Development and validation of an original magneto-chromatography device for the whole blood determination of hemozoin, the paramagnetic malaria pigment

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ABSTRACT

Background: Malaria remains the first global parasitic endemic disease with more than 400,000 deaths per year; there is a definite need for prevention measures and treatments, but also for rapid and low-cost diagnostic methods. Hemozoin, a detoxification polymer formed from heme by the parasite, and a likely biomarker of infection, has enticed many studies aiming at its whole blood determination. But, to our best knowledge, no accurate, precise and sensitive analytical method has been developed that could be implemented in endemic regions.
Objectives: Our group recently proposed a macroscopic trapping-dissolution method based on the paramagnetic properties of hemozoin crystals. The present paper further develops the concept into a workable fluidic device, validating an instrumental method that could be applied to the diagnosis of malaria.
Results: In the newly developed integrated on-line system, the paramagnetic crystals are successively trapped through a superparamagnetic microbeads gradient field, dissolved by an alkaline solution, losing magnetic properties, eluted and quantified by spectrophotometry at 405 nm. The analysis time is comprised between 10 and 15 min. The performances of the method have been evaluated both on aqueous suspensions of β-hematin (a synthetic pigment with physical and paramagnetic properties analogous to those of hemozoin) and *Plasmodium* cultures, including the response function, linearity, precision, trueness, accuracy and quantification limits. From β-hematin suspensions and *Plasmodium falciparum* 3D7 cultures, the limit of detection would correspond to 80 and 55 parasites/µL whole blood, respectively (0.05 and 0.033 µg hemozoin/mL).
Conclusion: In the absence of a reference method for the determination of hemozoin, its value as a malaria biomarker remains a matter of heavy debates. The newly developed magneto-chromatographic on-line system can discriminate the presence and absence of hemozoin in a sample but also accurately and precisely determine its level; application to whole-blood samples from strictly graded patients will allow to precise the usefulness of hemozoin for malaria diagnosis and/or prognosis.

1. Introduction

Malaria is the first parasitic endemic disease in the world, with 219 million cases and 435,000 deaths reported in 2017 according to the World Health Organization (WHO); there was no significant progress in reducing global malaria impact between 2015 and 2017 with the most malaria cases (92%) still registered in the WHO African Region [1]. Malaria remain a major blockage for the economic and social development of endemic countries [2]. The continued importance of malaria in the world is due to a series of factors that include the resistance of mosquitoes to insecticides, the chemoresistance of *Plasmodium*, the availability and affordability of quality antimalarials, the lack of

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effective vaccines and intensive worldwide exchanges [3]. In endemic regions, especially in remote areas, the lack of sanitary units and frequent presumptive treatments, in which all fever cases are treated as malaria, lead to the risk of *Plasmodium* chemoresistance emergence and to frequently inappropriate medications. Indeed many fever etiologies, i.e. dengue, influenza, typhoid, tuberculosis or pneumonia are difficult to differentiate from malaria only on the basis of symptoms [4]. Early and proper support, through diagnosis, is now considered as a major pillar in the fight against the disease [5].

WHO-through its *"Test-Treat-Track"* policy recommended to use a selective treatment of confirmed malaria cases [6] which relied on the availability of diagnostic tests that should be robust, sensitive, inexpensive and widely deployable in rural areas [6,7]; all malaria cases should be confirmed before antimalarial medication to avoid drug resistance, side effects, and depletion of drug precious stockpiles, particularly of artemisinin derivatives [8]. This strategy is progressively catching up with the use of membrane immunochromatographic methods, so-called *"rapid diagnostic tests"* (RDTs), a fast technique (~15 min) that does not require sample pre-treatment [9] or highly trained staff [10]. Between 2010 and 2015, the percentage of children with a fever who sought care at a public health facility in 22 African countries underwent a malaria RDT, increased approximately from 29% to 10% [7]. To diagnose malaria with usual methods faces to major constraints that limit their convenience. Reliable and quantitative assessment is performed by microscopy (thick and thin blood smears) and needs: to operate with a functioning microscope, to recruit skilled operators particularly for low parasitemia, and the degree of the sensitivity during the counting will depend on the state of tiredness of the microscopist [11]. Routinely, in endemic areas, the microscopy detection limit is estimated at about 88 parasites/μL [5]. The possibility to identify *Plasmodium* species is a major feature of microscopy; nonetheless, identification difficulties arise in the presence of parasites altered by presumptive treatment or in cases of very low parasitemia or staining problems [12]. RDTs are helpful in establishing an emergency diagnosis in endemic areas [13]; but obtained results are only qualitative or semi-quantitative, can be unreliable for low parasitemia (detection limit, ~100 parasites/μL, depending on species, strain and diagnostic device). They require special storage conditions and the results depend on the quality of products, strict adherence to manufacturer's indications, host genetic background and parasite strain [14–16]. The RDT detection principle is usually based on an immune antigen–antibody reaction; *P. falciparum* histidine-rich protein 2 (HRP2) is the most encountered and, less frequently, on *Plasmodium* lactate dehydrogenase (pan-pLDH common to all species; *Pv*-pLDH and *Pf*-pLDH, specific to *P. vivax* and *P. falciparum*, respectively). HRP2 presents a median blood clearance (up to 4 weeks) quite long compared to pLDH (1 week). This point causes a problem when you have to detect recurrent malaria infections [17] and also different HRP2 variants nowadays spread in some regions [18]. The monitoring and implementation of microscopy [11] and RDTs [19], is far from to be systematic, although the WHO has settled good practices and qualification procedures. Molecular methods based on polymerase chain reaction (PCR) techniques are the most sensitive methods (in theory, less than 1 parasite/μL), also allowing a formal identification of malaria parasites [20]. They however remain complex and costly, with a response time ranging from 3 to 16 h [4], so far, unsuitable for routine use in Southern countries [21].

Based on these ascertainments, it exists a real demand for a cheap and accessible diagnostic tool able to provide a reliable result about low parasitemia (< 200 parasites/μL) [22].

The erythrocytic stage of *Plasmodium* degrades hemoglobin, yielding free heme; in the presence of oxygen, the ferrous form (Fe^{2+}) of iron readily oxidizes to ferric form (Fe^{3+}) [23], generating reactive oxygen species, $O_2{\cdot}^-$, ${\cdot}OH$ and H_2O_2, toxic to the parasite [24]. Hemozoin, the detoxification polymer formed from heme in the digestive vacuole of the parasite [25], has enticed many studies aiming at its whole blood determination. Hemozoin is both a therapeutic target for antimalarial

drugs [26] and a likely biomarker of infection, possibly diagnosing malaria [27–29]. Hemozoin and its synthetic analog, β-hematin, have analogous structural properties, although their immunogenic properties may differ because of differing crystal sizes and/or shapes [26,30–32]. Both are paramagnetic due to their Fe^{3+} ions in high-spin configurations content [33–36]; the magnetic susceptibilities of hemozoin and β-hematin have confirmed the paramagnetic nature of their crystals [37].

Our group recently proposed a macroscopic trapping-dissolution method to isolate hemozoin, based on these crystals paramagnetic properties [38]. The present paper further develops the concept into a workable fluidic device, validating an instrumental method that could be applied to the diagnosis of malaria.

2. Description of the flow device

Roch *et al.*, [38] have shown that more than 80% of β-hematin and hemozoin (from *Plasmodium* 3D7) could be trapped using superparamagnetic microbeads placed in a magnetic field, then recovered by removing the magnetic field, dissolved and quantified. Based on these data, a magneto-chromatographic system for on-line trapping, detection and quantification has been developed, according to Fig. 1. The flow system consists in a dual syringe pump (KR Analytical, UK) generating two flows of eluents, water and 1 M sodium hydroxide, at 0.5 mL/min, connected to a TitanMX switching valve (Rheodyne, USA). The water line is equipped with a septum injector (Zuspritzstelle MF 1542, Germany) allowing to inject samples into the flow system, using 1-mL polypropylene syringes (BD Plastipak, Belgium). The magnetic filtration column is composed of a polypropylene tubing (5 cm × 1 cm i.d.) containing 0.5 mm diameter steel microspheres (Netzsch, Germany) and placed in the gap of a 0.45 T permanent magnet (MidiMACS, Miltenyi Biotec, Germany). A Z-type flow cell (Fia-Zcell-Peek, Ocean Optics, USA) is used as a support for the detection system that consisted in a light diode emitting at 405 nm (Led T-1 UV 3TZ, Farnell, Belgium) focalized through a pinhole aperture to the flow cell and photodetector (Sglux-Toc"Blue4-Photodetector, Farnell, Belgium). A home-made electronic device ensured system control, power supply, valve switching, photodetector amplification (sensitivity levels arbitrarily set from 1 to 7) and connection to a Windows-7 based laptop equipped with PCLab 2000 (Velleman, Belgium). Data were collected in the form of chromatograms and areas under the curve (AUC) were measured using GraphPad Prism 5.02 (GraphPad Software, San Diego, USA).

3. Methodology

All reagents and solvents were obtained from Chem-Lab (USA); culture medium components were from ATCC (American Type Culture Collection). All solutions and suspensions were prepared using water purified on a Milli-Q system (Millipore, USA).

3.1. Synthesis of β-hematin

The hemozoin synthetic analogue β-hematin (MW, 634 g.mol^{-1}) was synthesized from pork hemin (Sigma Life Science, > 98% pure) according to Slater's method [30]. 0.3018 g of hemin dissolved in 10 mL of NaOH 0.4 M were acidified to pH 4.0 with propionic acid and incubated at 70 °C for 18 h. The precipitate was washed in distilled water, 0.1 M sodium bicarbonate (pH 9.1) and distilled water, filtered through filter paper, dried at 37 °C for 24 h and crushed in a porcelain mortar. The powder was stored in small capped vials at 4 °C. The crystals were examined by scanning electronic microscopy (SEM XL20, Philips, Eindhoven, Netherlands; magnification: 25,000 X) and identified by infrared spectroscopy (FTIR Brucker, Tensor 27).

3.2. β-Hematin aqueous suspensions

Stock suspensions (100 μg/mL) were prepared by suspending 5 mg

Fig. 1. Simplified diagram of the magneto-chromatography device.

Fig. 2. Microscopy scan and IR spectrum of synthesized β-hematin.

of β-hematin powder in 50 mL of water, ultrasonicating for at least 20 min. Different dilutions (0.05 to 4 μg/mL) were prepared from this suspension. 300 μL of each suspension were required for flow injection analysis.

3.3. Lysis buffer

All samples of red blood cells required a lysis before analysis. The lysis solution was prepared according to Smilkstein *et al.*, [39]. A solution of 12.11 g of Tris Base in 60 mL of water was acidified to pH 7 with 1 M hydrochloric acid, completed to 100.0 mL with water and added with 10 mg of saponin and 1 mL of Triton-X. The lysis was carried out with a ¼ factor, i.e. 750 μL of blood were added with 250 μL of lysis solution and maintained 5 min at 37 ˚C.

3.4. Plasmodium culture

Plasmodium falciparum (chloroquine-sensitive strain 3D7, originally isolated from a patient living near Schiphol airport, The Netherlands; ATCC, USA) asexual erythrocytic stages were cultivated *in vitro* according to the procedure described by Trager and Jensen [40] at 37 ˚C and under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and L-glutamine. The medium was supplemented with 1.76 g/L glucose (Sigma–Aldrich), 44 mg/mL hypoxanthine (Sigma–Aldrich), 100 mg/L gentamycin (Gibco) and 10% human pooled serum (A or O Rh+, obtained from Belgian Red Cross). Parasites were subcultured every 3–4 days with initial conditions of 0.5% parasitemia and 5% haematocrit.

Thin blood smears were performed daily to assess parasites growth

Table 1
Linearity study.

Linearity parameters	Aqueous suspension of β-hematin 0.05 – 2 μg/mL	*Plasmodium* culture medium 25 – 350 parasites/μL
n	27	27
Determination coefficient r^2	0.9949	0.9909
p for the Bartlet test (homoscedasticity)	1.822E-14	2.242E-11
Eventual transformation	Square root	Square root
Slope ± standard deviation (RSD %)	1.671 ± 0.0124 (0.743)	0.1375 ± 0.0018 (1.309)
Y-intercept ± standard deviation (RSD %)	0.1542 ± 0.01159	0.02264 ± 0.0227
p for the slope significance F-test	2.58E-68	7.11E-48
p for the lack of fit F-test	2.772E-17	0.669

n: number total samples

Table 2
Precision study.

Source of Variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio (F)	p value
β-hematin samples					
Interaction	10	0.002596	0.0002596	0.7742	0.6523 [ns]
Day	2	0.0001921	0.00009604	0.2864	0.7527 [ns]
β-hematin level	5	30.15	6.03	17,980	< 0.0001 [***]
Residual	36	0.01207	0.0003353		
Plasmodium samples					
Interaction	8	0.104	0.013	22.8	< 0.0001 [***]
Day	2	0.03243	0.01622	28.43	< 0.0001 [***]
Parasitemia level	4	22.55	5.638	9885	< 0.0001 [***]
Residual	30	0.01711	0.0005704		

ns: not significant, ***: very highly significant.

and parasitemia. From a parasitemia of 5%, it was proceeded to dilution with free-*Plasmodium* red blood cells (5% hematocrit in culture medium) to obtain 0.28, 0.20, 0.12, 0.04 and 0.02% parasitemia. A second dilution (1/4) was made with lysis buffer to obtain 0.07, 0.05, 0.03, 0.01 and 0.005% parasitemia, which correspond to 350, 250, 150, 50 and 25 parasites/μL, respectively.

3.5. Malaria-free blood spiked with β-hematin

Suspensions of 0.5, 1, 2, 5, 10, and 15 μg/mL were prepared by diluting the aqueous suspension of β-hematin (1 mg/mL) in whole blood; for example, 75 μL of 1 mg/mL suspension were diluted to 5.0 mL with malaria-free whole blood to yield a concentration of 15 μg/mL. Each sample was analyzed in triplicate over 1 day.

4. Parameters of validation and statistical analyses

To document the method performances, the response function, linearity, precision (coefficient of variation), trueness (bias), accuracy and quantification limits were evaluated. Decision rules were based on accuracy profiles, statistically integrating in a single graph major elements essential for validating the method, i.e. the bias, precision, risk and quantitation limits [41]. This approach not only simplifies the validation process of an analytical procedure, but also allows to monitor risks related to its application. All graphics were obtained with GraphPad Prism 5.02.

5. Results and discussion

5.1. Characteristics of synthesized β-hematin

β-hematin is a synthetic pigment with physical [30] and paramagnetic properties similar to those of hemozoin [42,43]; both crystals,

sometimes described as *"biominerals"* rather than *"polymers"*, consist of hydrogen-bonded chains of heme dimers reciprocally linked by propionate sidechains [30] and attain lengths on the order of 1 μm, which makes them clearly visible under a visible light microscope [44]. Our synthesis yield was estimated at 43% (w/w). From Fig. 2, the size and shape of synthesized crystals correspond to those described in the literature [42] and the IR spectrum presents the characteristic peaks of biological hemozoin with signals at 1210 cm^{-1}, 1664 cm^{-1} and 1712 cm^{-1} [45] which correspond to the vibrations of C–O (C-O-Fe), C=O (O=C–O–Fe) and the hydrogen bond between two hematin dimers, respectively [30].

5.2. Validation parameters of the on-line magneto-chromatography analysis device

5.2.1. Specificity of the method

The hemoglobin forms found in blood have no magnetic properties [46] except for deoxygenated hemoglobin and methemoglobin that contain four and five unpaired electrons, respectively, per heme group, making them paramagnetic [47]. In deoxyhemoglobin, the number of iron ions per molecule is only four and the overall magnetic moment is too tiny to significantly interact with magnetic field gradients; even considering a large aggregate of deoxyhemoglobin proteins, the very low amount of iron in the agglomerate (mass ratio = 0.0033) predicts a very low magnetization of the whole entities, contrarily to what is observed for hemozoin and β-hematin [38] As methemoglobin is produced by oxidative processes, especially the interaction with nitric oxide [48], there are only very low levels in the circulation [47]. All experimental series included blank samples (i.e. unspiked or non-parasitized samples); as no peaks were recorded upon valve switching and NaOH elution, the results are not affected by these forms of hemoglobin or other blood components. As some carry-over was observed
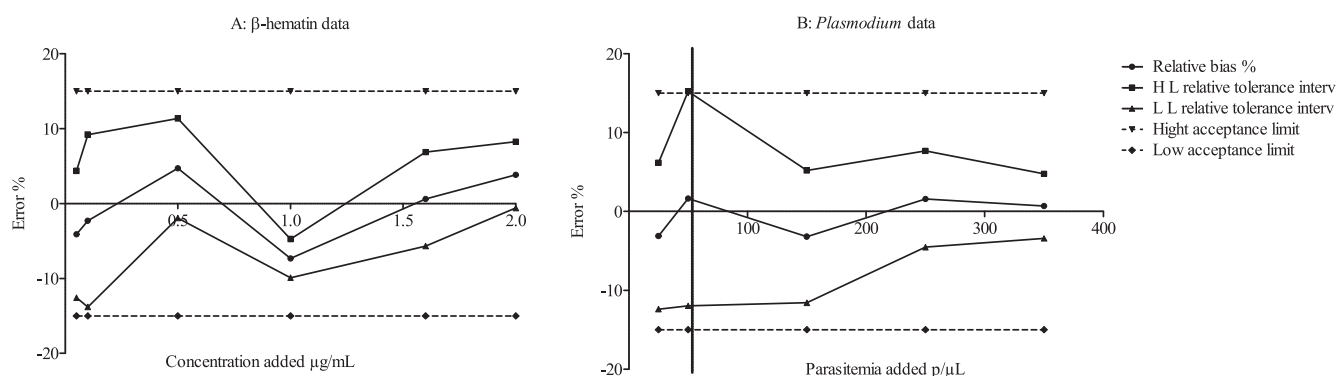

Fig. 3. Accuracy profile of *Plasmodium* and β-hematin sample (H L: high limit; L L: low limit; interv: interval).

Table 3
Validation parameters of transformed *Plasmodium* and β-hematin data.

	Conc added μg/mL	Mean Conc calculated μg/mL ± SD	Relative bias %	CV repeatability %	CV intermediate Precision %	Total error %	Maximum error observed %	Lower tolerance interval limit	Upper tolerance interval limit
β-hematin	0.05	0.05 ± 0.003	1.88	6.15	5.76	7.64	10.16	−9.65	13.86
	0.1	0.1 ± 0.006	0.23	5.95	5.69	5.48	9.23	−11.17	12.11
	0.5	0.54 ± 0.008	7.48	1.66	1.65	9.13	9.75	4.19	10.94
	1	0.91 ± 0.020	−8.53	2.16	2.10	10.63	10.73	−12.74	−4.12
	1.6	1.59 ± 0.029	−0.87	3.99	4.26	5.12	8.12	−9.38	8.22
	2	2.05 ± 0.044	2.58	3.43	3.64	6.22	7.40	−4.69	10.33
	Parasitemia parasites/μL	Mean parasitemia Calculated parasites/μL ± SD	Relative bias %	CV repeatability %	CV intermediate Precision %	Total error %	Maximum error observed %	Lower tolerance interval limit %	Upper tolerance interval limit %
Plasmodium	25	25.03 ± 1.38	0.14	3.51	6.25	6.37	9.3	−11.34	11.61
	50	51.04 ± 3.57	2.09	5.95	7.96	5.88	10.83	−12.26	16.43
	150	146.06 ± 7.83	−2.62	3.56	5.89	3.27	10.02	−13.42	8.18
	250	253.13 ± 5.94	1.25	1.83	2.67	1.42	4.02	−3.59	6.10
	350	350.05 ± 6.37	0.01	1.91	1.99	1.98	3.05	−3.98	4.00

Conc: concentration, SD: standard deviation, CV: coefficient of variation.

for the higher hematocrit samples, requiring repeated sham injections (injections of water, followed by valve switching) for extensive washing of the column.

5.2.2. Response function (linearity) of the method

The relationship between the response measured by the analysis device (i.e. the area under the curve, AUC) and the injected concentrations was investigated in triplicate on 3 different days at 6 levels of concentration (n = 54) for β-hematin and 5 levels of concentration (n = 45) for hemozoin; this for validation samples and n = 27 for standard samples Variances homogeneity of standard samples was established through Bartlett test; as homoscedasticity was not verified ($p < 0.05$) for the two types of samples, a square root transformation [49] was applied. Table 1 summarizes the linearity parameters. Both β-hematin and *Plasmodium* culture data fitted to a linear equation. The linearity range corresponded to 0.05–2 μg/mL for β-hematin samples and 25–350 parasites/μL for *Plasmodium* cultures (injection of cultures diluted at 5% hematocrit, yielding native hemozoin).

5.2.3. Precision of the method

The precision was estimated through the repeatability and intermediate precision at each concentration level as summarized in Table 2. A two-way ANOVA analysis considered two factors, "Day" and "Sample" (β-hematin level and parasitemia, respectively):

- For β-hematin, the factor "Day" ($p = 0.7527$) has no significant effect on measured concentration level. As expected, the factor "Sample" ($p < 0.0001$) has a significant effect on measured concentration; this is logical of course, as the concentration varies from sample to sample. This means that β-hematin concentrations of 0.05–4 μg/mL can be differentiated by the new device. As there is no significant interaction between the two factors ($p = 0.6525$), it can be concluded that there was no difference from day to day between the results obtained for all β-hematin aqueous samples.
- For *Plasmodium* samples, the two factors, "Day" and "Sample", and their interaction significantly impact the results ($p < 0.0001$). Here also, the different levels of parasitemia are logically discriminated by the "Sample" factor; but the significant effects of the factor "Day" and "Interaction" indicates a day-to-day variability in measured parasitemia. It should be noted that, for each daily analysis, the culture parasitemia was first assessed by microscopy evaluation (thin smears, Giemsa staining and examination over 100 microscopic fields) to then dilute the samples in the range of 25–350 parasites/μL. This significant effect of the factor "Day" may be

attributed to a day to day irregularity of the blood smears [9,50–52]; in our experiments, given the high workload of combining magneto-optical measurements and microscopy evaluation, the slides could not be repeated. To optimize the reproducibility of parasitemia evaluation, further experiments should aim at 3 measurements/slide for at least 2 operators as recommended by [53].

5.2.4. Accuracy profile and decision rules

The accuracy of a result expresses the closeness of agreement between the test result and the accepted reference value, equally called conventionally true value, and this, for each measurement [54]. The interpretation of accuracy was based on the bias of each concentration level for both β-hematin and *Plasmodium* samples. Given the biological nature of our samples, the maximum error was fixed, according to Rozet *et al.*, [55], to 15% of the true value. For accuracy profile drawing, we estimated the upper and lower limits of the tolerance interval using the trueness and precision at each concentration level. The tolerance interval, located within the acceptance limits (± 15%) with β-power of 90%, is visualized on an accuracy profile (Fig. 3), an interesting tool to evaluate the capability of a method [41,56,57]. For β-hematin samples, the fixed acceptance limits were verified for the full range of tested concentrations (Fig. 3 B). And for *Plasmodium* samples, only one value was out of the acceptance limits (Fig. 3 A). All the accuracy parameters are summarized in Table 3.

5.2.5. Quantification limits

5.2.5.1. As measured on β-hematin samples.
The accuracy profiles allowed to estimate the upper and lower quantification limits of the process. The intersections between the accuracy profile and the acceptance limits line (+/- 15%) define the lower (LQL) and upper (UQL) limits of quantitation of the procedure, respectively [41]. On the aqueous β-hematin square root transformed data accuracy profile, there was no intersection between accuracy profile and acceptance limits; the lowest and highest concentration levels tested were then defined as LQL (0.05 μg/mL) and UQL (2 μg/mL), respectively. According to the relationship *"hemozoin level - parasitemia"* proposed by Newman [27], 0.05 μg hemozoin/mL would correspond to 80 parasites/μL, a limit in accordance with diagnosis purpose. Then, the validity zone corresponds to the tested validation area (0.05–2 μg/mL).

5.2.5.2. As measured on Plasmodium-infected culture samples.
Applying the method to *Plasmodium* cultures (5% hematocrit) indicated an absolute need for red blood cells lysis. The chromatograms of blank (non-infected by *Plasmodium*) non-lysed samples (Fig. 4.1 A) presents 2
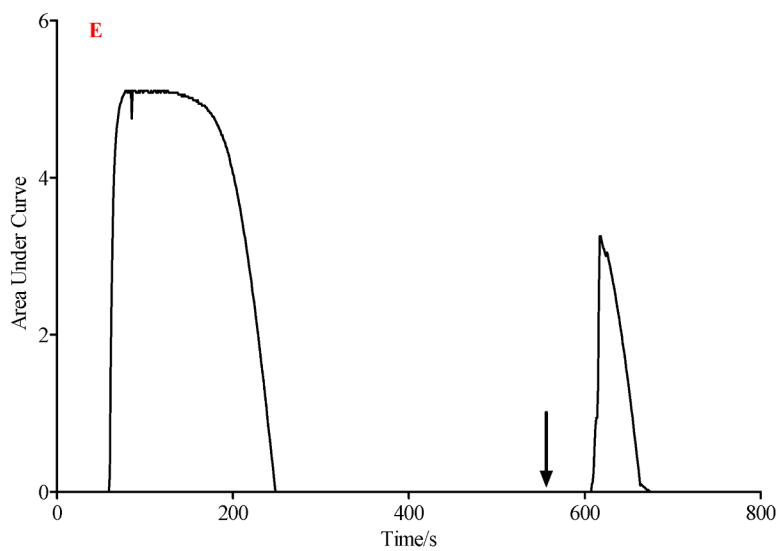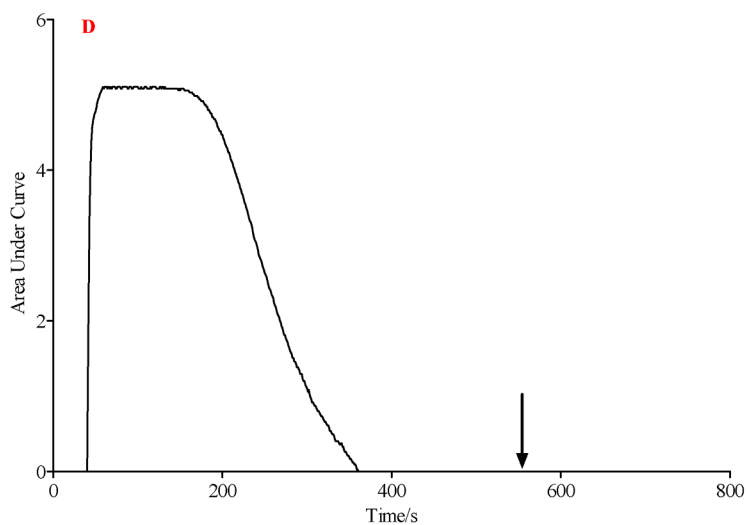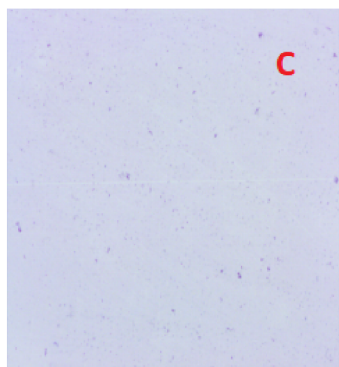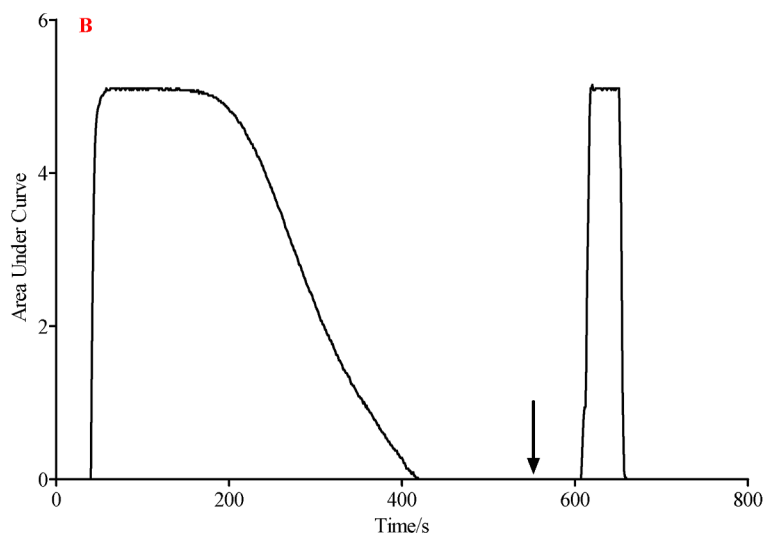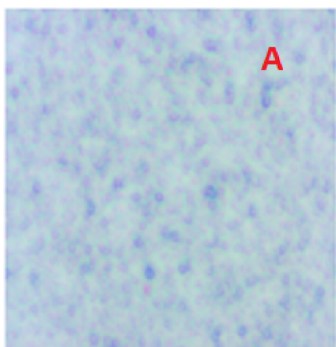
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Fig. 4. 1. *Plasmodium falciparum* 3D7 culture samples. The arrow indicates the valve switching moment. A: observation in optical microscopy of a non-lysed blood sample; B: chromatogram of a non-lysed blood; the passage of the blood in the system takes a long time and a huge peak is observed at the retention time of hemozoin; C: observation in optical microscopy of a lysed blood sample; D: chromatogram of a lysed blood sample, the passage of the lysate in the system takes less time and, in the absence of hemozoin in the lysate, no peak is observed at the NaOH passage; E: when hemozoin is present in the lysate (5 µg/mL), a peak is visible. 2. Linear regression of malaria-free blood spiked with β-hematin. 3. Chromatograms of whole blood samples spiked with hemozoin. A: 0.5 µg/mL of hemozoin. B: 5 µg/mL of hemozoin.

huge peaks, one at injection and one after valve switching (Fig. 4.1B), indicating a non-specific delay and trapping of erythrocytes, probably by the interstices and cavities of the packed microbeads. By contrast, the chromatograms of blank (non-infected by *Plasmodium*) lysed samples (Fig. 4.1C) presents a strongly reduced peak at injection (Fig. 4.1 D), indicating the importance of a pre-chromatographic lysis step. Interestingly, the chromatograms from *Plasmodium*-infected lysed samples present 2 peaks, one at injection and one after valve switching. This second peak is attributed to hemozoin selectively trapped by the magnetic field (Fig. 4.1 E). On these *Plasmodium* cultures (5% hematocrit), the LQL was estimated at 55 parasites/µL (~0.039 µg hemozoin/mL). The validity zone (55–350 parasites/µL) was more restricted than the tested validation domain.

5.3. Whole blood samples

Whole blood samples, spiked with β-hematin and then lysed, were analyzed with the on-line magneto-optical device. The data obtained from 6 concentration levels (0.5 to 15 µg/mL) fitted a linear regression (Fig. 4.2) with r^2 of 0.9902, indicating a close relationship between the calculated AUC and the β-hematin concentrations. The device makes it possible to discriminate β-hematin concentrations (Fig. 4.3). In this preliminary analysis, the lowest analyzed concentration was 0.5 µg/mL of β-hematin, which corresponds to about 800 parasites/µL [27]. These concentrations, tested in whole blood (hematocrit ~ 45%), are much higher than those validated for aqueous β-hematin suspensions and *Plasmodium* culture samples (hematocrit ~ 5%). The hematocrit appears to be an important parameter, yielding a carry-over of cells and/or cellular debris in the magnetic filtration column; a significant increase in background signal along injections prevents detecting low concentrations in β-hematin. This preliminary study highlights that *(i)* a single-use magnetic filtration device is needed to eliminate carry-over

problems; and *(ii)* the lysis process must be improved to make sure to free hemozoin from cell debris; indeed, the *in vivo* biosynthesis pathways of natural hemozoin, involving proteins [25] and/or lipids [58], probably results in a more complex environment of crystals, compared to spiked β-hematin. So, further analyses are needed to confirm these results, injecting in single-use magnetic devices and decreasing tested concentrations to estimate the achievable limits of quantification. To define an eventual bias introduced by spiking samples with extracellular β-hematin, we plan to test *(i)* whole blood spiked with parasitized red cells obtained through *in vitro* culture; and *(ii)* blood samples obtained from malaria patients. This will allow to appreciate the capacity of lysis to release hemozoin embedded in red blood cells.

6. Discussion

During a malaria infection, *Plasmodium* parasites consume about 50% of red blood cells hemoglobin [59] as a source of amino acids, leading to the formation of heme, which is quite toxic to them. To evade the toxicity of heme and survive, the parasites convert heme into an inert, insoluble, crystalline brown pigment known as hemozoin. During his meal, the parasite would convert More than 80% of the iron-porphyrin complexes into hemozoin [60,61]; during erythrocytes lysis, white blood cells capture quickly hemozoin which will be accumulated in organs (liver, spleen, bone marrow). Nevertheless, its fate and role in malaria infection remain still hypothetical [62]. Until now, no appropriate quantification method is described, and let the relevance of hemozoin levels as a biomarker for malaria diagnosis and prognostic in a "question mark" status [26]. Several questions indeed emerge.

1. To establish a clear relationship between whole-blood hemozoin level and parasitemia remains marked with some doubt. Considering that only a very small amount of hemozoin is present in
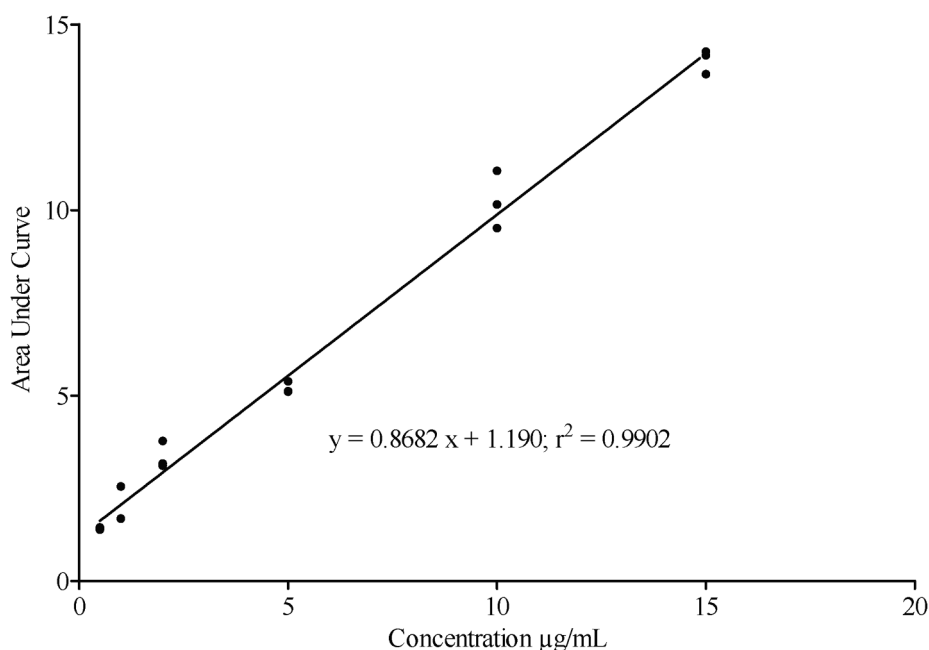

$$y = 0.8682 \, x + 1.190; \; r^2 = 0.9902$$
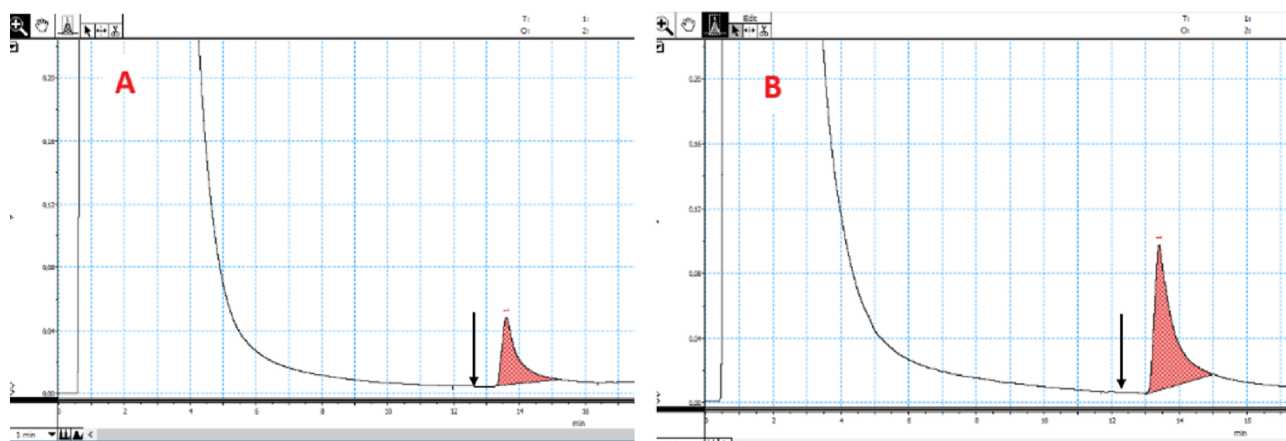
Fig. 4. (*continued*)

Fig. 4. (*continued*)

immature forms of the parasite, detected levels will depend on both *Plasmodium* cycle phase and parasitemia and hemozoin levels in white blood cells and serum [59]. Hemozoin is also sequestered in many organs of the host body such as liver, spleen, lungs, kidneys, brain, and heart [63]. In addition amounts of hemozoin produced may depend on the *Plasmodium* strain [58]. Whereas no differences have been recorded between *P. vivax* resistant and sensible strains, Pisciotta concluded that for, *P. berghei*, chloroquino-resistant (CR) strains produces fivefold less hemozoin that chloroquino-sensitive (CS) strains; in *P. berghei* CR-infected mice, the hemozoin was distributed in liver (93%), spleen (4%) and peripheral blood (3%); by contrast, in *P. berghei* CS-infected mice, the hemozoin was distributed in liver (54%), spleen (4%) and peripheral blood (42%). The evaluation of blood hemozoin was not representative of all body hemozoin but was nevertheless an index of the malaria infection.

2. On the other hand, in man, upon infection cure, the blood clearance of hemozoin, although not formally measured, is estimated at 9 days [64].

3. Other parasitic diseases (prevalent in endemic areas) produce hemozoin such as schistosomiasis [65]; in schistosomiasis infection, the appearance of blood in feces is a major disease sign and the diagnosis incorporates both the blood and faeces exams to establish the diagnosis.

4. Many diagnostic methods based on hemozoin consider only the hemozoin contained in red cells [27,28] whereas total blood hemozoin (red cells + leukocytes + serum) may be a more reliable diagnostic marker.

To develop a new strategy for whole blood hemozoin determination with accuracy and sensitivity will help to tackle these questions and evaluate the possibility to develop an hemozoin-based instrumental and rapid diagnostic test [8].

Current techniques such as microscopy and RDTs do not have satisfactory sensitivity or throughput, rendering them unsuitable for routine use in endemic areas [21].

The magneto-chromatographic method we developed combines in a single device the trapping, elution, detection and quantification of hemozoin, a possibly useful tool for the diagnosis and monitoring of malaria, also applicable to blood transfusion quality assurance [66]. Based on the magnetic [43] and solubility properties of hemozoin, it allows to isolate and quantify the marker polymer in biological samples. Using aqueous suspensions of β-hematin and *P. falciparum* (3D7 strain) cultures, LODs achieved were 80 and 55 parasites/μL, respectively, in a 15 min analysis time, lysis comprised. So, the detectability was slightly better than routine RDTs (~100 parasites/μL) [67], with about the same time of analysis.

Butykai *et al.*, using magnetic rotation-based measurements, reported a detectability of 0.015 μg hemozoin/mL of blood, a parasitemia of about 30 parasites/μL [28]. The sensitivity of this method varies from 77.2 to 88.8%, with specificity comprised between 72.5 and 74.6%. These diagnostic sensitivity and specificity of the magnetic rotation-based device were confirmed by Orban *et al.*, [68] who also showed, on a rodent *P. berghei* model, that the device is suitable for therapeutic monitoring and reinfections detection [69].

7. Conclusion

Malaria diagnosis in its early stages is vital to prevent the disease's spread, to provide medication at the right time, to control the disease in suspects and to reduce mortality [70]. The presence of hemozoin in malaria patient blood is a putative diagnostic biomarker.

The technology developed here is an on-line detection and quantification of hemozoin in blood samples. In a single analysis, it is possible to isolate hemozoin through its paramagnetic properties for spectrophotometric quantification. Our on-line technology has shown effectiveness with aqueous suspensions of β-hematin and *P. falciparum* 3D7 culture sample, with detection limits close to that of the microcopy reference method. The analysis time is relatively short, about 15 min, in line with the immunochromatographic rapid detection tests. In perspective, the device will be validated by analyzing malaria patients' blood in endemic areas of Burkina Faso and Democratic Republic of Congo. The aims are to work in real conditions so to establish the real performances (sensitivity, specificity, lysis buffer power, analysis time) of the device and verify its suitability on whole blood. Based on our encouraging data, the development is on-going, so our on-line magneto-optical technology becomes a significant support in the malaria disease management in endemic and non-endemic countries. In combination with other methods, this new device could add value to the common fight against malaria.

CRediT authorship contribution statement

Orokia Traore: Investigation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Moussa Compaore:** Writing - review & editing. **Philippe Okusa:** Investigation, Formal analysis, Validation. **François Hubinon:** Investigation, Formal analysis, Validation. **Pierre Duez:** Supervision, Project administration, Funding acquisition, Writing - review & editing. **Bertrand Blankert:** Supervision, Project administration, Funding acquisition, Writing - review & editing. **Martin Kindrebeogo:** Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

P. Okusa, B. Blankert and P. Duez are inventors for a patent based on the research described in the present manuscript (*"Malaria detection", British Patent Office, 29 October 2014*). B. Blankert and P. Duez are members of the board of Magnetrap sa (https://www.magnetrap.eu).

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