na-phosphate buffer (final concentration of 0.05 M) followed by gradual titration with NaOH until the desired pH 7.5 was reached.

Determination of total corrinoids and uncorrected Cbl

Our setup generally followed the binding assay described earlier (Lau et al., 1965) with some modifications. In short, a Cbl-containing solution (either calibrators or experimental samples) where pre-mixed with a radioactive tracer ($CN^{57}Cbl$) and both added to either HC or IF in a neutral solution of bovine serum albumin (BSA). The final mixture (1 mL) contained either HC or IF ≈ 25 pmol/L, BSA = 1 mg/mL, 0.1 M Na-phosphate pH 7.5 (or 0.2 M buffer, when adding 0.1–0.2 mL of pH 4.6 extracts), $CN^{57}Cbl$ ≈ 25 pmol/L (total counts 5000–8000 CPM per sample) and Cbl = 0–200 pmol/L. Each experimental sample was prepared in triple with different volumes of the test solution added, e.g. 5–20 μL of a four-fold diluted extract for Cbx measurements by HC; or 20–200 μL of an undiluted extract for Cbl measurements by IF. The binding mixtures were incubated at room temperature for ≈ 1.5 h (in one instance for 3 days), whereupon protein-free ligands were adsorbed on 0.2 mL of 5% charcoal (with BSA = 1%, Tris = 0.1 M, NaCl = 0.1 M, NaN_3 = 1 mM). The suspension with charcoal was vigorously shaken and stored for 5–15 min with periodical agitation. The samples were centrifuged on a benchtop centrifuge (20 000 g, 20 min), whereupon the supernatant (1.15 mL) was collected and mixed with 4 mL of a scintillation cocktail (OptiPhase HiSafe 3). Each mixture was counted for 2 min and compared to a calibration curve, prepared and counted in the same run. The related calculations are explained in two Sections Mathematical procedures ....

Separation of Cbx and Cbl by HPLC

The designed "ecological" HPLC gradient used only nontoxic solutions. The gradient included two solvents: A (0.1 M acetic acid, pH 2.8) and B (solvent A with 80% ethanol). They were mixed at the indicated time intervals as follows: (0 min, 92% A + 8% B), (20 min, 88% A + 12% B), (23 min, 10% A + 90% B), (28 min, 0% A + 100% B), (30 min, 92% A + 8% B), (40 min, 92% A + 8% B); with linear transitions between these points. The gradient was applied at a flow of 1 mL/min to a reverse phase column (Luna 3u C18(2), 150×4.6 mm, 100 Å, 3 μm, 400 m^2/g, Phenomenex) warmed to 40 °C. A mixture of cyanated standards (100 μL containing Cbi = 3 μM, fA = 1 μM, pB = 1 μM, Cbl = 2 μM) was used for calibration of the HPLC profile. Elution of the standards was monitored by their absorbance at 254 nm. Elution of fA, ready available from our previous works (Fedosov et al., 2007), was expected to coincide with fS, because both molecules have similar hydrophobicity indexes.

Separation of corrinoids in the two cricket extracts (obtained from insects fed on either a Regular or Organic diet) was done using the same procedure as for the standards. In all cases, 100 μL of an undiluted extract was injected into the HPLC system, whereupon 1 mL fractions were collected at a flow of 1 mL/min. To avoid any partial hydrolysis of Cbx and Cbl at pH 2.8, acidity of each HPLC-fraction was adjusted to pH 4.6 by adding NH_4OH to the final concentration of 0.05 M. Then the liquid phase was removed in a centrifugal evaporator (2 days, no heating). Such procedure is not expected to cause any degradation of the cyanated analytes (Pratt, 1972). The dry solids in each vial were dissolved in 0.5 mL of 0.2 M Na-phosphate buffer pH 7.5 with 0.1 mM KCN. After incubation for 20–30 min with periodical agitation, the samples were centrifuged and either stored in a refrigerator or immediately used for determination of Cbx and Cbl. The sample volumes (taken for the competitive binding assay) varied and corresponded to 50–120 μL for HC-analysis or 200–300 μL for IF-analysis.

Protein-free and protein-bound corrinoids in the experimental extracts

Organic homogenate in 0.15 M NaCl, pH 6.8 (not subjected to heating with cyanide) was centrifuged. The issuing supernatant (1.8 mL) was mixed with either 0.2 mL of a 5% charcoal suspension for a test sample or 0.19 mL of salt solution for the control sample (the latter volume is corrected for solids in charcoal). After 10 min of incubation, the samples were centrifuged, supernatants were heated with KCN, illuminated, and centrifuged once more. Afterward, all soluble fractions were tested for the total corrinoid content (ΣCor = Cbx + Cbl) in the HC-assay and compared to each other, as well as to the standard NaCl extract (derived from the homogenized solid particles heated with KCN). The quantity of ΣCor, precipitated with charcoal, was assumed to be the protein-free ΣCor fraction.

Interaction of Cbx and Cbl with immobilized IF

IF was immobilized on CNBr-activated Sepharose (1 mg of IF per 1 mL of settled matrix) according to the instructions of the manufacturer. The total binding capacity of IF-Sepharose was determined after incubation of 100 μL of the settled matrix with 150 μL of 30 μmol/L CNCbl (10 min, 22 °C). Afterward, the optical density (A_{361}) of the supernatant was examined and compared to that of CNCbl + Sepharose (without immobilized IF). Percentage of the difference was assumed as CNCbl bound to IF-Sepharose. The conjugated IF appeared to be not homogeneous in terms of its Cbl-binding properties and contained several fractions with different affinity for CNCbl. They were established in a binding assay using increasing concentrations of $CN^{57}Cbl$ = 10–400 nmol/L (a constant $CN^{57}Cbl$ ≈ 10 pmol/L with variable concentrations of "cold" CNCbl). The IF-fractions in the ready mixture (see next paragraph) included: (1) a high affinity IF ≈ 90 nmol/L, capable to bind all $CN^{57}Cbl$ = 10–90 nmol/L; (2) a low affinity IF ≈ 120 nmol/L, capable to bind only a part of $CN^{57}Cbl$ = 100–210 nmol/L; and (3) a very low affinity IF ≈ 150 nmol/L, which did not bind $CN^{57}Cbl$ = 220–400 nmol/L, and was calculated as a difference between the total binding capacity of IF-Sepharose and fractions 1 and 2.

Prior to the interaction with IF-Sepharose, the experimental sample (NH_4Acetate extract from Organic powder, heated with KCN at pH 4.6) was neutralized to pH 7.5, see Section Extraction procedure. Afterward, 1.87 mL of the neutral sample was mixed with 0.05 mL of IF-Sepharose slurry (1 vol of the settled matrix with 1 vol of liquid above Sepharose). In such way ≈ 90 nmol/L of a high-affinity IF was introduced (see

previous paragraph). After 30 min of incubation with constant agitation, IF-Sepharose was briefly centrifuged and settled for 15–20 min. Supernatant was collected and assessed for Cbx and Cbl. The precipitated IF-Sepharose particles were washed with 1.9 mL of 0.2 M Na-phosphate pH 7.5, and the issuing supernatant was examined on its content of Cbx and Cbl.

Effect of pseudo B12 in a competitive binding assay

Different concentrations of either cyanated pB or CNCbl were added to a standard binding mixture, used for determination of Cbl (see Section Determination of total corrinoids...). The binding reaction was initiated by addition of IF, whereupon the samples were incubated at room temperature for 1.5 h, 1 day, 3 days. The competitive binding was terminated by addition of charcoal, followed by measurement of radioactivity in the supernatants as described in Section Determination of total corrinoids....

Mathematical procedures, when assessing the uncorrected Cbl

The calibration points of $CN^{57}Cbl$ displacement by CNCbl (see Section Extraction and determination...) were approximated by a fitting function (equation 1a). The equations 1a and 1b connected the radioactive signal f with the total concentration of a test ligand (notated as x^*) in two direction with x^* as an independent variable in equation 1a and f as independent variable in equation 1b:

$$f = \Delta f_{(x^*)} + F_\infty = \frac{\Delta F}{1 + \left(\frac{x^*}{K_{1/2}}\right)^n} + F_\infty \quad (1a)$$

$$x^* = K_{1/2} \cdot \left(\frac{F_0 - f}{f - F_\infty}\right)^{1/n} \quad (1b)$$

Here $\Delta f_{(x^*)}$ is the function that describes specific response of radioactivity to changing x^*; F_∞ is the predicted signal at $x^* \to \infty$; F_0 is the starting signal at $x^* = 0$; ΔF is the maximal amplitude of specific response. All F-parameters are positive and they are connected to each other as $F_\infty = F_0 - \Delta F$. Other parameters of equations 1a and 1b include: $K_{½}$ – the coefficient of half-response (i.e. $x^*_{½}$ where $f = ½\cdot\Delta F + F_\infty$); n – the power coefficient, giving a sigmoid curve at $n > 1$ (in our tests $n \approx 1.5$ on average). The radioactivity (f) of calibration samples (with the known x^*) was used to construct the calibration curve, fitted by equation 1a to assess ΔF, F_∞, $K_{½}$, and n. The radioactivity (f) in test samples was used to calculate x^* according to equation 1b and the aforementioned fitting parameters. We should point out that dispersion of x^*-data (σ_x) changes along the calibration curve in contrast to the constant dispersion of f-data (σ_f). Therefore, the weighted mean (see equation 2) was calculated for a test sample from three measurements at different dilutions d_i using the normalized dispersion $\sigma_{i\%} = 100\%\cdot(\sigma_{x^*i}/x^*_i)$ of each result:

$$\mu_{x^*} = \frac{\sum d_i \cdot \frac{x^*_i}{\sigma^2_{i\%}}}{\sum \frac{1}{\sigma^2_{i\%}}} \quad (2)$$

Mathematical procedures, when assessing the corrected Cbl

The following biding schemes was used as a reasonably accurate general approximation of the competitive binding assay in question:

$$E + n \times S \leftrightarrow ES_n$$
$$E + n \times X \leftrightarrow EX_n$$
$$\sum \left(E + n \times Y_{(i)} \leftrightarrow EY_{(i)n}\right)$$

Here, E stands for the binding protein (e.g. IF); S is a tracer-substrate (e.g. $CN^{57}Cbl$) kept at a constant concentration; X represents the competing ligand of interest (e.g. non-radioactive CNCbl); while $Y_{(i)}$ corresponds to the interfering ligand(s), e.g. pseudo B12 + factor S. The latter pool of $Y_1 + Y_2 + \ldots$ can be treated as either a single ligand (if all Y_i have similar affinities for E) or several ligands (if the affinities are different). Ligands Y with a very low affinity (in comparison to the best binding constituent(s) of Y-set) can be discarded. A power coefficient $n > 1$ was introduced to the schemes. In a formal solution, n reflects the number of ligand molecules, simultaneously binding to E. This assumption does not reflect the true binding mechanism but allows to approximate the calibration curve with a complex shape caused by (i) comparable concentrations of the binding sites and the ligands; (ii) absent equilibration, reached only after days of incubation; (iii) plotting the measured signal *vs* the total ligand concentration (to use the curve as a calibration chart). Based on the suggested binding schemes, the decrease in the specific signal of ES_n can be expressed via equation 3:

$$\Delta f_{(x)} = \frac{\Delta F \cdot es_n}{e + es_n + ex_n + \sum ey_{(i)n}} = \frac{\Delta F \cdot \left(\frac{s}{K_s}\right)^n}{(1 \approx 0) + \left(\frac{s}{K_s}\right)^n + \left(\frac{x}{K_x}\right)^n + \sum \left(\frac{y_i}{K_{yi}}\right)^n} \quad (3)$$

The function $\Delta f_{(x)}$ in equation 3 is expressed via the concentrations of all protein forms (e, es_n, ex_n, and $\Sigma ey_{(i)n}$) and the maximal response (ΔF) obtained at $s \to \infty$ and $es_n \to e_{total}$. The function $\Delta f_{(x)}$ is also a part of the fitting equation 1a, where it substitutes for $\Delta f_{(x^*)}$. The new parameters of equation 3 (K_s, K_x and K_{yi}) can be interpreted as the apparent dissociation constants of the complexes ES_n, EX_n and $EY_{(i)n}$. They were used (together with the respective total ligand concentrations) to express the ratios of protein species, e.g. $ex_n/e = (x/K_x)^n$. The expression $(1 \approx 0)$ reflects a low representation of free protein (e) after some time of incubation. The exact meaning of K-parameters might vary. For instance, if all binding reactions are regarded as irreversible (e.g. $E + n \times X \to EX_n$), then the binding rate constants substitute for the equilibrium dissociation constants, e.g. $(1/K_x)^n = k_{+x}$, etc. Such case adequately describes the initial phase of the Cbl-binding (e.g. 0–10 min at picomolar concentrations). In contrast, if all reactions approach the equilibrium (after days of incubation), then K-parameters are close to the true dissociation constants. The situation in-between (e.g. 1–2 h of incubation) gives a mixed meaning of all K-values, which does not, however, affect a formal solution of the equation.

The concentration of interest x (Cbl in the current context) was expressed via other elements of equation 3 and presented as the final equation (4):

$$x = \frac{x^*}{D}; \quad D = \left\{1 + \sum \left(\frac{y_i/x}{y_{i,1/2}/x_{1/2}}\right)^n\right\}^{\frac{1}{n}} \quad 4$$

Here $\times$ stands for the "true" (corrected) Cbl, x^* is the preliminary (uncorrected) measurement of Cbl (see equation 1b), and D (denominator) is the new correction coefficient. Calculation of the latter requires knowledge of: (y_i/x) – a ratio of concentrations for Y_i and X present in the experimental sample (e.g. Cbx/Cbl); as well as $(y_{i,½}/x_{½})$ – a ratio between the half-effect concentrations of the respective ligands ($x_{½} = s\cdot(K_x/K_s)$ and $y_{i,½} = s\cdot(K_{yi}/K_s)$), when the increasing X or Y displace S from the binding site of E. The values of $x_{½}$ and $y_{i,½}$ can be found in two separate competitive reactions $E + X + S$ and $E + Y_i + S$ (Fig. 5), conducted under the same conditions as the test assay (Fig. 2). The third parameter (n) is a power coefficient of the calibration curve, see the description of equation 1. If the ligands S and X are two chemical twins (like $CN^{57}Cbl$ and CNCbl), then $K_{½}$ in equation 1 becomes equal to s (the tracer concentration, kept constant in all assays). If X is a different molecule (e.g. a fluorescent derivative of Cbl), then $K_{½} = s\cdot(K_x/K_s)$. The most simple case of equation (4) implies presence of only one interfering ligand Y, or alternatively several Y_i with similar affinities for E (all regarded as a single ligand).

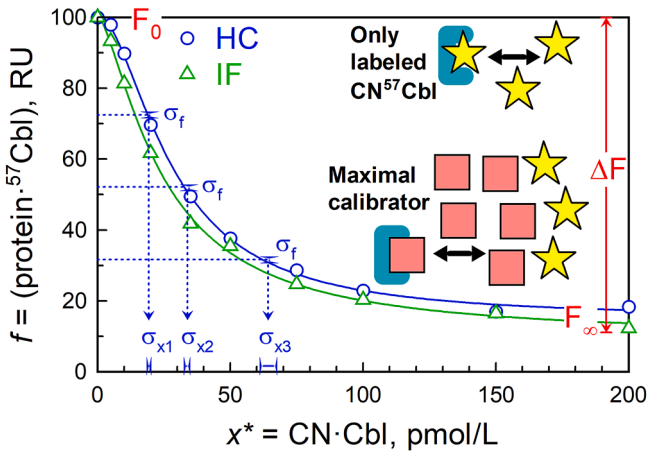

Fig. 2. Examples of the calibration curves and measurements of the ligands. Blue circles or green triangles indicate protein-bound radioactivity (RU, relative units) at different concentrations of a non-radioactive analyte (e.g. CNCbl), examined with help of HC or IF, respectively. A thick brace "[" schematically depicts the ligand-capturing protein, which preferentially binds either $CN^{57}Cbl$ (★) or the nonradioactive analyte (■), depending on a low or high concentration of the latter. The solid curves (blue and green) show the fitting function (equation 1) with the following parameters for HC (n = 1.8, $K_{½} = x_{½}$ = 28 pmol/L) and IF (n = 1.5 and $K_{½} = x_{½}$ = 25 pmol/L). Other parameters (F_0, ΔF, F_∞) are indicated graphically in red. Blue dashed lines connect radioactive counts in the binding mixture with the corresponding tracer concentrations (HC-measurements). Small blue segments approximately depict a constant error on f-axis ($f \pm \sigma_f$) and variable errors on x-axis ($x \pm \sigma_x$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Software

A freeware KyPlot 6 (KyenceLab Inc, Japan, https://www.kyenslab.com/en-us/) was used during fitting and calculations.

Results and discussion

Protein-free and protein-bound corrinoids in the untreated cricket extracts

Employing the HC-binding assay (Section Determination of total corrinoids...), the balance between protein-free and protein-bound total corrinoids (ΣCor = Cbl + Cbx) was determined in saline homogenates of cricket powders without addition of KCN (see Section Protein-free and protein-bound...). The amount retrieved in supernatants (or calculated for related pellets) is expressed as percentage of all corrinoids measured after inclusion of KCN treatment, see Section Extraction and determination.... The insoluble debris retained 18% of ΣCor, while 82 % partitioned to the liquid phase. Adsorption of the extracted corrinoids on charcoal precipitated 67 % of ΣCor, while a small fraction (15 %) stayed in the supernatant (presumably representing the protein-bound ligands).

Denaturation and digestion of different Cbl-specific binding proteins in food is necessary for the subsequent binding of Cbl to IF in the intestine (Fedosov, 2012; Obeid et al., 2015). Therefore, a high content of free corrinoids makes them readily available for the gastro-intestinal uptake by humans. It seems that most Cbl-binding proteins in the cricket powder were denatured by a lengthy heating during manufacturing, see Section Insect housing and processing. We should, however, mention that heating in the absence of cyanide does not affect the unspecific cobalt-coordination of HOCbl or HOCbx to protein residues, like histidine, thiols, and amines (Pratt, 1972; Fedosov et al., 2018). Therefore, the protein-bound Cbl and Cbx (accounting for 33 % at pH 7.5) are most probably attached to soluble and insoluble macromolecules via these unspecific cobalt-contacts. Such type of connection does not significantly constrain the multi-point interactions of Cbl and Cbx with the specific binders (HC and IF, present in human digestive tract), as was shown during the removal of HOCbl from its casein-complexes initiated by HC (Fedosov et al., 2019) or IF (Fedosov et al., 2018). Thus, >90 % of HO[^{57}Co]Cbl got transferred to the specific proteins after 30 min incubation at 37 °C. Therefore, all the cricket corrinoids are likely to be highly available for intestinal absorption in humans.

Extraction and determination of total corrinoids. A preliminary assessment of Cbl

Extraction of all endogenous corrinoids involved heating of the test homogenates with cyanide to (i) liberate Cbx and Cbl from their specific and unspecific residual complexes; and (ii) convert them to chemically stable CNCbl and CNCbx (Frost, Lapidus, Plaut, Scherfling & Fricke, 1952; Watanabe et al., 2022). The extraction was carried out in several variations using different buffers, all of them giving similar results, see Section Separation of pure chemical samples. The dilution factor of the original samples was assessed based on the weights of insect powder: buffer (g: g) and varied from 1: 30.7 to 1: 34.4. The extracts were further diluted (if necessary) to fit the scale of the calibration curve for ΣCor and Cbl (Fig. 2).

Total corrinoids (ΣCor) were examined by the HC-binding assay, while an exploratory assessment of Cbl was undertaken by the IF-binding assay. In the latter case, the readings were expected to be overestimated, because of a combined response from Cbl (a major signal) and Cbx (a minor signal). The degree of Cbx interference was examined at a later stage of this study (Section Effect of pseudo B12...), after collection of additional information about the sample composition.

In each binding assay, the calibration standard (CNCbl) or the test ligand(s) competed with a radioactive tracer ($CN^{57}Cbl$) for the binding to the specific protein (HC or IF). This caused a high/low protein-bound radioactivity at a low/high concentration of the nonradioactive ligands. Examples of the calibration curves, as well as schemes of the ligand interaction and readings for an experimental sample, are shown in Fig. 2. The fitting of calibration curves revealed that the Cbl-concentration of half-response corresponds to 20–30 pmol/L (expected to be equal to $CN^{57}Cbl$ in the binding mixture). The binding proteins IF and HC were apparently in a slight excess of $CN^{57}Cbl$. In such case, small quantities of the test ligand do not displace the tracer but bind simultaneously to the superfluous protein sites, which causes a small lag at very low concentrations of "cold" CNCbl. A complex overall shape of the curve affected the precision of Cbl-measurements in different parts of the calibration chart, see blue segments on x-axis (Fig. 2). Different reliability of measurements at different dilutions was taken into account, see Section Mathematical procedures ... uncorrected Cbl and equation 2.

Standard deviation of f-values (σ_f) varied in different experiments from 1 % to 1.5 % of relative units (see an example in Fig. 2). This corresponds to the limits of detection for Cbl ≤4 pM (IF-based analysis) and Cbx ≤7 pM (HC-based analysis) in the binding mixture. Both values were derived from $f = F_0 - 3{\cdot}\sigma_f$ on the respective calibration curves. Limits of quantification (stipulated as Cbl or Cbx at $f = F_0 - 10{\cdot}\sigma_f$) were found to be Cbl ≤10 pM and Cbx ≤15 pM for IF- and HC-based methods, respectively. A normalized average error ($\sigma_{i\%}$) of a single Cbl measurement corresponded to approximately ±5% of its value (e.g. 20±1 pM), if not reaching plateau of the calibration curve (Fig. 2). The outcome of ligand binding is insensitive to moderate changes in pH (e.g. 7.5±1) and ionic strength (e.g. 0.2–0.4 M).

All measurements of ΣCor and Cbl in the cricket samples are summarized in Table 1 and Supplementary Fig. S1. HC-based measurements of ΣCor are presented as pmol/g, or μg/100 g, or %. The values of pmol/g are more accurate, because they are derived from displacement of a known picomolar quantity of $CN^{57}Cbl$ from the binding site of HC, which efficiently interacts (mol per mol) with both Cbl and nearly all Cbx. Presentation of the results as μg/100 g is less accurate, because of a difference between the molecular masses of Cbl and Cbx, as well as

Table 1
Total corrinoids and Cbl in cricket protein powders.

Ligand	ΣCor	Cbl	ΣCor	Cbl	ΣCor	Cbl
Source	pmol/g	pmol/g	μg/100 g [b]	μg/100 g [b]	%	%
Regular powder	298 ±12	≤ 6.0 ±0.6	40.4 ±1.6	≤ 0.81 ±0.08	100 ±4.0	≤ 2.0 ±0.2
"true" Cbl [a]	n.a.	5.4±0.5	n.a.	0.75 ±0.05	n.a.	1.8 ±0.2
Organic powder	441 ±16	≤ 16.9 ±1.4	59.7 ±2.2	≤ 2.29 ±0.19	100 ±3.6	≤ 3.8 ±0.3
"true" Cbl [a]	n.a.	16.3 ±1.3	n.a.	2.20 ±0.15	n.a.	3.7 ±0.3

All results present mean ± SEM values, see also Fig. S1; n.a. – not applicable.
[a] Results of the IF-assay corrected for interference from Cbx.
[b] All μg values were calculated using M_r = 1355 for Cbl, though M_rs of different Cbx are not identical: 1317 for pB, 1331 for fA, 1392 for fS (all cyano-forms), 1042 for baseless Cbi (dicyano-form).

between different analogues in a mixed Cbx-pool. The approximate assessment of μg-values (traditionally used in food industry) was done using the molecular mass of CNCbl (see subscript "b" of Table 1). Likewise, all "raw" IF-based determinations of Cbl are notated as ≤ pmol/g, or ≤ μg/100 g, or ≤ % (Table 1). These Cbl-values contain an inherent overestimate due to an additional signal from Cbx. Yet, we could confirm already at this stage that the main fraction of corrinoids in the two edible cricket products largely consisted of Cbl-analogues, because the measurements by HC (ΣCor = 40–60 μg/100 g) exceeded by far the measurements performed by IF (Cbl ≤2.3 μg/100 g). This observation agrees with two recent reports, where the composition of corrinoids was examined by more elaborate methods (Schmidt et al., 2019; Okamoto et al., 2021), but contrasts with the high values of Cbl listed on the nutrition declarations of various insect products, reviewed by Finke (2008), Finke (2015). Such contradictory information underlines the necessity of appropriate detection methods, which can discriminate between Cbl and Cbx and give realistic values for the bioactive vitamin B12.

Separation of pure chemical samples of Cbx and Cbl by HPLC

A calibrating separation of CN-forms of several Cbx-species and Cbl (all available from our previous studies (Fedosov et al., 2007) was performed by HPLC in Fig. 3 (repeatability of elution time ±0.11 min, peak area ±2%). It revealed that three "complete" corrinoids (factor A, pseudo B12 and Cbl) were eluted at the end of the chromatogram (Fig. 3), while the baseless corrinoid dicyano-Cbi (with upper and lower CN-groups) was eluted earlier as two widely separated peaks. Splitting of dicyano-Cbi into two species at acidic pH is caused by dissociation of one of the two cyanide anions from either "upper" or "lower" surface of Cbi, giving approximately equimolar mixture of "CN-up" and "CN-down" isoforms. Such effect has previously been described (Pratt, 1972).

Separation of Cbl and Cbx in cricket extracts

Fractions were collected from the chromatographic separation of corrinoids in raw extracts and the amount of ΣCor or Cbl determined employing the HC– or IF-binding assay. The results are shown in Fig. 4. It should be noticed that optical registration of pure corrinoids (254 nm) occurred earlier by approximately 1 mL (Fig. 3). The difference between standards and test samples was of technical nature, because the optical measurements (Fig. 3) took place earlier than collection of the samples (Fig. 4), which involved additional tubing.

Quantification of two HPLC profiles by the HC-assay (Fig. 4, each point measured in duplicate or triplicate) demonstrated that the major corrinoid in the two cricket powders corresponded to pseudo B12 (pB). Its fraction was equal to mean ± SEM = 38±3 % (n = 3) in the Regular

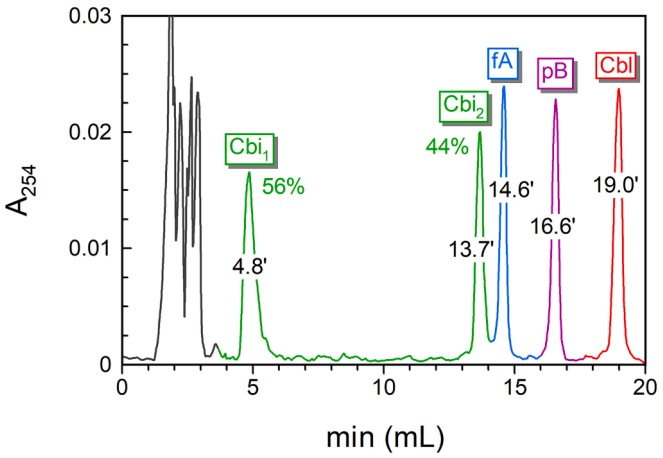

Fig. 3. HPLC profile of corrinoid standards. The mixture contained several cyanated Cbx and Cbl: gray record – injection noise and residues of the original organic groups of pseudo B12 and factor A, detached during cyanolysis; green record – a baseless corrinoid cobinamide (Cbi) eluted as two isoforms (Cbi_1 and Cbi_2); blue peak – factor A (fA); magenta peak – pseudo B12 (pB); red peak – Cbl. The structures of all compounds are shown in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

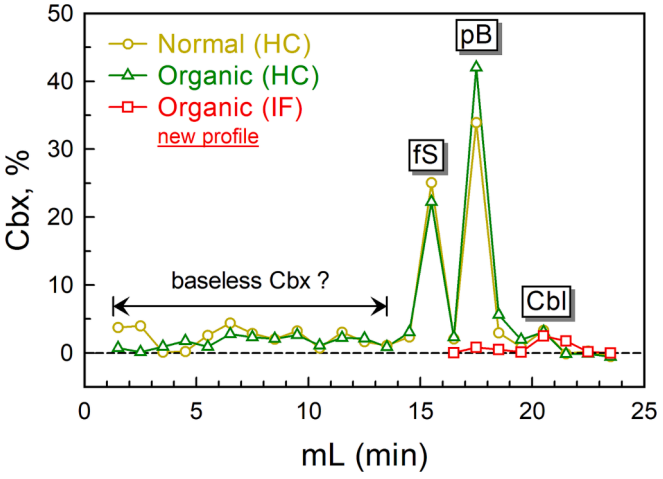

Fig. 4. HPLC profiles of cricket extracts. Regular powder (–○–, dark yellow) contained pseudo B12 (pB) = 38±3 %, factor S (fS) = 27±1 %, multiple unidentified (baseless?) Cbx ≈ 21 %, and Cbl ≈ 3±1 %, according to HC-measurements. Organic powder (–Δ–, green) contained pseudo B12 (pB) = 49±3 %, factor S (fS) = 27±1 %, multiple unidentified (baseless?) Cbx ≈ 30 %, and Cbl ≈ 3±1 %, according to HC-measurements. A separate HPLC profile of Organic extract (–□–, red) was measured by IF, adding 3.5-fold more material from the Cbl-containing fractions to the competitive binding assay. These measurements gave Cbl = 4.2±0.3 % and a small signal in pB-fractions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

powder and at 49±2 % (n = 3) in the Organic powder. The second largest HPLC peak coincided with factor S (fS) or factor A (fA), and it accounted for 28±1 % and 26±1 % in the Regular and Organic samples, respectively. Elution of fS is indistinguishable from fA in our settings, but the presence of fS should be expected according to the literature (Okamoto et al., 2021). The fraction of Cbl ≈ 3±1 % was very low, and its assessment by the HC-assay was rather rough. The amount of Cbl was further specified in the Organic sample by the IF-measurements, where 3.5-fold more of the fraction material was added. The assay gave a better precision of Cbl = mean ± SEM = 4.2±0.3 % (n = 3). The HPLC data for

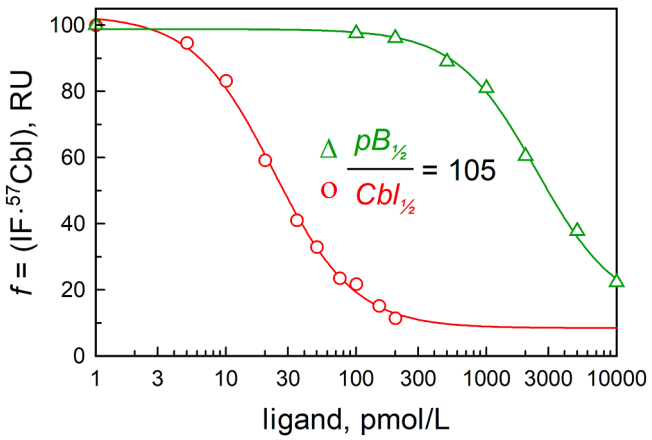

Fig. 5. Displacement of ^{57}Cbl from IF by either pB (Δ green) or Cbl (○ red) after 1.5 h incubation. Both fittings were done by equation 1 with a stipulated power coefficient of $n = 1.4$ (the average of n = 1.5 and 1.3 for Cbl and pB curves, respectively). The concentrations of half-effect corresponded to $Cbl_{½} = 23.3$ pmol/L (for Cbl) and $pB_{½} = 2450$ pmol/L (for pseudo B12). The *x*-axis (the ligand concentrations) is presented on a logarithmic scale, and the *y*-axis shows IF-bound radioactivity in relative units (RU). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cbl showed a good correspondence with the measurements obtained by the IF- and HC-tests, i.e. Cbl ≤2 % in the Regular powder and Cbl ≤3.8 % in the Organic powder, Table 1. It should be noted that precision of the above HPLC data depended not on the chromatographic method itself but on the volumes of HPLC fractions taken for analysis by the competitive binding assay (Section Extraction and determination... and Fig. 2). Thus, the HPLC peaks were examined at the concentrations of analytes well above the limits of quantification (Section Extraction and determination...): pB and fS in the range of 20–60 pM during the HC-based assay, and Cbl in the range of 16–23 pM during the IF-based assay.

Apart from the well- defined peaks of pB, fS and Cbl, both cricket samples contained small quantities of various compounds, resembling baseless Cbx (Fig. 4, elution at 1.5–13.5 mL). The probability of an overall zero level on this stretch of the HPLC profile was very low: $p = 9\times10^{-5}$ for the Regular powder and $p = 2\times10^{-5}$ for the Organic powder. The unidentified corrinoids might represent: (i) various metabolic precursors of the "complete" corrinoids during their bacterial synthesis in insects; or (ii) the products of decay, generated in course of a lengthy heating during the manufacturing of the cricket powders (see Section Insect housing and processing).

Effect of pseudo B12 on Cbl-measurements in the current IF-assay

Precise determination of Cbl by IF in a competitive biding assay (if excessive analogues are present) requires knowledge about the relative affinities of these analogues for IF (see theory in Section Mathematical procedures ... corrected Cbl and practical examples in Section Correction of Cbl measurements). In the current work only pure pB was tested, because its derivatives (e.g. fA, fS etc.) are expected to have similar apparent affinities for IF (Kolhouse & Allen, 1977; Stupperich et al., 1991; Fedosov et al., 2007). Increasing concentrations of pB decreased the binding of radioactive ^{57}Cbl to IF, though the potency of pB was much lower than that of Cbl (Fig. 5). The ratio of half-effect concentrations for these two ligands corresponded to $pB_{½}/Cbl_{½} = 105\pm13$ after 1.5 h of incubation (Fig. 5), 124±28 (after 24 h, not shown), and 148±6 (after 68 h, not shown). Such drift from small ratios to large ratios in course of incubation reflects a slow transition of the preliminary binding complexes to the species of final equilibrium. The initially formed quantities of IF·Cbx and IF·Cbl are usually quite comparable (e.g. IF·Cbl ≈ IF·pB), because of the comparable binding rate constants (e.g. $k_{+Cbl} \approx k_{+pB}$) (Fedosov et al., 2007). Yet, a continued incubation leads to a gradual redistribution in favor of a tighter complex (e.g. IF·Cbl). This transition ends by a high excess of IF·Cbl above IF·pB due to their largely different equilibrium dissociation constants ($K_{Cbl} \ll K_{pB}$). The kinetics of such competition was explored earlier (Fedosov, Petersen & Nexo, 1995; Fedosov et al., 2007) and revealed that a high excess of Cbx above Cbl might require days of incubation before the true equilibrium is reached. Considering the full equilibration not very practical, the time of incubation becomes an important factor. Thus, a short incubation of the binding mixture is expected to give a higher impact of analogues on Cbl-measurements, while a longer incubation would give a lower impact (if all other conditions are the same).

Correction of Cbl measurements for presence of its analogues

The previously obtained "raw" measurements of Cbl (Table 1) were adjusted after division by the correction coefficient $D \geq 1$ (equation 4). Its value was determined considering two additional ratios of (i) the half-effect concentrations $y_{½}/x_{½} = pB_{½}/Cbl_{½} = 105$ (see Section Effect of pseudo B12...); and (ii) the ligand concentrations $y_i/x = (pB + fS)/Cbl = (38 + 27)/2 = 32$ for the Regular powder and $(49 + 27)/4 = 19$ for the Organic powder, see Fig. 4. Presence of incomplete analogues (Fig. 4) was ignored, because their affinity for IF is extremely low (Stupperich and Nexø, 1991); Fedosov et al., 2007). The results of corrections are summarized in Table 1 in the rows notated as "true" Cbl, referring to the content of Cbl without a false increment added by the analogues.

We found that the interference of an excessive pB (or fS) with the Cbl-measurements by IF was minimal at pB/Cbl < 30 and $pB_{½}/Cbl_{½} > 100$, at least in the described setup with the incubation time of 1.5 h. Yet, a shorter incubation (e.g. 10 min), or a lower difference in the apparent affinities of Cbx *vs* Cbl, or a higher proportion of Cbx/Cbl might increase the error. A consideration of the incubation time is of special relevance in the automatic methods, such as those used in hospital laboratories. It should be noticed that these routine procedures have a fast turnaround time (e.g. 27 min for all procedures, Pirkle, 2018) with a fixed ligand-binding time of approximately 10 min. Such short incubation is expected to lower the ratio of apparent half-effect concentrations ($y_{½}/x_{½}$), as was discussed in Section Effect of pseudo B_{12}.... A simple assessment of D-coefficient in equation (4) demonstrates that $y_{½}/x_{½} = 21$ (instead of 100), a 30-fold excess of an analogue, and a power coefficient of $n = 1.4$ (as in Fig. 5) would give $D = 2.004$ and a 2-fold overestimate of Cbl in a "raw" IF measurement. Therefore, an accurate planning of the automated B12 measurements is required including: (i) some knowledge about Cbl and Cbx composition in the examined material (many samples are already described in the literature); and (ii) analysis of the displacement curves (like those in Fig. 5), produced under the exact conditions of the automated (or manual) competitive assay.

Interactions of IF with Cbx and Cbl in relation to their intestinal uptake

We explored whether Cbx in cricket powder might possibly bind to IF at physiological concentrations of the binding species. For this purpose, a neutralized extract from Organic cricket powder was mixed with IF conjugated to Sepharose (Section Interaction of Cbx and Cbl ...). The prepared suspension contained 12.5±0.8 nmol/L of corrinoids and ≈ 90 nmol/L of a high-affinity IF. In comparison, a typical concentration of IF in gastric juice is of approximately 50 nmol/L, reviewed by Fedosov (2012). IF-Sepharose absorbed ≈ 78 % of Cbx and all Cbl, as was found by the HC– and IF-assays, respectively. Washing of the matrix released ≈ 22 % of Cbx but no Cbl, indicating a low/high stability of the respective protein-ligand complexes.

The above experiment introduces an issue of potential clinical concern. Even though the ingested non-Cbl corrinoids are generally not considered available during human intestinal uptake, there is no direct proof of this belief. Some observations indicate that if non-Cbl corrinoids are absorbed systemically in high quantities they might act as

antagonists, inhibiting cellular uptake or metabolic pathways dependent on the Cbl co-factor (Bito et al., 2020). Animal experiments are somewhat contradictory as to whether antagonism of absorbed Cbx exists. Thus, simultaneous oral administration of a mixture (CN^{57}Cbx + CN^{58}Cbl = 5 pmol + 5 pmol) was accompanied by a low but detectable absorption of Cbx in rabbit kidneys, liver, lung, and heart that varied from 2 to 90 % compared to the uptake of CN^{58}Cbl (Kolhouse & Allen, 1977). The most relevant analogues (pB and fA) were accumulated at the respective levels of 13 % and 9 %. On the other hand, a continuous infusion (1.5 nmol/h) of naturally-occuring analogues (HOCbx) to rats did not significantly disturb their Cbl-dependent metabolism (Stabler, Brass, Marcell, & Allen, 1991). Moreover, feeding of rats with Spirulina powder seemingly increased Cbl status of test animals (Madhubalaji, Rashmi, Chauhan, Shylaja & Sarada, 2019), despite a high content of pB and a low content of Cbl in this product (Watanabe et al., 1998). It appears, therefore, that the overall impact caused by ingestion of excessive analogues to humans can be established only after a clinical study.

Irrespective of the (in)ability of various Cbx-compounds to penetrate the defensive mechanisms of human Cbl-uptake, the decrease of Cbx and increase of the "active" Cbl in insect products is desirable. Our observations indicate that quantities of Cbl can indeed be manipulated, as demonstrated by the feeding-housing programs (Regular *vs* Organic), which affected the levels of both Cbl and ΣCor (Table 1 and supplementary Fig. S1). Similar observations were reported by Okamoto et al. (2021), who suggested chow-dependent variations in the microbiome, inhabiting the digestive system of crickets. Therefore, the choice of feed potentially becomes a driving factor for optimization of Cbl/Cbx-content in insect products. Another issue concerns preservation of the produced Cbl. Thus, comparison of the whole crickets to the manufactured protein powders (Schmidt et al., 2019; Okamoto et al., 2021), as well as our measurements, apparently imply partial destruction of both Cbx and Cbl during processing. In the current work, we observed presence of several unspecified Cbx-compounds (Fig. 4) different from the two main corrinoids (pB and fS) and Cbl, and, most likely, representing products of their thermal decomposition.

Conclusions

We confirm that the "vitamin B12" contents listed on the nutritional declarations of the cricket products do not always reflect the physiologically active Cbl but correspond rather to the sum of Cbl and inactive analogues (Cbx). Establishing this distinction is very important since the total corrinoid content of some foodstuffs is largely comprised of B12 analogues (not available for the support of B12 metabolism), and a prime example of which are the edible cricket powders we examined.

During our measurements a competitive binding assay with two Cbl-specific proteins was used. This method is nearly unexplored by the food industry, though it offers the considerable convenience of allowing the use of crude food extracts. The two main forms of Cbx (pseudo B12 and factor S) were identified by HPLC and showed a limited binding to IF in a competitive binding assay. In addition, minor quantities of hydrophilic analogues were detected. These are apparently baseless corrinoids produced via decay of the major analogues and Cbl during heating of insect powders. The presence of these additional analogues was missed in other assays, which involved purification and concentration of Cbl-like molecules with a higher hydrophobicity index.

The ability of Cbx-ligands to mimic Cbl can be either reduced or amplified, depending on a long or short incubation, respectively. To subtract the irrelevant signal, we present a method for assessment of Cbl on the background of excessive Cbx using a suitable correction factor. Experimental tests showed that a 30-fold excess of pseudo B12 and factor S in comparison to Cbl gave a marginal overestimate of Cbl in an uncorrected IF-assay as compared to a corrected IF-assay, if the time of incubation corresponded to 1.5 h. Yet, the setup of IF-measurements (especially in automated machines with a fixed time scale) should be carefully planned. Our observations underscore the need to avoid the assessment of Cbl content in food products by methods that do not distinguish different corrinoids. Compromised B12 status and its potential adverse effects on health is now recognized as a common phenomenon among the elderly (Hughes et al., 2013), highlighting the importance of accurate assessment of the Cbl content of foodstuffs.

The most suitable procedures appear to be the specific binding assay (in one or another implementation) or an HPLC analysis. Yet, the two approaches pursue different purposes. While the HPLC helps to identify the individual species of the analogues, the binding assay just distinguishes between Cbl and the pooled analogues. The food industry is largely concerned with the latter issue, making the binding assay more practical.

Finally, we include a cautionary note about a possible role of Cbx in a physiological context. The excess of IF (that often exceeds the amounts of Cbl and Cbx that are present in the gastrointestinal tract) provides an opportunity for interactions of IF with Cbx. Such interaction raises some concerns as to whether Cbx in novel food products may hinder absorption and/or utilization of Cbl by human cells. The currently available data from animal models do not give reasons for a serious concern, but future studies in humans are needed.

CRediT authorship contribution statement

Sergey N. Fedosov: Investigation, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Ebba Nexo:** Conceptualization, Resources, Writing – review & editing. **Christian W. Heegaard:** Resources, Writing – review & editing, Project administration. **Jarrod Goldin:** Resources, Funding acquisition, Writing – review & editing. **Joel B. Mason:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100824.

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