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ORIGINAL ARTICLE

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Optimal wavelength for the clot waveform analysis: Determination of the best resolution with minimal interference of the reagents

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Abstract

Introduction: Clot waveform analysis (CWA), a new methodology to assess coagulation process, can be usefully applied in various clinical settings. However, its clinical use is limited mainly because of the absence of standardization. No consensus exists regarding the wavelengths at which CWA has to be performed what is crucial for the sensitivity of the CWA.

Objectives: The primary aim of this study is to determine which wavelength is the most sensitive and specific for CWA. Interindividual baseline absorbance will also be assessed as the impact of reagents from the intrinsic, extrinsic, and common coagulation pathway will be determined.

Methods: Plasma samples were screened at wavelengths from 280 to 700 nm to provide absorbance spectra in clotted and nonclotted plasma. The interindividual variability of baseline absorbance was obtained by screening plasma from 50 healthy individuals at 340, 635, and 671 nm. The inner-filter effect of reagents was assessed in plasma or serum when appropriate at the same wavelengths. The reagents were those commonly used for activated partial thromboplastin time, prothrombin time, thrombin time, and dilute Russell's viper venom time.

Results: Clotted plasma has higher absorbance value than nonclotted plasma ($P < 0.01$). The absorbance of all type of samples is higher at 340 nm than at >600 nm ($P < 0.01$). The interindividual variability at the different wavelengths was around 25%. However, except with the STA®-CKPrest® and STA®-NeoPTimal®, the reagents do not have a significant effect on the baseline absorbance.

Conclusions: Wavelengths above 650 nm are recommended to perform CWA. Most of the commercialized reagents can be used for CWA.

1 | INTRODUCTION

The most widely known global coagulation assays are the thrombin generation test (TGT), the viscoelastometric assays (TEG, ROTEM, ClotPro) and more recently the sonic estimation of elasticity via resonance (SEER) sonorheometry.[1,2] They analyze the kinetic of the formation of an end-point of the coagulation cascade (ie, thrombin for TGT or fibrin clot for viscoelastometric assays, clotting assays, and SEER sonorheometry) and allow the detection of several coagulation abnormalities.[3,4]

Up to now, the principle of clot waveform analysis (CWA) is mainly used to provide routine coagulation test results such as the prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT). Indeed, the principle of CWA is to measure and record the time required for a plasma to clot. However, the CWA could also be considered as a global coagulation test, similarly to thromboelastography. This technique assesses changes in light absorbance that occur during the process of fibrin clot formation and gives more information on the fibrin formation than the simple clotting time assays.[5] The obtained curves present a sigmoid profile, called "waveform." From this waveform, the clotting process can be categorized into three parts: (i) the first phase called *precoagulation process*, matching to signal of the onset of coagulation (a-b); (ii) after the onset of coagulation, the light absorbance increases with the formation of fibrin, this step is defined by a slope in the waveform and can be called *the coagulation phase* (b-c); (iii) finally, at the end of

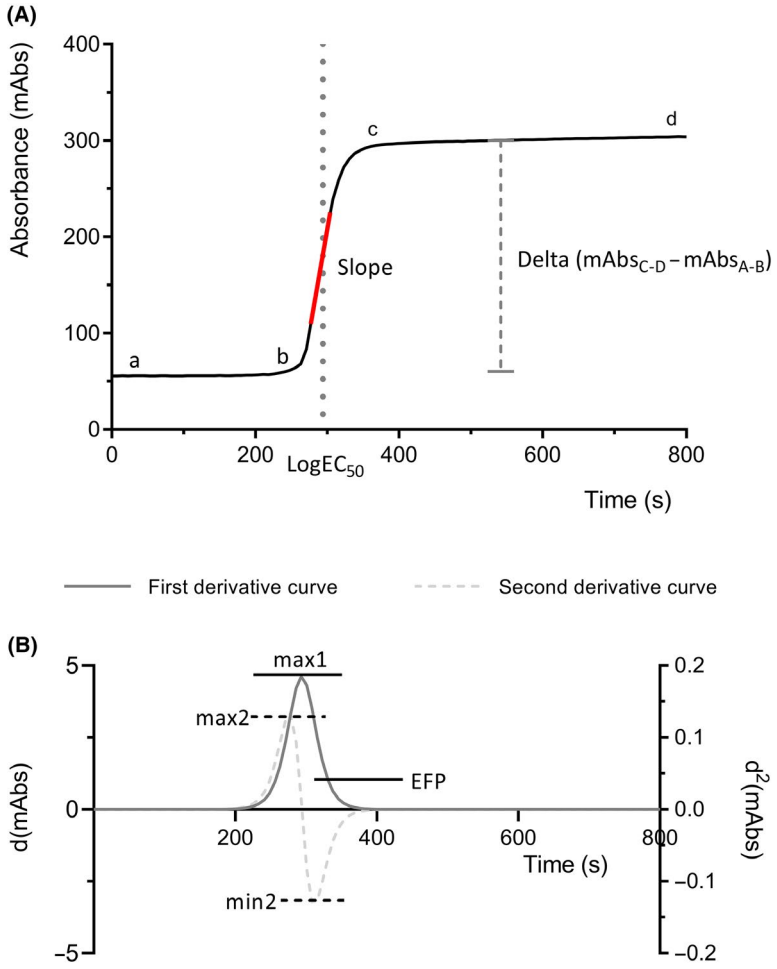

FIGURE 1 Schematic clot waveform (A) and first and second derivative curves (B). A, The curve presents a sigmoid profile, called "waveform" which represent the light absorbance of the sample over time. From this waveform, the clotting process can be categorized into three parts: (i) the first phase is *precoagulation process*, matching to signal of the onset of coagulation (a-b); (ii) after the onset of coagulation, the light absorbance increases with the formation of fibrin, this step is defined by a slope in the waveform and can be called *the coagulation phase* (b-c); iii) finally, at the end of coagulation, the absorbance tends to stabilize (c-d), and this phase can be called *the postcoagulation phase*. With the initial curve, a sigmoidal dose-response curve, an idea of the appearance formation of the fibrin clot can be depicted. Information such as (i) *the delta*, which describes difference between top (maximum absorbance) and bottom (minimum absorbance), (ii) *the slope* which describes the steepness of the curve, and (iii) *the LogEC$_{50}$*, which defines the *X* value when the response is halfway between bottom and top can be extracted. This parameter defines also the time where the maximum concentration of fibrin is produced. B, The waveform is mathematically processed by an algorithm to obtain the first and second derivative curves. The first derivative (dA/dt) reflects the coagulation velocity (max1) and endogenous potential fibrin (ie, area under the curve, EFP), whereas the second derivative (d^{2}A/dt) reflects the acceleration and deceleration of coagulation (max2, min2). [Colour figure can be viewed at wileyonlinelibrary.com]

coagulation, the absorbance tends to stabilize (c-d), and this phase can be called *the postcoagulation phase* (Figure 1).[6] The waveform can be mathematically processed by an algorithm to obtain the first and second derivative curves. The first derivative (dA/dt) reflects the coagulation velocity (max1) and endogenous potential fibrin (ie, area under the curve, EFP), whereas the second derivative (d^2A/dt) reflects the acceleration and deceleration of coagulation (max2, min2).[7]

The CWA demonstrated its potential in abnormalities of blood coagulation such as hemophilia, disseminated intravascular coagulation (DIC), sepsis, cirrhosis, and lupus anticoagulant.[8-12] In these clinical settings, the in-depth analysis of the clot waveform may provide additional information than simple clotting tests.

The fact that the method is not well standardized limits its routine use in clinic.[6,13] In particular, the wavelength at which the analysis is performed, the choice of the reagent or interferences from plasma samples (lipemic or hemolytic samples) could impact CWA results and interpretation.[14] The aim of this work is to determine (a) which wavelength is the most sensitive and specific to distinguish nonclotted and clotted plasma samples, (b) the interindividual variability of baseline absorbance value in healthy subjects at the preferred wavelength, and (c) the impact of reagents from the intrinsic, extrinsic, and common coagulation pathway on baseline absorbance. The final goal is to provide grounds for recommendations about wavelengths and reagents that should be used to perform CWA which is the first step for the implementation of new techniques, methodologies, analyzers, and algorithms for the assessment of the coagulation process.

2 | MATERIALS AND METHOD

The study protocol is in accordance with the Declaration of Helsinki and the recruitment of the healthy volunteers has been approved by the Ethical Committee of the CHU UCL Namur, Yvoir, Belgium (approval number: B03920096633). The sampling conditions and the preparation of the biological material are described below.

2.1 | Blood sample collection and plasma preparation

Fifty-eight healthy volunteers were included in this study for the constitution of a normal pooled plasma (NPP), and fifty of these volunteers were further included for the collection of the corresponding individual plasma (IP). The exclusion criteria were thrombotic and/or hemorrhagic events, antiplatelets and/or anticoagulants medication, pregnancy and uptake of drugs potentially affecting the platelet and/or coagulation factor functions during the 2 weeks prior to the blood drawn. Blood was taken by venipuncture in the antecubital vein with a 21-gauge needle (Venoject®, Terumo, Belgium) and collected into 0.109 mol/L sodium citrate tubes (9:1 v/v) (Venosafe®, Terumo, Belgium). The NPP and IPs were obtained by double centrifugation. The whole-blood samples centrifuged at 1500 *g* during 15 minutes. The supernatant is collected without disturb the buffy coat. The collected plasma is re-centrifuged at 1500 *g* during 15 minutes. The supernatant is then aliquoted for IPs or pooled for the NPP and stored at −80°C without any delay.

2.2 | Blood sample collection and serum preparation

Six healthy volunteers were included for the constitution of a normal pooled serum (NPS) and the collection of the corresponding individual sera (IS). The exclusion criteria were the same as for the plasma. Blood was taken by venipuncture in the antecubital vein a 21-gauge needle (Venoject®) and collected into Z serum sep clot activator tubes (Vacuette®, Greiner Bio-One, Belgium). The NPS and IS from six patients were obtained by simple centrifugation. Blood samples are left in vertical position in 30 minutes at room temperature to allow coagulation process. The clotted whole-blood samples were centrifuged at 2000 *g* during 15 minutes. The supernatant is then aliquoted for ISs or pooled for the NPS and frozen at −80°C without any delay.[15,16]

2.3 | General procedure of the clot waveform analysis

With the aim of standardizing the CWA and further comparing the fibrin formation vs the thrombin formation, we adapted the methodology of the CWA to the methodology of the calibrated automated thrombogram (CAT).[17] In brief, the fibrin clot formation is initially measured from 80 µL of NPP mixed with 20 µL of inducers of the intrinsic or extrinsic pathway of coagulation in a 96-well microplate (Thermo Scientific®, Brussels, Belgium) and incubated for 3 minutes at 37°C in a Tecan® Infinite M200 pro microplate reader (Tecan®, Mechelen, Benelux). The coagulation process is initiated by the addition of 20 µL of $CaCl_2$ at 100 mmol/L (LabChem®, Pennsylvania) and monitored during 15 minutes. As preheating conditions are known to affect the coagulation process,[18] the microplate, the plasma and serum samples were preheated at 37°C for 5 minutes before experiment. All tests were performed in triplicate on the same microplate.

2.4 | Absorbance spectra of coagulated and noncoagulated plasma

The NPP was screened at wavelength from 280 to 700 nm, by step of 1 nm on a TECAN® microplate reader (Tecan®) to provide absorbance spectra of clotted and nonclotted plasma. The coagulation process was triggered by either the intrinsic pathway of the coagulation using Actin FS® (Siemens, Marburg, Germany) or by the extrinsic pathway using PPP reagent® or PPP reagent Low® (Thrombinoscope BV, Maastricht, The Netherland's). The latter reagents were chosen because they do not contain calcium, contrary to conventional PT reagents used on routine analyzers (eg, Dade Innovin®, HemosIL ReadiPlasTin®). This is necessary to allow the triggering of the coagulation by exogenous $CaCl_2$.

As mentioned above, 20 µL of reagent was mixed with 80 µL of plasma in a microplate and incubated for 3 minutes at 37°C before the addition of the triggering solution. For clotted plasma, the coagulation process was initiated by the addition of 20 µL of a homemade solution of $CaCl_2$ at 100 mmol/L (LabChem®, Pennsylvania) while 20 µL of physiological saline (Kela Pharma NV, Sint-Niklaas, Belgium) was added in nonclotted plasma to harmonize the dilution ratio between the two conditions. The signal-to-noise ratio (clotted plasma absorbance/nonclotted plasma absorbance) between the two conditions (coagulated and noncoagulated) was then calculated at different wavelengths.

2.5 | Choice of wavelengths

According to the manufacturer recommendations and the abilities of the routine analyzers, wavelengths of 340, 635, and 671 nm were used to compare the interindividual variability and the inner-filter effect of the reagent on the light absorbance of the coagulation process.

2.6 | Interindividual variability of plasma samples

To assess the interindividual variability on baseline absorbance, 50 IPs were screened. To allow measurement of baseline absorbance, the 20 µL of reagent and the 20 µL of $CaCl_2$ were replaced by 40 µL of phosphate buffer saline (PBS) without calcium or magnesium (Lonza BioWhittaker, Walkersville). This prevents the coagulation process and follows the dilution ratio of the CWA test. Thus, 80 µL of IP was mixed with 40 µL of PBS and absorbance was measured at 340, 635, and 671 nm.

2.7 | Measurement of the inner-filter effect of reagents on plasma

To investigate the inner-filter effects of reagents on plasma samples, the NPP was mixed with reagents from the intrinsic pathway (aPTT). To avoid triggering coagulation process, the 20 µL solution of $CaCl_2$ 100 mmol/L was replaced by PBS. Thus, 80 µL of NPP was mixed with 20 µL of reagent and 20 µL of PBS in the microplate and absorbance was measured at 340, 635 and 671 nm.

2.8 | Interindividual variability of serum samples

As reagents for prothrombin time (PT), thrombin time (TT), reptilase time (RT), and dilute Russell's Viper venom time (DRVVT) contain calcium ions, the adjunction of PT, TT, RT, or DRVVT reagents on plasma form a clot within 15-40 seconds, which makes impossible the assessment of the inner-filter effect of these reagents on plasma. Thus, to circumvent this problem, plasma samples were replaced by serum samples to avoid activation of the coagulation process. The baseline absorbance of the serum samples was compared to the baseline of plasma, and the interindividual variability was assessed using the serum from six healthy individuals. These sera were assessed at the same wavelengths than the IP samples using the same procedure which means that 80 µL of IS were mixed with 40 µL of PBS to preserve the dilution ratio.

2.9 | Measurement of the inner-filter effect of reagents on serum

To investigate the inner-filter effects of reagents on serum, NPS was screened with reagents from the extrinsic (PT) or common pathway (TT/RT/DRVVT). Twenty µl of reagent mixed with 80 µL of NPS and 20 µL of PBS in microplate and screened at same wavelengths as the NPP samples.

2.10 | Statistical analysis

Statistical analysis was performed using GraphPad version 5.0 (GraphPad Prism software, San Diego, CA). Results of absorbance are presented as the mean of the triplicate ± SD. The comparison between the absorbance spectra of clotted and nonclotted plasma has been calculated as the signal-to-noise ratio. The signal-to-noise ratio indicates the ratio between the absorbance obtained once the plasma has clotted (postcoagulation phase) on the baseline value (in absence of the clot—precoagulation phase). This reflects the sensitivity toward the clotting process. The benefit of a high signal-to-noise ratio allows a good discrimination between the baseline absorbance from maximum absorbance. It has been used to determine the most sensitive and specific wavelength in order to distinguish coagulated plasma from noncoagulated. For the baseline absorbance of IPs and ISs, results are presented as the mean ± SD.

For the measurement of the inner-filter effect of reagents on plasma, the baseline absorbance of NPP + reagents was compared to baseline of IPs. For the measurement of the inner-filter effect of PT, TT, RT, and DRVVT reagents on serum, the baseline absorbance of NPS + reagents was compared to baseline of ISs. A Kruskal-Wallis test has been used in order to assess the statistical difference between these groups (ie, IP vs NPP plus intrinsic reagents and IS versus NPS plus extrinsic or common reagents). A multiple comparison test using the Dunn's principle has been used to compare absorbance of tested reagents. An unpaired *t* test, with 95% confidence intervals, was then used to assess the statistical difference for each reagent. Results are considered statistically different when *P*-value is <0.05. Scatter dot plot was used to visualize if absorbance of the reagents is inside the mean absorbance of IP or IS ± SD. The interference of the reagent is considered statistically significant if the absorbance is outside the mean of IP or IS ± SD.

3 | RESULTS

3.1 | Absorbance spectra of coagulated and noncoagulated plasma

The absorbance spectrum of clotted and non-clotted plasma is shown in Figure 2. As expected, the coagulated plasma has higher absorbance value than non-coagulated plasma ($P < 0.05$). The baseline

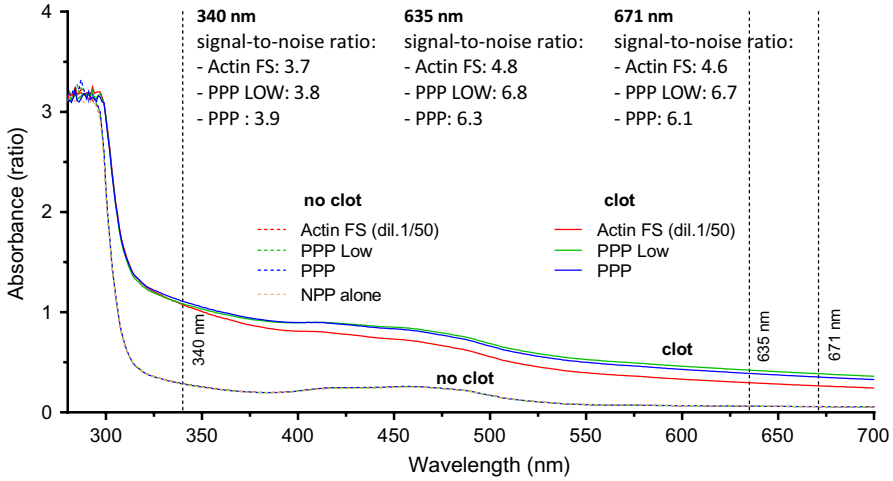

FIGURE 2 Absorbance spectrum of nonclotted and clotted plasma. Triggering of the intrinsic pathway of the coagulation was performed using Actin FS® while the extrinsic pathway was triggered using PPP reagent® or PPP reagent Low®. For clotted plasma, the coagulation process was initiated by the addition of 20 µL of a home-made solution of $CaCl_2$ at 100 mmol/L while 20 µL of physiological saline was added in nonclotted plasma to harmonize the dilution ratio between the two conditions. The coagulated plasma has higher absorbance value than noncoagulated plasma, and the absorbance of clotted and nonclotted samples is higher at 340 nm than 635 nm or 671 nm. A summary of signal-to-noise ratio is calculated for the intrinsic and extrinsic pathway at 340, 635, and 671 nm. The signal-to-noise ratio is higher at 635 nm and 671 nm than 340 nm and higher with inducers from the extrinsic pathway of the coagulation (ie, PPP Reagent® and PPP Reagent Low®) [Colour figure can be viewed at wileyonlinelibrary.com]

absorbance (mAbs) of nonclotted samples at 340, 635, and 671 nm equal to 287.9, 61.8, and 57.2 for Actin FS®. Once clotted, plasma showed absorbance values equal to 1074.2, 296.5, and 265.2 mAbs for the same reagent (Table 1). A difference of the delta absorbance (maximum − minimum absorbance) is observed between inducers of the intrinsic and the extrinsic pathway for clotted plasma (Table 1).

However, due to very low absorbance of noncoagulated plasma at 635 and 671 nm, the signal-to-noise ratio is higher at 635 nm and 671 nm than 340 nm whatever the reagents (Figure 2). Of note, the signal-to-noise ratio was higher with inducers of the extrinsic pathway of the coagulation (ie, PPP Reagent® and PPP Reagent Low®; Figure 3).

3.2 | Measurement of interindividual variability on plasma and serum

The variability on 50 IPs for the three tested wavelengths was ranging from 24% to 26%. The variability on six ISs for three tested wavelengths was ranging from 38% to 40%.

3.3 | Measurement of inner-filter effect of reagents on plasma

The inner-filter effect of aPTT reagents on nonclotted plasma is summarized in Table 2. Except for STA®-CKPrest®, the reagents used in this study have no significant effect on the baseline absorbance ($P < 0.01$; Figure S1). The intra-reagent coefficients of variation (CV) were below 5% for absorbance, except for STA®-CKPrest® (<5% at 340 nm and >10% at 635 and 671 nm). The mean absorbance, SD, and CV (%) of reagents are shown in Table 2.

3.4 | Measurement of inner-filter effect of reagents on serum

The use of serum does not have a significant impact on the baseline absorbance compared to plasma ($P > 0.05$). The inner-filter effect of reagents, containing calcium ions, assessed on serum is shown on Figure S2. Except for STA®-NeoPTimal® ($P > 0.05$ at 340 nm,

TABLE 1 Summary of results of absorbance measurements (mAbs) of nonclotted and clotted samples for the intrinsic and extrinsic pathway of the coagulation

Summary of results of absorbance measurements (mAbs)						
	Nonclotted plasma			Clotted plasma		
Wavelength (nm)	Actin FS®	PPP reagent low®	PPP reagent®	Actin FS®	PPP reagent low®	PPP reagent®
340	287.3	283.5	284.3	1074.2	1084.5	1109.4
635	61.8	62.2	62.2	296.5	422.4	389.4
671	57.2	57.9	57.8	265.2	386.2	352.9

The intrinsic pathway was assessed by Actin FS® while the extrinsic pathway was assessed using PPP reagent® or PPP reagent Low®.

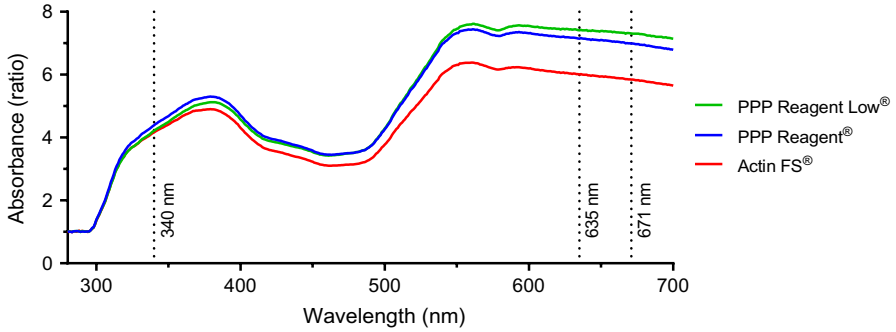

FIGURE 3 Spectrum of the signal-to-noise ratio. The signal-to-noise ratio is higher at 635 nm and 671 nm than 340 nm and higher with inducers of the extrinsic pathway of the coagulation (ie, PPP Reagent® and PPP Reagent Low®) [Colour figure can be viewed at wileyonlinelibrary.com]

$P < 0.05$ at 635 and 671 nm), reagents used in this study do not have a significant effect on the baseline absorbance of serum ($P > 0.05$). Compared to the range of measurement (mean ± 1*SD) obtained with the ISs, only STA®-NeoPTimal® showed a disturbance on the baseline absorbance. The intra-reagent coefficients of variation (CV) were below 3% for absorbance, even for STA®-NeoPTimal®. The mean absorbance, SD, and CV (%) of reagents are shown in Table 2.

4 | DISCUSSION

The clot waveform analysis can be considered as a global hemostasis assay as for to thrombin generation test (TGT), thromboelastographic test (TEG/ROTEM), or sonic estimation of elasticity via resonance (SEER) sonorheometry.[2,19] The TGT is an assay that measures the thrombin formation over time with fluorescence or absorbance detector. The TEG/ROTEM and SEER sonorheometry measure viscoelastic properties of the fibrin clot.[13,20] They both tempt to detect, thanks to an optical, a mechanical or a resonance readout, the evolution of the coagulation process over time following the addition of a triggering reagent. The CWA reflects the whole process of the clot formation. It is based on an optical method which measures the absorbance of a plasma sample over time.

Previous studies reported CWA as a potential method for the assessment of coagulation disorders such as hemophilia, disseminated intravascular coagulation (DIC), sepsis, cirrhosis, or lupus anticoagulant.[21] In hemophilia, CWA reflects the balance between procoagulant and anticoagulant factors and was described to predict better the bleeding tendency than the determination of FVIII:C alone [13,22]. In sepsis and DIC patients, the pattern of waveform differs from pattern of healthy patients. The pattern is a biphasic waveform characterized by the absence of initial plateau and the presence of two slopes (Figure S3). However, CWA lacks from standardization and further studies are needed to demonstrate the clinical benefit of CWA in the diagnosis and the management of these pathologies.[8]

The purpose of this article is to propose a standardization of CWA methodologies in term of wavelength, reagents or even the quality of the tested sample (lipemic or hemolytic samples) which is today a specific limitation of this test.[13,6] Some limitations, inherent to the methodological design of this study, have to be recognized such as the use of serum for the assessment of reagents from the extrinsic pathway or the impossibilities to assess the reagents from all manufacturers. However, we evaluated the most widely used reagents on the market.

4.1 | Determination of optimal wavelength

Currently, and according to the analyzer, different wavelengths can be used to perform CWA. The Destiny Max®, Destiny Plus® and DT 100® (Diagnostica Stago, Asnières-sur-Seine, France) have a system of photo-optical detection at 340, 405, 635, and 705 nm; the ACL-Top family series (Instrumentation laboratories, Lexington) have a system at 405 and 671 nm, and finally, the hemostasis systems from Siemens Healthineers (Marburg, Germany) have detectors at 405, 575, and 660 nm for Sysmex CS-5100® and CS-2000i®/CS-2100i® and at 340, 405, 575, 660, and 800 nm for Sysmex CS-2500®. All these hemostasis analyzers have a system for detection of hemolytic, lipemic, and icteric (also called "HIL") plasma samples in addition to clot formation. According to our results, clotted and nonclotted plasma samples have a higher absorbance at 340 nm than other wavelengths, suggesting in a first idea that this wavelength could be optimally used to perform CWA. However, our results also show that the signal-to-noise is higher at wavelengths >550 nm. In addition, previous studies reported that HIL interferences can significantly interfere with the fibrin coagulation process at 340 nm. [23,14,13] Thus, according to our results and the literature, the most sensitive and specific wavelengths seems to be >550 nm but even at these wavelengths, HIL interferences can still occur. A more consolidated feature in modern coagulometers is the possibility to use multi-wavelength detection (eg, simultaneous analysis at 340, 405, 575, 660, and 800 nm), which would enable selection of the appropriate absorbance for specific types of interfering substances such as lipemia, bilirubin, or hemoglobin. This allows to process those samples with a modest degree of photometrical interference. Thus, we recommend that CWA should be performed at wavelengths >650 nm for at least two reasons: (a) to avoid HIL interferences and (b) to have a high signal-to-noise which in definitive will provide a better sensitivity for the parameters that can be assessed in CWA.

4.2 | Interindividual variability

Nowadays, no recommendations exist about the interindividual variability. Our results show that interindividual variability on 50 IPs for the three tested wavelengths was ranging from 24% to

TABLE 2 Summary of impact of the reagents from the intrinsic, extrinsic and common pathway on the baseline absorbance (mAbs) of plasma and serum

Plasma	340 nm			635 nm			671 nm		
	Mean ± SD	CV (%)	*P*-value (<0.05)	Mean ± SD	CV (%)	*P*-value (<0.05)	Mean ± SD	CV (%)	*P*-value (<0.05)
Reagents of the intrinsic coagulation pathway									
Actin FS®	330.6 ± 4.0	1.20%	0.4798	62.3 ± 1.0	1.61%	0.9754	57.4 ± 0.8	1.33%	0.9525
SynthAFax®	291.9 ± 5.209	1.78%	0.9003	63.9 ± 0.9	1.41%	0.8443	59.1 ± 0.6	0.93%	0.7961
SynthASil®	273.9 ± 4.8	1.74%	0.6079	58.5 ± 1.5	2.52%	0.7111	54.0 ± 1.1	2.04%	0.7232
Cephen®	300.1 ± 1.6	0.53%	0.9585	70.8 ± 2.6	3.68%	0.3552	64.8 ± 0.9	1.32%	0.3409
STA®-PTT-A®	359.5 ± 0.3	0.08%	0.1872	75.5 ± 0.9	1.15%	0.1581	69.6 ± 0.9	1.32%	0.1277
STA®-Cephascreen®	331.8 ± 3.6	1.08%	0.4640	63.3 ± 1.4	2.19%	0.8942	58.4 ± 1.3	2.17%	0.8625
STA®-C.K.Prest®	778.8 ± 36.0	4.63%	<0.001	374.0 ± 37.8	10.12%	<0.001	355.8 ± 38.7	10.88%	<0.001
Reagents of thrombin generation test									
PPP Reagent Low®	267.0 ± 3.5	1.30%	0.5091	57.5 ± 0.8	1.33%	0.6367	53.4 ± 0.7	1.33%	0.6634
PPP Reagent®	269.0 ± 3.0	1.10%	0.5365	57.7 ± 0.6	1.10%	0.6518	53.3 ± 0.9	1.74%	0.6546
PPP Reagent High®	266.2 ± 1.8	0.68%	0.4982	57.0 ± 0.3	0.54%	0.5948	53.4 ± 1.0	1.85%	0.6634
Serum									
	340 nm			635 nm			671 nm		
	Mean ± SD	CV (%)	*P*-value (<0.05)	Mean ± SD	CV (%)	*P*-value (<0.05)	Mean ± SD	CV (%)	*P*-value (<0.05)
Reagents of the extrinsic coagulation pathway									
ReadiPlasTin®	416.9 ± 6.9	1.66%	0.7583	73.5 ± 0.6	0.75%	0.7804	67.0 ± 0.7	1.08%	0.8981
RecombiPlasTin 2G®	421.8 ± 1.9	0.44%	0.7188	72.7 ± 0.9	1.20%	0.7483	66.1 ± 1.0	1.51%	0.8584
Dade Innovin®	385.3 ± 1.3	0.34%	0.9724	71.8 ± 0.8	1.05%	0.7155	65.2 ± 0.6	0.85%	0.8161
PT HTF®	437.3 ± 4.8	1.10%	0.5995	91.2 ± 1.5	1.62%	0.5404	82.6 ± 0.6	0.76%	0.4452
PT Excel®	455.6 ± 7.5	1.64%	0.4745	91.7 ± 0.4	0.44%	0.5242	83.0 ± 0.7	0.87%	0.4313
PT Excel S®	449.5 ± 1.6	0.34%	0.5142	90.7 ± 0.4	0.42%	0.5554	83.0 ± 0.3	0.39%	0.4302
STA®-Neoplastin Ci®	477.1 ± 4.4	0.92%	0.3514	100.4 ± 0.8	0.75%	0.2950	91.2 ± 0.1	0.06%	0.2252
STA®-Neoplastin Ci Plus®	478.8 ± 10.9	2.28%	0.3434	100.9 ± 1.0	0.99%	0.2848	91.5 ± 1.6	1.74%	0.2199
STA®-Neoplastin R®	408.8 ± 1.6	0.38%	0.8258	79.5 ± 0.6	0.69%	0.9790	72.1 ± 0.3	0.37%	0.8660
STA®-NeoPTimal ®	560.3 ± 8.2	1.46%	0.0942	128.5 ± 2.8	2.17%	0.0349	116.9 ± 2.9	2.47%	0.0238
Reagents of the common coagulation pathway									
DRVV Screen®	410.2 ± 1.8	0.43%	0.8140	81.0 ± 0.6	0.75%	0.9195	70.3 ± 0.5	0.70%	0.9474
DRVV Confirm®	449.8 ± 5.6	1.23%	0.5121	86.8 ± 1.5	1.68%	0.6945	79.0 ± 1.3	1.65%	0.5729
STA®- Reptilase®	395.5 ± 1.5	0.38%	0.9392	73.0 ± 1.3	1.79%	0.7599	66.7 ± 1.8	2.67%	0.8829
STA®-Thrombin®	426.4 ± 3.8	0.89%	0.6819	79.4 ± 1.1	1.43%	0.9831	72.1 ± 1.0	1.41%	0.8660
HemosIL® Thrombin Time	478.2 ± 2.6	0.54%	0.3458	85.2 ± 0.5	0.59%	0.7521	77.0 ± 0.5	0.60%	0.6521

The mean, standard deviation (SD) and coefficient of variation (CV) (%) for reagents are shown. A reagent with a *P*-value < 0.05 means that reagent has a significant effect on the baseline absorbance of plasma or serum. Except STA®-C.K-Prest® and STA®-NeoPTimal®, reagents do not have significant impact on the baseline absorbance ($P > 0.05$).

26% and it must be taken into account that rejecting all samples showing an abnormal color needs to be reconsidered because the process of taking new samples is time-consuming and causes additional laboratory operating costs as well as patient discomfort.[24] In order to limit the rejection of samples, hemostasis systems should be able to detect types of interferences and adapt the wavelength for the clot formation. The use of a reference plasma as calibrator could be useful to compare plethora of patients (anticoagulants, factor Xa inhibitors, DIC, and hemophilia patients) considering the pattern of clot waveform is different (Figure S3).[6]

4.3 | The inner-filter effect of reagents on plasma and on serum

The impact of aPTT reagents has been studied on nonclotted plasma (Table 2 and Figure S1). As the CWA is based on a photo-optical detection method, the appearance of the plasma samples and reagents could impact the output signal. Sevenet and Depasse[13] proposed to use standardized colorless and nonopaque reagents in order to limit interferences on plasma samples. In this study, aPTT reagents are composed of a surface activator (eg, ellagic acid, silica or micronized silica, kaolin, polyphenolic activator) and platelets substitute (cephalin, synthetic phospholipids) which are summarized in Table S1. Some aPTT reagents are colored and results showed that solely STA®-CKPrest® impacted significantly the output signal. The STA®-CKPrest® is an aPTT reagent containing kaolin and cephalin and is used to explore the factors of the intrinsic pathway of coagulation.[25] STA®-CKPrest® is widely available to determine aPTT on analyzers based on a mechanical detection of the clot. However, our results showed STA®-CKPrest® present a large variability in absorbance measurements and we consider that this reagent should not be used to perform CWA due to this variability. Otherwise, we found no interference for all other reagents tested in this study even those which are colored.

The impact of reagents investigating the extrinsic (PT) and common (TT, RT, DRVVT) coagulation pathway was measured on serum. PT reagents are composed of a source of tissue factor (eg, thromboplastin), phospholipids and calcium ions, TT reagents contain a human or bovine thrombin, RT reagents are composed of a thrombin-like enzyme while DRVVT reagents are composed of Russel's viper venom, phospholipids, and calcium ions (Table S1). Among reagents tested, some reagents are colored and results showed that only STA®-NeoPTimal® impacted significantly the output signal. Ultimately, except STA-NeoPTimal® and reagents containing kaolin such as STA®-CKPrest®, reagents from the intrinsic, extrinsic or common pathway could be used to perform the CWA.

5 | CONCLUSION, RECOMMENDATIONS, AND PERSPECTIVES FOR CLOT WAVEFORM ANALYSIS STANDARDIZATION

Nowadays, CWA possibilities are widely underused compared to current interpretations we have of such tests, for example aPTT, PT, TT. Nevertheless, CWA seems to possess a promising future in the clinical field.[13] The present study confirms that the choice of the wavelength is crucial for CWA. Based on our results, wavelength >650 nm should be considered in order to avoid HIL interferences[23] and to obtain a high signal-to-noise providing a better sensitivity and specificity although the current analyzers are able to detect the presence of HIL interferences in plasma samples (and could be able to choose the appropriate wavelength for CWA). The study confirms also that despite colorful appearance of reagents, most of reagents currently on the market can be used to perform CWA. Further investigations should be planned to confirm our conclusion about STA®-CKPrest® (aPTT reagent) and STA®-NeoPTimal® (PT reagent) and their use to perform a CWA. The avoidance of colorless reagents is no longer recommended using our procedure and methodologies but can be useful to limit interferences, especially in methodologies where the amount of reagents in the final reaction mixture is higher.

Future perspectives of the use of the clot waveform analysis could be the optimization and the harmonization of this new methodology whatever reagents and the quality of the samples. In this study, ratio dilution of plasma samples was 2:3 compared to current analyzers (1:3). Therefore, another perspective for CWA should be the harmonization of dilution ratios between hemostasis systems to avoid impact not only on the overall visual aspect of the coagulation process but also on other parameters of CWA such as velocity and delta for which further investigations are required. Finally, the current analyzers are able to detect the presence of HIL interferences in plasma samples. The last perspectives to consider would be the development of hemostasis systems able to use multi-wavelength detection (eg, simultaneous analysis at 340, 405, 575, 660, and 800 nm), which would enable selection of the appropriate wavelength for specific types of interfering substances with the quality of the patients samples such as lipemia, bilirubin, or hemoglobin.

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CONFLICT OF INTEREST

Among the authors, J. Douxfils is CEO and founder of QUALIblood sa and reports personalfees from Stago and Daiichi-Sankyo, outside the submitted work. F. Mullier reports institutional fees from Stago, Werfen, Nodia, Sysmex and Bayer. He also reports speaker fees from Boehringer Ingelheim, Bayer Healthcare and Bristol-Myers Squibb-Pfizer, all outside the submitted work. The other authors have no conflicts of interest to disclose.

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REFERENCES

1. Ferrante EA, Blasier KR, Givens TB, Lloyd CA, Fischer TJ, Viola F. A novel device for the evaluation of hemostatic function in critical care settings. *Anesth Analg*. 2016;123:1372-1379.
2. Lance MD. A general review of major global coagulation assays: thrombelastography, thrombin generation test and clot waveform analysis. *Thromb J*. 2015;13:1.
3. Hemker HC, Giesen P, AlDieri R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb*. 2002;32:249-253.
4. Whiting D, DiNardo JA. TEG and ROTEM: technology and clinical applications. *Am J Hematol*. 2014;89:228-232.
5. Braun PJ, Givens TB, Stead AG, et al. Properties of optical data from activated partial thromboplastin time and prothrombin time assays. *Thromb Haemost*. 1997;78:1079-1087.
6. Shima M, Thachil J, Nair SC, Srivastava A, Scientific and Standardization Committee. Towards standardization of clot waveform analysis and recommendations for its clinical applications. *J Thromb Haemost*. 2013;11:1417-1420.
7. Abraham SV, Rafi AM, Krishnan SV, et al. Utility of clot waveform Analysis in Russell's viper bite victims with hematotoxicity. *J Emerg Trauma Shock*. 2018;11:211-216.
8. Downey C, Kazmi R, Toh CH. Novel and diagnostically applicable information from optical waveform analysis of blood coagulation in disseminated intravascular coagulation. *Br J Haematol*. 1997;98:68-73.
9. Hussain N, Hodson D, Marcus R, Baglin T, Luddington R. The biphasic transmittance waveform: an early marker of sepsis in patients with neutropenia. *Thromb Haemost*. 2008;100:146-148.
10. Milos M, Coen HD, Zupancic-Salek S, Zadro R. New quantitative aPTT waveform analysis and its application in laboratory management of haemophilia A patients. *Haemophilia*. 2014;20:898-904.
11. Minami H, Nogami K, Yada K, et al. Emicizumab, the bispecific antibody to factors IX/IXa and X/Xa, potentiates coagulation function in factor XI-deficient plasma in vitro. *J Thromb Haemost*. 2018;17:126-137.
12. Ruberto MF, Marongiu F, Sorbello O, Civolani A, Demelia L, Barcellona D. Are prothrombin time and clot waveform analysis useful in detecting a bleeding risk in liver cirrhosis? *Int J Lab Hematol*. 2018;41:118-123.
13. Sevenet PO, Depasse F. Clot waveform analysis: Where do we stand in 2017? *Int J Lab Hematol*. 2017;39:561-568.
14. Nagant C, Rozen L, Demulder A. HIL interferences on three hemostasis analyzers and contribution of a preanalytical module for routine coagulation assays. *Clin Lab*. 2016;62:1979-1987.
15. Adcock DM, Favaloro EJ, Lippi G. Critical pre-examination variables in the hemostasis laboratory and their quality indicators. *Clin Biochem*. 2016;49:1315-1320.
16. Dargaud Y, Wolberg AS, Gray E, Negrier C, Hemker HC, Subcommittee on Factor VIII, Factor IX, and Rare Coagulation Disorders. Proposal for standardized preanalytical and analytical conditions for measuring thrombin generation in hemophilia: communication from the SSC of the ISTH. *J Thromb Haemost*. 2017;15:1704-1707.
17. Hemker HC, Giesen P, Al DR, et al. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003;33:4-15.
18. De Smedt E, Hemker HC. Thrombin generation is extremely sensitive to preheating conditions. *J Thromb Haemost*. 2011;9:233-234.
19. Viola F, Mauldin FW Jr, Lin-Schmidt X, Haverstick DM, Lawrence MB, Walker WF. A novel ultrasound-based method to evaluate hemostatic function of whole blood. *Clin Chim Acta*. 2010;411:106-113.
20. Mauldin FW Jr, Viola F, Hamer TC, et al. Adaptive force sonorheometry for assessment of whole blood coagulation. *Clin Chim Acta*. 2010;411:638-644.
21. Toh CH, Giles AR. Waveform analysis of clotting test optical profiles in the diagnosis and management of disseminated intravascular coagulation (DIC). *Clin Lab Haematol*. 2002;24:321-327.
22. Siegemund T, Scholz U, Schobess R, Siegemund A. Clot waveform analysis in patients with haemophilia A. *Hamostaseologie*. 2014;34(Suppl 1):S48-52.
23. Lippi G, Ippolito L, Favaloro EJ. Technical evaluation of the novel preanalytical module on instrumentation laboratory ACL TOP: advancing automation in hemostasis testing. *J Lab Autom*. 2013;18:382-390.
24. Hernaningsih Y, Akualing JS. The effects of hemolysis on plasma prothrombin time and activated partial thromboplastin time tests using photo-optical method. *Medicine (Baltimore)*. 2017;96:e7976.
25. Margolis J. The kaolin clotting time; a rapid one-stage method for diagnosis of coagulation defects. *J Clin Pathol*. 1958;11:406-409.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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