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# Simultaneous determination of free biliverdin and free bilirubin in serum: A comprehensive LC-MS approach

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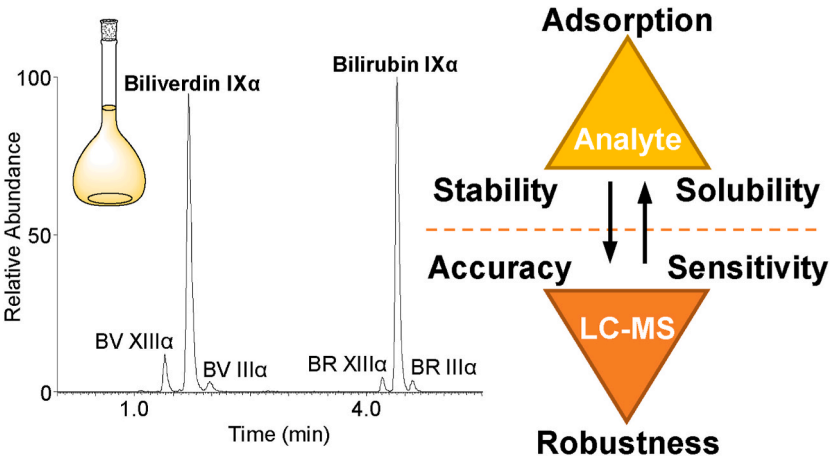
## HIGHLIGHTS

- Common sources of analytical error in the analysis of free bilirubin/biliverdin revealed.
- The first validated LC-MS method for the determination of bilirubin/biliverdin in serum.
- The LC-MS method offers excellent sensitivity, accuracy, selectivity, robustness, and high sample throughput.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Background:* Prognosis, diagnosis, and treatment of several diseases strongly rely on the sensitive, selective, and accurate determination of specific biomarkers in relevant biological samples. Free biliverdin and free bilirubin represent important new biomarkers of oxidative stress, however, the lack of suitable analytical methods for their determination has hindered progress in biomedical and clinical research.

*Results:* Here, we introduce a first comprehensive approach for robust and simultaneous determination of these bilins in serum using liquid chromatography – mass spectrometry (LC-MS). The developed analytical method exhibits linearity for both analytes within the concentration range of 0.5–100 nM, with limits of detection and quantitation determined at 0.1 nM and 0.5 nM, respectively. Moreover, several analytical pitfalls related to the intrinsic molecular structures of free bilirubin and free biliverdin and their trace concentration levels in biological samples are discussed here in detail for the first time. We have shown that the solubility, chemical

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stability, and affinity of these bilins to various materials strongly depend on the solvent, pH, and addition of stabilizing and chelating agents. Finally, the validated LC-MS method was successfully applied to the analysis of both bilins in fetus bovine serums, yielding higher free bilirubin/biliverdin ratios compared with previously reported values for human serum.

*Significance:* Failure to recognize and address the challenges presented here often leads to substantial analytical errors and consequently biased interpretation of the obtained results. This pertains not only to LC-MS, but also to many other analytical platforms due to the compound-derived sources of error.

## 1. Introduction

Bilirubin (BR) and biliverdin (BV) are natural pigments belonging to the group of bilins. From the perspective of vertebrate metabolism, BV is an intermediate and BR is the end-product of heme catabolism. More importantly, BR is recognized as one of the most potent endogenous intracellular antioxidants [1]. Upon reaction with reactive oxygen species, it converts to BV and is subsequently reduced back to BR by biliverdin reductase, thus, completing the cycle. Moreover, BR has anti-inflammatory properties [2] and exhibits anti-thrombotic activity [3]. Several epidemiological studies demonstrated that elevated serum BR levels are associated with reduced risk of developing various cardiovascular diseases (CVD) or type 2 diabetes mellitus [4]. Recently, bilirubin has been described with endocrine actions and metabolic roles, such as decreasing adiposity [5]. In blood, BR is present in various chemical forms, namely, conjugated with glucuronic acid (direct BR), unconjugated but bound to serum albumin (indirect BR), and unconjugated-unbound (free BR). From a physiological point of view, the bioactive form is the free BR, however, its routine measurement in a clinical setting has yet to be implemented. Actually, the free forms of both pigments (and their relative concentration ratios) represent potentially important medical biomarkers as various physiological roles of BV and BR continue to be uncovered.

The advancement of biomedical research involving free BR and free BV has always been hindered partially due to the absence of robust (bio) analytical methods for their determination in biological fluids and tissues. Both compounds have carboxylated tetrapyrrole chemical structures which govern their complex physicochemical properties. Notably, despite extensive chemical research, their pKa values have still not been clearly determined. In contrast to BV, the structure of BR is substantially stabilized by intramolecular hydrogen bonds, which are largely responsible for its poor solubility in most aqueous and organic solvents [6,7]. At least 3 positional (IIIα, IXα, and XIIIα) [8] and 4 geometric isomers (4*Z*,15*Z*-, 4*E*,15*Z*-, 4*Z*,15*E*-, and 4*E*,15*E*-) of BR are known [9], and by analogy, so are many isomers of BV [10]. However, the naturally occurring forms are 4*Z*,15*Z*-bilirubin IXα (referred to here as "bilirubin" – BR) and 4*Z*,10*Z*,15*Z*-biliverdin IXα (referred to here as "biliverdin" – BV) (Fig. S1). Nevertheless, the IIIα and XIIIα positional analogues are frequently encountered as typical impurities in commercial BR and BV analytical standards, leading to analytical bias [11]. Bilirubin is highly susceptible to degradation and geometric isomerization in the presence of light, but not BV since, only recently, a 5*Z*,10*Z*,15*E* geometric analog of naturally occurring 5*Z*,10*Z*,15*Z*-biliverdin IXα was first proposed [12]. A comprehensive and systematic evaluation of the experimental factors (solvent composition, temperature, light, pH, salinity, oxidants, additives, and metals) that affect free BR and BV determination remains to be carried out. Moreover, although the significance of sample and standard stability prior to and during chemical analysis is of paramount importance, it is often underestimated [13,14].

The diagnostic value of total and conjugated BR in clinical practice has been found to be overstated in certain cases, but low-sensitivity analytical methodologies for the determination of these species at micromolar levels in serum or bile are still considered the gold standard [15]. On the other hand, there is an increasing interest in direct measurement of low concentrations of free BR and free BV, but this has proven to be quite challenging, and requires reliable analytical solutions. Indirect HPLC-UV methods involving derivatization can be used to determine free BR and BV, but such approaches are accompanied by poor method accuracy and reproducibility [16]. The most frequently used peroxidase measurement of free BR was long considered accurate and reliable, but it is now clear that this assay suffers from the lack of selectivity in the presence of conjugated bilirubins and BR photoisomers which are readily found in biological matrices [15,17]. Sensitive methods (LOD <100 nM) based on ligand-binding fluorescent sensors provide fast indirect measurements of BR through its protein-binding dynamic equilibrium, but these methods can be prone to calibration errors and, most importantly, they lack selectivity [18–20]. A related metal-organic framework sensor was shown to enable a low 0.59 pM BR detection limit, but it could not be used for BV analysis [21]. Actually, the simultaneous determination of the free forms of BR and BV in biological matrices is a formidable task [15–22]. There is only one report describing a direct, accurate, and simultaneous determination of free BV and free BR in biofluids and is based on HPLC in combination with ultra-sensitive thermal lens spectrometric (TLS) detection [11,23]. However, the TLS detector is not commercially available and represents a rather cumbersome piece of instrumentation, as the complex detection optics need regular fine-tuning to ensure high sensitivity; this negatively affects the robustness and reproducibility of such a setup and hinders its wide applicability. The stability of sample and standard solutions presents another important analytical challenge [13]. During sample preparation and/or analysis, BR and BV can readily convert into a number of isomeric species as well as oxidation products [24–26]. If these species cannot be distinguished, they must be separated prior to their determination to ensure the quality of the experimental data (free of artefacts or interferences) and the validity of these results [15]. Only proper consideration of all major sources of error can perhaps prevent the frequent erroneous biological interpretations [27–29].

Reliable and sensitive analytical methods for the determination of free BV and free BR are needed to capture the true value of these species as they provide valuable insights into the delicate balance between antioxidant and pro-oxidant mechanisms in the body. Alterations in the BR/BV ratio may indicate pathological conditions characterized by increased oxidative stress, inflammation, or impaired hemostasis. In addition, this parameter can also serve as a biomarker for monitoring disease progression and evaluating the efficacy of pharmacotherapy. Liquid chromatography–mass spectrometry (LC-MS) has been extensively used in biomedical and biochemical research for years and is emerging as a practical and valuable metabolic phenotyping tool for molecular diagnostics and personalized medicine [30–36]. The main aim of this work was to develop and validate a fast, selective, and sensitive LC-MS/MS method for the determination of free BV and free BR in a model biological fluid – serum. However, during the course of this study many important compound-specific sources of analytical error were identified, critically evaluated, and strategies for their mitigation were proposed.

## 2. Experimental section

Chemicals and materials used in this study are listed in the Supplementary material, along with an in-depth description of the experimental design used for the development of the LC-MS method. The Supplementary material also gathers the experimental details on the

stability study of BV and BR in different solutions.

### 2.1. Preparation of standard and sample solutions

Stock BV and BR solutions (100 μM) were prepared in the absence of direct light by using DMSO which was sparged with argon beforehand. Stock solutions were further diluted with DMSO down to a 1 μM concentration. Further dilutions were made by a diluent consisting of DMSO:$H_2O$ (1:1, *v/v*) which contained 0.1 mg $mL^{-1}$ ascorbic acid (Diluent 2).

The first steps of sample preparation were carried out as described previously [11]. Briefly, a fresh set of crude fetal bovine serum (FBS) was filtered consecutively 3 times through the same AMICON 10 kDa regenerated cellulose membrane from Merck Millipore (Burlington, MA, USA) to saturate the membrane. The filtrates from the first two filtrations were discarded, but the third filtrate was collected and processed further. As demonstrated previously, the membrane is saturated with bilins after only two consecutive filtrations, as the concentrations of bilins in the third, fourth, and fifth filtrate do not vary [11]. For the analysis of BR, the filtrate (300 μL) was mixed with DMSO (300 μL), ascorbic acid was added (10 μL; 6 mg $mL^{-1}$), the solution was mixed and finally transferred into an amber HPLC vial. For the analysis of BV, the filtrate (300 μL) was acidified with formic acid (10 μL) and extracted twice with chloroform (300 μL). The organic phases were pooled, the solvent evaporated under a stream of $N_2$, and the solid residue was re-constituted in Diluent 2 (300 μL) and transferred into an amber HPLC vial. Samples were then analyzed by the LC-MS/MS method developed here.

### 2.2. LC-MS/MS method for the simultaneous determination of free BV and free BR

LC-MS/MS analyses were carried out using an UHPLC Accela 1250 system (Thermo Finnigan), coupled to an LTQ Velos ion trap MS system (Thermo Finnigan) using a heated ESI source in positive mode. The UHPLC system consisted of a quaternary pump Accela Pump, a thermostated autosampler Accela Autosampler with a 25 μL loop, and a photodiode-array detector Accela PDA Detector. Xcalibur (2.1.) software was used for collection and evaluation of the data. The separation of analytes was achieved on a Kinetex C18 EVO column from Phenomenex (100 × 2.1 mm i.d., 1.7 μm), which was equipped with an EVO C18 (2 × 2.1 mm i.d.) guard column. Full loop injections (25 μL) were made and DMSO:ACN (1:3, *v/v*) was used as a needle wash solvent. The mobile phase consisted of 5 mM AmF pH 3 (solvent A) and a mixture of 200 mM ammonium formate (AmF) pH 3, water, and ACN (2.5:7.5:90, *v/v*; solvent B) and the following step gradient was used: 0–2 min (48% B), 2–2.1 min (48–93% B), 2.1–4.5 min (93% B), 4.5–4.6 min (93–100% B), 4.6–5 min (100% B), 5–5.1 min (100–48% B), 5.1–7.5 min (48% B). Flow rate was set to 0.8 mL/min. Autosampler temperature was maintained at 15 °C and the column oven temperature at 35 °C. The MS parameters were set as follows: ESI heater temperature: 400 °C; transfer capillary temperature: 350 °C; sheath gas: 70 arbitrary units (a.u.); auxiliary gas: 20 a.u.; sweep gas: 0 a.u.; spray voltage: 3 kV; S-lens RF level: 69%. Data were acquired in positive SRM mode using an isolation width of 3 *m/z* and activation amplitude of 0.25. A transition 583.2 → 297.2 was used for identification and quantitation of BV with a normalized collision energy of 35 and activation time of 25 ms. For the analysis of BR, transition 585.2 → 299.0 was used with a normalized collision energy of 27 and activation time of 10 ms.

### 2.3. Validation of the LC-MS/MS method

To show that the developed LC-MS/MS method was fit-for-purpose, the method was validated by considering the following parameters: precision and intermediate precision, linearity, limit of detection, limit of quantitation, selectivity, matrix effects, accuracy, and robustness. The detailed validation protocol is given in the Supplementary material.

### 2.4. Implementation of the developed LC-MS/MS method for the analysis of BV and BR in serum

Commercial serum samples (gamma irradiated FBS and heat inactivated FBS) were prepared in triplicate according to the analytical procedure described above and BV and BR were determined by using the developed and validated LC-MS/MS method.

## 3. Results & discussion

Biliverdin and bilirubin are hydrophobic molecules that differ only by one double bond. In a biological setting (e.g., in animal and human serum), they are mainly conjugated with glucuronic acid or bound to serum albumin. However, it is noteworthy that small fractions of total BV and BR are also present in free form. Importantly, free BR is the bioactive form and plays a critical role in various physiological processes, including antioxidant and anti-inflammatory activities. Consequently, the measurement of both free BR and its precursor, free BV, holds particular clinical importance for assessing real-time serum antioxidant status. Free BV and free BR behave quite differently from their conjugated or bound analogues and these physicochemical properties (hydrophobicity, solubility, stability, etc.) make their analytical determination quite challenging. Here, we provide a step-by-step LC-MS/MS method development and highlight the obvious and not so obvious methodological pitfalls that can introduce significant bias into analytical results and can undermine the validity of the underlying biological interpretations that usually follow.

### 3.1. Method development

Due to the intrinsic lipophilicity of both analytes and the recognized robustness and reproducibility of reversed-phase mode of chromatography, a C18-based UHPLC column was used for the separation and quantitation of free BV and BR while keeping them well resolved from their IIIα and XIIIα positional isomers – common impurities of commercially available reference standards [11]. The selection of the organic mobile phase modifier (acetonitrile, methanol, tetrahydrofuran, or a combination thereof) had no significant influence on the chromatographic resolution of BV, BR, and their isomers. Use of acetonitrile was eventually preferred because, in contrast to other tested solvents, it gives lower system backpressures at high mobile phase flow rates, thus, reducing the overall analysis time and increasing analytical throughput. Both analytes contain multiple ionization sites, therefore, the effects of different mobile phase additives on the separation and detection sensitivity were tested next (Fig. S2). No chromatographic peaks for either of the analytes were observed when mobile phase additives were omitted. When 0.1% formic acid was used, signals for BV and BR appeared, but XIIIα and IIIα isomers did not separate from BV IXα. Better results were obtained with 5 mM AmF buffer in the mobile phase. Higher ionic strengths gradually decreased the sensitivity of MS detection with no discernible improvements in the resolution of chromatographic peaks. Further, changes in mobile phase pH affected the selectivity for BV and BR, but not in the same way (Fig. S3). A baseline resolution of BV IIIα/BV and BV XIIIα/BV was indicated at acidic conditions, however, at the same time resolution between BR and its isomers was <1.3. On the other hand, all BR isomers could be fully resolved by increasing the pH, but the problematic pair BV IIIα/BV completely co-eluted. Thus, acidic mobile phase containing AmF was selected for further improvement of the separation. By lowering the column temperature from 50 °C to 20 °C, the resolution between the most critical pairs of analytes improved on account of an increase in selectivity and retention factor (Fig. S4). As a compromise between sample throughput and resolution, a column temperature of 35 °C was selected and with an optimized step gradient program, a baseline separation of BV, BR, and their positional isomers

was achieved in 7.5 min – the time inclusive of the column re-equilibration period (Fig. 1).

Although both studied bilins contain chromophores that absorb light in the visible region of the spectrum, UV detection and quantitation was practical only in the 100 nM concentration range or above. Therefore, MS detection was preferred as it enabled the determination of both BV and BR at physiologically relevant (low nM) concentrations. Despite possessing two carboxylic functional groups the sensitivity for BV and BR in positive ion mode at 1 nM level was approximately 2–3–fold higher relative to the negative ion mode (Table S1). The order of MS analysis on the sensitivity was also evaluated with the relevant transitions listed in Table S2. Bilirubin failed to be detected when using SIM mode due to a high MS background, but both analytes were observed when using MS/MS in SRM mode with S/N equal to 9.5 and 31 for BR and BV, respectively. Employing MS/MS/MS in SRM mode offered no additional gain as there was no improvement in S/N and, compared to the MS/MS acquisition, the absolute signal intensity was roughly 5-fold lower, decreasing the detection sensitivity. The developed LC-MS/MS method for qualitative and quantitative determination of BV and BR was also validated to demonstrate its applicability to biological samples (*vide infra*). But first, a number of important analytical challenges are addressed in the next section that were identified during the method development stage and later on during validation. Care should be taken as these issues can often be overlooked, however, they negatively affect the accuracy and robustness of an analytical method, especially when structurally complex species such as the two bilins studied here are at play [6,17,22,27].

### 3.2. Analytical pitfalls and how to avoid them

During the first stages of LC-MS/MS method validation we stumbled across many analytical issues such as poor repeatability, low linearity, poor analyte stability, etc., which severely undermined the applicability of the initially developed method. Therefore, the reasons behind these issues were explored by conducting a series of experiments.

#### 3.2.1. Solubility and non-specific adsorption

Free BV, and especially BR, is well known for its poor solubility in many liquid solvents as a consequence of its limited intermolecular interactions with the solvent and a lack of chemical modifications, respectively, which are known to markedly increase solubility in biological settings (cells, blood serum, etc.). Aqueous solubility of free BR remains ambiguous and BR is often simply referred to as being insoluble. However, there are a few, but rather inconsistent, studies which estimate BR's aqueous solubility to be within a surprisingly broad range of 7 nM–2000 nM at physiological pH [7,37]. Having two carboxylic functional groups, solubility of BR shows strong pH dependence and increases significantly under alkaline conditions, but so does its degradation rate [38]. Only a few solvents such as chloroform, dichloromethane, and particularly DMSO, sufficiently solubilize these bilins at mM levels or higher. However, diluting DMSO solutions of BR and BV with water to more closely match a typical injection solvent in RP LC may lead to analyte precipitation and analytical error [39]. Thus, the effect of water fraction in the diluent on possible pigment loss from standard solutions was estimated in the concentration range of 1 nM–5 μM (Fig. 2).

With an average recovery of 96%, there was minimum-to-no loss of analytes observed when standard solutions contained at least 50% DMSO(aq) (*v/v*), but at 10% DMSO(aq) (*v/v*), recoveries were much lower. The lowest recoveries were obtained at 1 nM and 10 nM concentrations, whereas only 30% loss due to precipitation was observed at 5 μM level for BV, but not for BR. These results show that preparation of bilin solutions in highly aqueous and unbuffered solvents should preferably be avoided in quantitative analysis. Especially at physiologically relevant low nanomolar concentrations the measurement error could amount to as much as 60%, but this difference cannot be ascribed to poor solubility of the analyte. Arguably, adsorption of BV to glass HPLC vial walls could at least partially explain the phenomenon (Fig. S5). However, it is more likely that BV and BR bind to metal ion impurities within the LC-MS system flow path (autosampler needle, injector, stainless steel tubing, LC column, or ESI needle) as both analytes represent good chelating agents [40,41]. Additional research is needed to support this hypothesis, which was outside of the scope of this study. For the preparation of free BV and BR solutions, DMSO and 50% DMSO

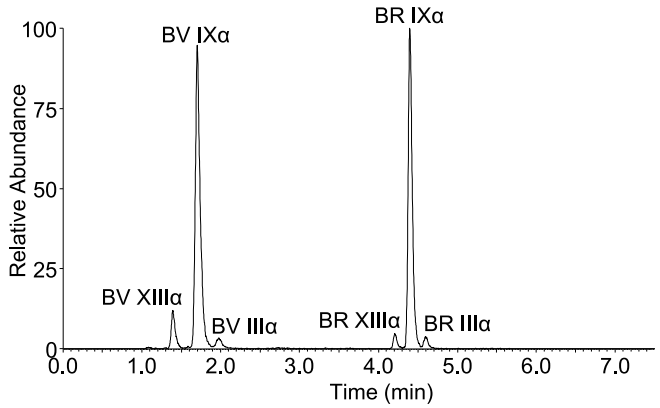


**Fig. 1.** Separation of BV and BR (10 nM) along with their positional isomers found in commercially available standards. Chromatographic conditions are described in detail in Experimental section.

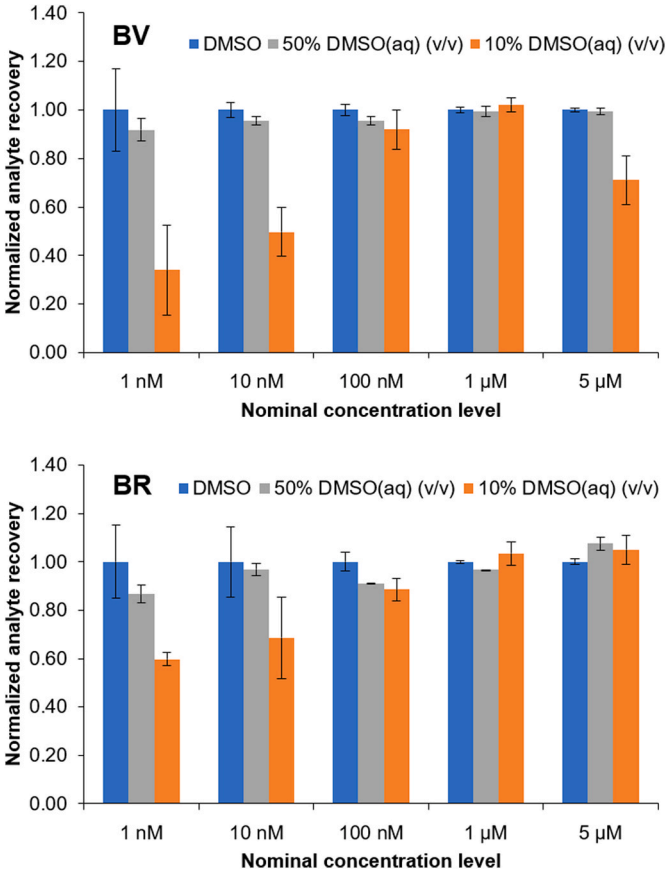


**Fig. 2.** Analyte loss as a function of solvent composition of standard solutions. Each standard solution was prepared by a 10x dilution of a corresponding solution in DMSO and was centrifuged prior to LC-MS/MS analysis. Data were normalized to DMSO at each concentration level.

(aq) (*v/v*) proved suitable, giving higher recovery and precision relative to 10% DMSO(aq) (*v/v*) (Fig. 2).

### 3.2.2. Injection solvent and injection volume

Inappropriate injection solvents are known to cause distortion of peak shapes in LC, which in turn negatively impact specificity, precision, sensitivity, and quantitation accuracy of an analytical method. As reported in the previous section, highly aqueous mixtures can cause BR and BV precipitation at high concentrations or non-specific analyte adsorption in the low nanomolar concentration range and are, thus, not preferred. On the other hand, when large 15 μL and 25 μL injections of BV and BR in 100% DMSO were made, chromatographic peaks became significantly distorted (Fig. S6). Additionally, with a melting point of 19 °C, DMSO solutions solidify in an HPLC autosampler tray at temperatures often used for biological samples (below 20 °C). Thus, we find that using 50% DMSO(aq) (*v/v*) as the injection solvent gives a good balance between sensitivity and robustness. The selection of a suitable injection solvent can also govern the stability of BR as shown in the next section.

### 3.2.3. Stability of BV and BR in solution

The developed LC-MS/MS method is reasonably fast (analysis cycle time ~ 8 min), but in order to support a high-throughput of samples, the latter should be sufficiently stable for longer periods of time in the LC autosampler. In analogy, the same applies to standard solutions of BV and BR. Since BR and BV are chemically labile compounds, their stability in solution was evaluated through a systematic forced degradation study. Both pigments were separately exposed for 48 h to different experimental conditions to assess the influence of light, elevated temperature, pH, solvents, and selected stabilization measures on analyte stability.

Bilirubin is extremely sensitive to light with reported half times of as low as 17–63 min, but its degradation rate depends strongly on the irradiation source and other experimental conditions [42]. Here, BV and BR were exposed to direct daylight or to irradiation at 366 nm. As expected, BR degraded very rapidly under both conditions, whereas the retention of 48% was observed for BV after 48 h under direct daylight as well as 366 nm (Fig. S7). Control samples, which were kept in the dark, demonstrated that in the absence of light, BV was stable for at least 48 h, whereas BR degraded, but at a slower rate, showing approximately 60% retention after 48 h. Thus, working under reduced lighting conditions during standard/sample preparation and analysis is critical to minimize photodegradation. Temperature, on the other hand, did not present a major factor (Fig. S8). Biliverdin was stable for 48 h within 5 °C–40 °C interval. Degradation kinetics of BR at 5 °C was relatively slow with the measured 73% retention after 48 h, but at 40 °C the compound degraded much faster as only 20% of initial BR remained. Solution pH is another important parameter to keep in mind, not so much for the preparation of standard solutions of bilins, but for the preparation of sample solutions of biofluids as discussed later on. Here, standard solutions of BV and BR showed similar degradation patterns at pH 3, pH 5, and pH 9, but differed in kinetics (Fig. S9). Both compounds were most stable at pH 3 (recovery after 48 h for BV and BR was 82% and 104%, respectively) and least at pH 5. They also degraded under alkaline conditions, which indicates that a commonly used procedure for dissolution of bilins in 0.005–0.1 M NaOH may introduce significant analytical error [12,38]. Moreover, pH experiments revealed a unique effect that pH has on the chromatographic signals of bilins. Relative to the highest peak areas observed at pH 9, the peak area for BV at pH 3 at time 0 was lower by 72%, however, BR was less severely affected with a reduction of 37% under the same conditions. Therefore, since solubility/adsorption and stability of bilins proportionally and inversely correlate with pH, respectively, these effects should carefully be evaluated when adjusting the pH of the dissolution solvent.

The selection of the diluent also plays an important analytical role as it must successfully dissolve the solute and at the same time prevent distortion of the chromatographic peak shape. Even more, the solvent should have no detrimental effects on the stability of analytes. Biliverdin was reasonably stable for 48 h in the three tested solvents: 10% DMSO (aq) (*v/v*), 50% DMSO(aq) (*v/v*), and 43% ACN(aq) (*v/v*), with recoveries >81% (Fig. S10). On the other hand, BR was more labile. The initial rate of degradation for BR was highest in 10% DMSO(aq) (*v/v*), but after 48 h the recovery was in the range of 12–20%, regardless of the solvent used. Thus, the use of certain stabilizers could not be avoided. Since oxidation is one of the main degradation pathways for BR, experiments were carried out where: (i) the test solvent (10% DMSO(aq) (*v/v*)) was either sparged with argon to reduce the dissolved oxygen or (ii) ascorbic acid (an antioxidant) was added to the solvent prior to the preparation of standard solutions. The effect of EDTA addition to the injection solvent was also assessed since it was previously reported that EDTA can effectively mask metal impurities within the LC-MS flow path, reducing their catalytic effect in the reaction of bilin oxidation [43]. No significant changes were observed for BV (Fig. 3). On the other hand, all three attempts to stabilize BR were successful, but only to a certain extent. Sparging the solvent with argon slowed down the degradation of BR by a factor of two. Ascorbic acid (0.1 mg $mL^{-1}$) kept the concentration of BR constant for nearly 4 h, but after that the pigment started to degrade rapidly. Solutions of BR containing EDTA (1 mg $mL^{-1}$) retained more than 74% of BR after 48 h (Fig. 3). However, EDTA simultaneously

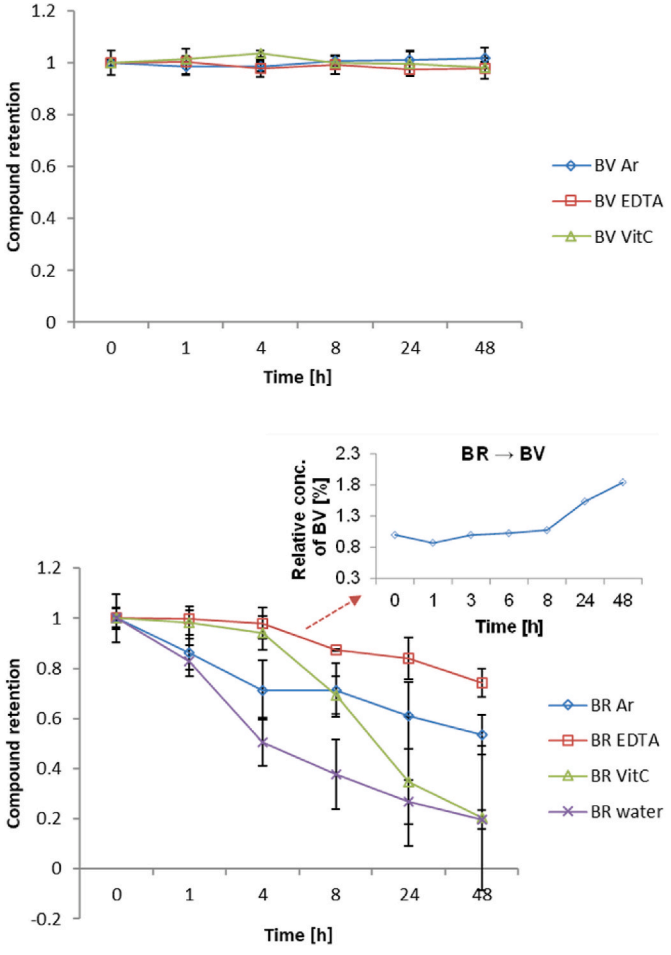


**Fig. 3.** Degradation of BV (top) and BR (bottom) under different conditions (solution sparged with Ar, solution containing EDTA (1 mg $mL^{-1}$) or ascorbic acid (0.1 mg $mL^{-1}$)). Conditions: incubation in the dark, T = room temperature, solvent = 10% DMSO(aq) (*v/v*), c = 100 nM, vessel = capped amber HPLC vial. Controls were prepared in the same manner, but no additives were used and the solution was not sparged with argon. Inset: increase in BV concentration due to BR oxidation in the presence of EDTA (1 mg $mL^{-1}$).

induced partial oxidation of BR to BV, introducing analytical bias which is reported here for the first time (inset of Fig. 3).

Between the two bilins, BV exerted higher stability in solution and particular stabilization was not required. In contrast, BR proved very labile. Shielding it from UV irradiation is obviously necessary, but additional stabilization of BR is also advised when the standard solutions are not analyzed immediately. Removing oxygen from the solution by an inert gas significantly reduced the degradation of BR, while ascorbic acid served as a shielding antioxidant. The latter is also a complexing agent for metal ions that could also assume the role of EDTA [44]. Various amounts of ascorbic acid (5–1000 μg $mL^{-1}$) were added to the BV and BR standard solutions in search of potential issues that could affect the robustness of the developed LC-MS/MS method (Fig. S11a). When using 50% DMSO(aq) (*v/v*) as an injection solvent, peak areas for BR (10 nM injections) were quite constant across the entire ascorbic acid concentration interval. The same was true for BV up to a concentration of 1 mg $mL^{-1}$, where the recorded peak area was 32% lower in comparison to the average value from other measurements. This reduction could tentatively be explained by partial BV precipitation as ascorbic acid shifts the pH towards acidic values where solubility of BV decreases. In the case of 10% DMSO(aq) (*v/v*) the results were more complex. The analyte peak areas for both BV and BR first increased with the amount of added ascorbic acid, but then decreased at the highest concentration levels (Fig. S11b). Ascorbic acid acts as a complexing agent and, arguably, masks metal ions in the LC-MS flow path which catalyze the oxidation (and degradation) of BV and BR [24]. Since the latter is more prone to oxidation, the effect of added ascorbic acid was proportionally larger. At highest concentrations, however, we believe that the protective effect of ascorbic acid is counterbalanced by a decrease in the pH of the solution, which we have shown above to decrease the bilin solubility. As expected, these effects were more evident in highly aqueous injection solvents, whereas they could be considered negligible when using 50% DMSO(aq) (*v/v*) due to a more efficient BV and BR solvation. Thus, 50% DMSO(aq) (*v/v*) that contains ascorbic acid (0.1 mg $mL^{-1}$) and that has been rigorously sparged with argon was selected as the optimum dissolution solvent for BV and BR analysis.

#### 3.2.4. Filtration of BV and BR solutions

Due to the intricate solubility properties of BV and BR and due to heavy matrices of common biological samples, solutions should be made clear of any precipitates or undissolved matter prior to LC-MS/MS analysis. For this purpose, the suitability of five commonly used membrane filters (polytetrafluoroethylene – PTFE, regenerated cellulose – RC, glass microfiber – GMF, polyvinylidene difluoride – PVDF, and cellulose acetate – CA) was assessed for the clean-up of 10 nM and 100 nM standard solutions of BV and BR. Again, solutions prepared in 10% DMSO(aq) (*v/v*) gave the most complex results (Fig. S12). It is evident that all five membrane filters bind BV as well as BR, but to different extent, so their use is discouraged under these conditions. When filtering BV and BR solutions prepared in 50% DMSO(aq) (*v/v*), the recovery of analytes improved and adsorption could be considered negligible except in the case of PVDF membrane filter which strongly retained BR, but not BV. Nonetheless, only RC membrane gave recoveries of BV and BR in the range of 99–110% at both concentration levels and could thus potentially be used to filter the solutions. Finally, an explanation for the unusually high recovery obtained for BR when using GMF filter membrane (186–213%) is warranted. The signal enhancement was caused by an unknown species, which leached from the filter (despite prior membrane washing) and caused this strong matrix effect in the MS (Fig. S12). Centrifugation of samples at high speeds is perhaps the most viable approach to remove particulate matter in this case.

### 3.3. Method validation

The developed LC-MS/MS method was validated in terms of linearity, limit of detection (LOD), limit of quantitation (LOQ), system precision, intermediate precision, selectivity, matrix effect, recovery, and robustness (stability of sample and standard solutions). The results are presented in Table 1.

The validation of the LC-MS/MS method revealed a good linear response in a broad concentration range that includes the physiologically relevant range for biofluids such as serum (1–50 nM). The method was found to be selective for both analytes using SRM mode of MS/MS acquisition, but minor matrix effects were observed for both BV (ion enhancement) and BR (ion suppression). The method can be considered precise and accurate with low relative standard deviation at the quantitation limit of 0.5 nM. Standard solutions and sample solutions were sufficiently stable in the presence of ascorbic acid in 50% DMSO(aq) (*v/v*) to enable a high-throughput analysis of 180 samples per day. It should be noted though, that two distinct sample preparation procedures had to be used for this particular analysis of BV and BR in serum, because using a single procedure for both analytes resulted either in a high matrix effect for BV (ME% = 151) or low method accuracy for BR (recovery = 62%), respectively. In addition, acidification of serum with formic acid proved to be a key step that enabled the partitioning of BV from crude, although ultrafiltered, serum (pH ≈ 7.4) into chloroform. As demonstrated by good recoveries of BV and BR listed in Table 1, acidic treatment, which was used during sample preparation, did not introduce any analytical bias into the measurement. Potentially interfering serum species such as bilin glucuronides, which could lead to the overestimation of BR and BV through deconjugation (hydrolysis), are stable at low pH used here and could be non-enzymatically hydrolyzed only in alkaline media [45,46]. The excellent performance of the developed LC-MS/MS method, supported by the validation data (Table 1), was only obtained after closely examining and overcoming the many analytical challenges discussed here. To the best of our knowledge, this is the first validated LC-MS method that enables the simultaneous identification and determination of free BV and free BR in serum.

Finally, by applying the validated method to the determination of free BV and BR in two model serum samples (gamma irradiated (GI) and heat inactivated (HI) FBS), certain similarities between bovine and reported human serums could be observed (Table S3). The concentration levels of free BR in human and fetal bovine serums are comparable, ranging from 5 to 12 nM [23]. The ratio between the determined free BR and BV, however, is much larger for the FBS serums analyzed here (>12) in comparison to the human serum (2.5–4.5), on account of low levels of

**Table 1**
LC-MS/MS method validation parameters.

| Validation parameter | BV | BR |
|---|---|---|
| Linearity range (nM) | 0.5–100 | 0.5–100 |
| [a]Slope (a) | 4406 | 3910 |
| [a]Intercept (b) | 2078 | 845 |
| $R^2$ | 0.9992 | 0.9999 |
| LOD (nM) | 0.1 | 0.1 |
| LOQ (nM) | 0.5 (RSD = 4.8%) | 0.5 (RSD = 1.9%) |
| System precision (at 10 nM) | RSD = 1.2% | RSD = 1.0% |
| Intermediate precision (at 10 nM) | RSD = 8.7% | RSD = 8.6% |
| Selectivity | No signals found in BV retention time window | No signals found in BR retention time window |
| Matrix effect (%) | 109.7 | 90.1 |
| Recovery (%) | 96.2 (2 nM)<br>111.0 (10 nM)<br>95.7 (20 nM) | 100.8 (2 nM)<br>96.6 (10 nM)<br>96.5 (20 nM) |
| Standard solution stability (10 nM) | R = 95.7% (72 h) | R = 96.9% (72 h) |
| Sample solution stability (10 nM) | R = 95.5% (24 h) | R = 103.9% (72 h) |

[a] Linear regression model was used to fit the data: $y = ax + b$, where: $y$ is the peak area, $x$ is the concentration of the analyte (nM), $a$ is the slope and $b$ is the intercept.

BV detected in bovine serums. The difference could be attributed to the interspecies variations, or to the fact that human serum samples were analyzed immediately after blood collection, whereas commercial bovine serums were firstly treated with either heat or gamma rays to remove potential microbiological agents, and then frozen for long-term storage before using them in the experiment. Further research, which falls outside of the current scope, is needed to confirm this hypothesis.

## 4. Conclusions

Evidence is accumulating that changes in concentration levels of free BV and free BR within human tissues or biofluids could provide information about the progression of many pathologies, or simply reflect the response to a particular treatment or other external stimuli. To fully exploit the prognostic and diagnostic value of these two biomarkers in the clinical setting, it is imperative to employ accurate, sensitive, and robust analytical approaches capable of measuring them in the low nanomolar range. Here, we have introduced the first validated LC-MS-based method for the simultaneous determination of free BV and BR. With a wide linearity range (0.5–100 nM), good precision, high recovery (95.7–111.0%), and low limit of quantitation (0.5 nM), the method is suitable for its intended purpose. Nonetheless, there are potential pitfalls, primarily related to the unique physicochemical properties of BV and BR, which are most apparent in highly aqueous and unbuffered media (e.g., water content >90%, v/v). Many issues can be avoided by using dilution/injection solvents that contain at least 50% DMSO (*v/v*) while analyte degradation in standard and sample solutions can be reduced by preventing oxidation of analytes, thus increasing throughput. With some adjustments, our developed method holds promise for extension to biological tissues and other biofluids such as urine, saliva, and lacrimal fluid, which are all obtained through non-invasive sampling methods. To the best of our knowledge, there is only one research study showing the LC-MS separation and detection of BV, BR, and their positional isomers within a 25-min analysis run time, but the reported method is not suitable for quantitative analysis [47]. Understanding the impact that sample/standard preparation and individual experimental parameters have on the determination, stability and solubility of free BR and free BV should also facilitate the future development of robust analytical methods. Moreover, the results presented here provide a critical retrospect of the already reported experimental data and their interpretation.

## CRediT authorship contribution statement

**Alen Albreht:** Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing, Investigation. **Mitja Martelanc:** Conceptualization, Methodology, Writing – review & editing. **Lovro Žiberna:** Formal analysis, Investigation, Writing – review & editing.

## 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.

## 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.aca.2023.342073.

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