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A novel low-cost approach for the semi-quantitative analysis of carbohydrate-deficient transferrin (CDT) based on fluorescence resonance energy transfer (FRET)

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ABSTRACT

Background and aim: The increase of the carbohydrate-deficient transferrin (CDT) as results of an heavy intake of alcohol for at least two weeks, is a well-known biochemical modification since the middle '70s. Notwithstanding the first commercial kit for the diagnosis of chronic alcohol abuse based on this biomarker was commercially accessible already thirty years ago, only expensive analytical methods are currently available for its determination. The present paper shows a new approach intrinsically sensitive and specific, based on a specific derivatization of transferrin, and not requiring sophisticated instrumentation.

Methods: The proposed procedure is based on a selective chelation of terbium (III) by transferrin followed by detection using an characteristic Fluorescence Resonance Transfer Energy (FRET) phenomenon (ex 298 nm - em 550 nm).

Results: The proposed procedure showed a limit of detection of 2.5 pmol/mL and a reproducibility intra-day and inter-days < 15% and 20%, respectively. The results obtained analyzing 40 serum samples using the developed method, were compared with those obtained with HPLC-Vis and an $R^2 = 0.8854$ was found.

Conclusions: Considering its main features (low-cost, ease of operation, minimum need of instrumentation) the present method is suitable for application in screening contexts and in non-strictly regulated environments (e.g. clinical diagnosis) as well as in developing countries or remote areas.

1. Introduction

Human transferrin (hTf) is an iron carrier serum glycoprotein constituted of 679 amino acids in single chain, with a molecular mass of 79.6 kDa. hTf is characterized by two similar lobes (lobe-C and N), each containing a site binding one ferric ion. The peptidic sequence binds mono-, bi- or tri- antennary carbohydrate chains containing *N*-acetylglucosamine, mannose, galactose and terminal sialic acid residues. This carbohydrate-related micro-heterogeneity produces different glycoforms with different isoelectric point (pI), related to the number and composition of the saccharide chains. The most represented glycoform (about 80%) contains two bi-antennary N-glycans for a total of 4 terminal residues of sialic acid (tetrasialo-Tf, pI 5.4). Other glycoforms with a different number of sialic acid residues represent the remaining part of the pattern [namely, disialo-Tf (pI 5.7), trisialo-Tf (pI 5.6), pentasialo-Tf (pI 5.2) and hexasialo-Tf (pI 5.0)]. Minor glycoforms with a single residue of sialic acid (monosialo-Tf) or lacking any residue (asialo-Tf) are also reported [1,2].

Since the middle '70s, it is well known that heavy consumption of alcohol (at least 50–80 g of ethanol per day) for two weeks or more, results in an increase of the Tf glycoforms with reduced glycosylation, and particularly asialo- and disialo-Tf [3–5]. These two components, and particularly disialo-Tf, are referred as Carbohydrate-Deficient Transferrin (CDT) and today are widely used in the diagnostics of alcohol abuses in clinical, occupational and forensic contexts [1,2].

Nowadays the analysis of CDT is based on different approaches,

Abbreviations: hTf, Human transferrin; Tf, Transferrin; CDT, Carbohydrate-Deficient Transferrin; FRET, Fluorescence Resonance Energy Transfer; POC, Point-of-Care; HPLC, High Performance Liquid Chromatography; TRIS, Tris-hydroxy-methyl-amino methane; BIS-TRIS, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; SPE, Solid-phase extraction; LOD, Limit of detection; ROC, Receiver operating curve; AUC, Area under the curve

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including immunoassay, multi-capillary electrophoresis or ion exchange liquid chromatography. Although instrumentally sophisticated and characterized by high automation and high productivity these techniques show important operative costs and require well equipped laboratories and specifically trained personnel.

This technological approach, because of analytical complexity and costs, is therefore limited to fully equipped laboratories, excluding alcohol detoxification centers and private doctors' offices. Also, hospitals and laboratories located in peripheral regions or in developing countries can hardly afford the implementation of CDT analysis.

In the present work, a new simple and low-cost fluorescence-based analytical method is presented as a potential tool for CDT analysis, not requiring sophisticated instrumentation. The method is based on an original analytical approach offering high sensitivity and specificity by means of the selective interaction between transferrin and terbium (III) (i.e. fluorescence resonant energy transfer) [Patent: WO2015135900A1]. This analytical mechanism was first applied to HPLC, achieving high sensitivity and selectivity in CDT determination also in complex biological matrices, e.g. whole blood, cadaveric blood, cerebrospinal fluid and dried blood spots [6]. Taking advantage of this first experience, in the present work we have developed an alternative application tailored for screening purposes, not requiring sophisticated instrumentation and/or expensive reagents, such as immunometric methods.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. Pure human apo-transferrin, tris-hydroxy-methyl-amino methane (TRIS), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BIS-TRIS), terbium chloride hexahydrate, hydrochloric acid, sodium hydroxide, sodium hydrogen carbonate and sodium chloride were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA). Weak anion exchange DEAE Sepharose™ Fast Flow and strong anion exchange Q Sepharose™ Fast Flow were purchased from GE Healthcare (Chicago, IL, US). Desferoxamine (Desferal®; Novartis AG, Basel, Switzerland) was purchased from Novartis Farma SpA. A model Purelab1 Chorus 3 water purification system (Elga Veolia, High Wycombe, United Kingdom) was utilized to obtain ultra-pure water. Empty SPE cartridges (1 mL) and inert frits were purchased from Agilent Technologies (Santa Clara, CA, US).

2.2. Samples

Blood samples were collected by venipuncture in Vacutainer serum tubes. After clotting at room temperature, serum was obtained by centrifugation at 1730*g* and the supernatant was collected and stored at −20 °C until analysis. For the present study, the samples were selected in order to cover a wide range of CDT concentrations (from 1 to 9%).

2.3. Home-made anion exchange cartridges

Before the analysis, the CDT separation cartridges were prepared by pouring 200 μL of Q Sepharose™, or DEAE Sepharose™, into 1 mL SPE cartridges containing an inert frit. The resin-filled cartridges were washed with 1.5 mL of a 24% water/ethanol mixture and then conditioned with 1.5 mL of 20 mmol/L BIS-TRIS buffer, pH 5.90. The device was ready to be used when the excess of liquid had flowed down through the cartridge (process taking about 3 min).

2.4. Sample pre-treatment and ion-exchange extraction

After saturation with 1 μL of a 10 mmol/L terbium (III) solution in water, 20 μL of serum was added with 480 μL of 20 mmol/L BIS-TRIS buffer, pH 5.9. The resulting 1:25 diluted sample was poured into the cartridge and let flow through by gravity. The drops were collected in a tube containing 20 μL NaOH 1 M (to obtain a final pH of the eluate around 8–9). The collected fluid was then analyzed with a stand-alone cuvette fluorimeter. Alternatively, CDT in the eluate was separated with anion-exchange HPLC with fluorimetric detection [6].

2.5. Fluorescence analysis

The fluorescence analysis was performed by analyzing the complex terbium (III)-transferrin. The principle on which this procedure is based is reported in [6]. Briefly, by exciting at 298 nm, the fluorescence of the transferrin, which represents the antenna in sensitizing process [ref], is modified and enhanced by adding the trivalent ion terbium. This phenomenon is the result of an intermolecular energy transfer process showing an intense and identifiable emission centered at 492, 550, and 586 nm. Among these emission signals, the strongest one (550 nm) is the selected wavelength for the present study.

Fluorescence spectra were recorded on a model RF 6000 spectrofluorimeter (Shimadzu Europe, Duisburg, Germany). The excitation wavelength was set at 298 nm, and the emission was set at 550 nm wavelength, or scanned within the range 450–700 nm. The results were expressed as absolute arbitrary units.

In the feasibility study of a Point-of-Care device, the excitation was exerted with the xenon lamp (150 W) of an old fluorimeter fitted with an optical filter at 300 nm (10 nm bandwidth). The emission radiation was recorded by the camera of a smartphone (OnePlus mod. 5 T - OnePlus, Shenzhen, China) after removal of the native protein fluorescence with a high-pass filter (460 nm) attached in front of the objective.

2.6. HPLC with fluorescence detection

The chromatographic system used for studying the CDT glycoforms present in the eluate, was composed of a Shimadzu LC-10 CE equipped with an RF-10AXL double monochromator fluorimeter. The column was a ProPac strong anion exchange (50 mm × 4 mm i.d.), 5 μm particle size (Thermo Fisher Scientific, Waltham, MA, US). A binary mobile phase was used with gradient elution. Mobile phase A was TRIS 20 mmol/L pH 8 added with terbium (III) chloride 50 mmol/L and desferoxamine 0.04 mmol/L, and mobile phase B was TRIS 20 mmol/L pH 8 added with NaCl 0.5 M. The flow rate was 2 mL/min. The column temperature was kept at 50 °C. The elution linear gradient was as follows: 0% B – 18% B in 5 min. After the separation, the column was cleaned and regenerated by washing for 3 min with NaCl 1.0 mol/L and then conditioning for 3 min at 0% B. The injection volume was 10 μL. The fluorimeter was set at excitation and emission wavelengths of 298 and 550 nm, respectively.

3. Results

In short, the proposed method was based on three steps:

a) functionalization of transferrin glycoforms with terbium (III);
b) isolation of functionalized CDT glycoforms on the basis of their charge on home-made ion exchange cartridges;
c) detection with a stand-alone fluorimeter.

In the method optimization, step a) did not require specific changes in comparison with the procedure reported in the article describing the use of FRET for CDT analysis with HPLC-FL [6].

Differently, the procedure of step b) was optimized in order to find the best conditions for isolating a suitable amount of asialo- and disialo-Tf from the whole Tf in the serum samples. A weak and a strong anionic exchange resin were tested, particularly DEAE and Q Sepharose™ (GE Healthcare - Chicago, IL, US), respectively. In this procedure, the content of the eluates from the extraction cartridges obtained under

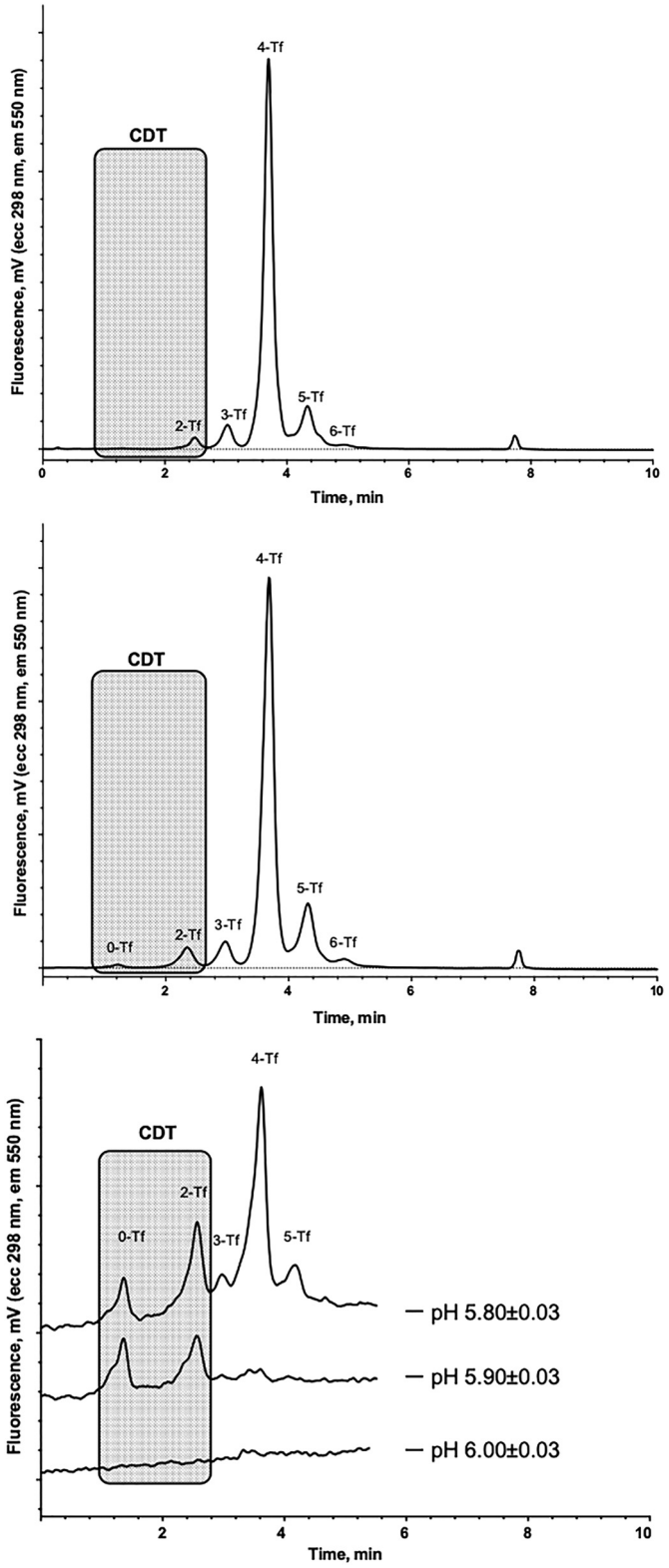

Fig. 1. Study of the best pH to isolate asialo-Tf and disialo-Tf. A) HPLC-FL analysis of a normal serum sample; B) HPLC-FL analysis of a CDT positive serum sample; C) HPLC-FL analysis of a serum sample treated with the proposed procedure within pH values of 5.8–6.0.

different conditions was analyzed with HPLC-FL. This method proved to be sensitive and selective enough for giving a picture of the CDT components passed through the cartridge after the loading of the serum samples previously saturated with terbium (III) and diluted with buffer.

The best results in terms of selective isolation of the major CDT components from human serum were achieved using Q Sepharose™, i.e. the strong anion exchanger, which was adopted for the further experiments.

After preliminary tests with BIS-TRIS buffers in a pH range 5.5–6.5, a 20 mmol/L BIS-TRIS buffer solution at a pH of 5.9 was chosen for cartridge equilibration and sample dilution (Fig. 1). Under the described conditions the average total recovery ($n = 6$) of asialo-Tf and disialo-Tf, measured with HPLC-FL, was 26.2% (SD ± 1.1) and 27.4% (SD ± 1.5) for CDT levels of 1.5% and 4.6%, respectively.

3.1. Assessment of method performances

The performances of the method were evaluated in terms of limit of detection (LOD), intra-day and inter-day precision and accuracy. Also, the developed method was used for analyzing 40 serum samples. The results were then compared with those obtained using an HPLC-Vis method (detection wavelength 460 nm) routinely in use in our laboratory [7].

The limit of detection (LOD) was calculated as the lowest amount of transferrin showing a fluorescence signal with a signal-to-noise ratio ≥ 3. Under these conditions the LOD resulted 2.5 pmol/mL.

The reliability of the cartridge pre-treatment was also evaluated in terms of reproducibility. The analysis of two serum samples, with CDT concentration of 1.5% (normal value) and 4.9% (high value) [measured with HPLC-Vis], was replicated five times in the same day and for five non-consecutive days. Using the fluorescence intensity as the analytical signal, the intra-day imprecision in the different 5 days resulted in the range between 6.6% and 14.2% and the inter-days imprecision was better than 20% (Table 1).

Notwithstanding the semi-quantitative-use of the proposed method, in an attempt to verify the analytical accuracy by comparison with an internationally accepted method for CDT analysis, 40 human serum samples were analyzed by using the proposed procedure and the results were compared with those obtained with the HPLC-Vis routinely in use in our center. A neat correlation was observed between the two methods, described by the equation $y = (14.97 \pm 0.87SD)\ x - (11.44 \pm 2.77SD)$, where x is the independent variable (%CDT-HPLC-Vis) and y is the dependent variable (absolute fluorescence of the eluates) (Fig. 2). The coefficient of correlation was highly significant ($R^2 = 0.8854$). The accuracy of the method was also evaluated by using the receiver operator curve (ROC) analysis, the results are depicted in (Fig. 3a) showing an area under the curve (AUC) equal to 0.8488. However in view of the potential application of the proposed method to real cases, it was decided to provide the user with a normalization parameter. Since, to the best of our knowledge, no commercial standards of pure asialo-Tf and/or disialo-Tf are available, an apo-Tf solution containing the whole pattern of glycoforms was used to produce a terbium (III)-Tf complex of standard concentration. Based on the fluorescence of the CDT negative and positive samples (as confirmed with HPLC-Vis), Tf solutions were prepared containing 650 µmol/L and 800 µmol/L to be used as references, being the former a sort of cut-off for the "negative" samples and the latter the cut-off for the "positive" ones. Samples showing fluorescence in between were interpreted as in a "grey zone", for which a confirmation analysis was mandatory. Using these criteria (and consequently excluding the results in the grey zone), the accuracy of the method was re-evaluated with ROC analysis achieving a AUC of 0.9525 (Fig. 3b).

3.2. Feasibility study of a point-of-care device

Considering the simplicity of the analytical procedure, not requiring sophisticated instrumentation and the interest in clinical settings for the so-called Point-of-Care testing (POCT) [8], it was preliminarily investigated a possibility of offering a possibility of detection with low-cost instrumentation. For this purpose, the double monochromator

Table 1
Precision intraday (n = 5) and interday (n = 25).

	IntraDay - Day 1 (n = 5)	IntraDay - Day 2 (n = 5)	IntraDay - Day 3 (n = 5)	IntraDay - Day 4 (n = 5)	IntraDay - Day 5 (n = 5)	InterDay (n = 25)
Negative sample (%CDT 1.5), RSD%	6,6	10,7	10,4	9,6	8,9	19,4
Positive Sample (%CDT 4.9), RSD%	11,2	9,2	9,4	10,0	14,2	18,6

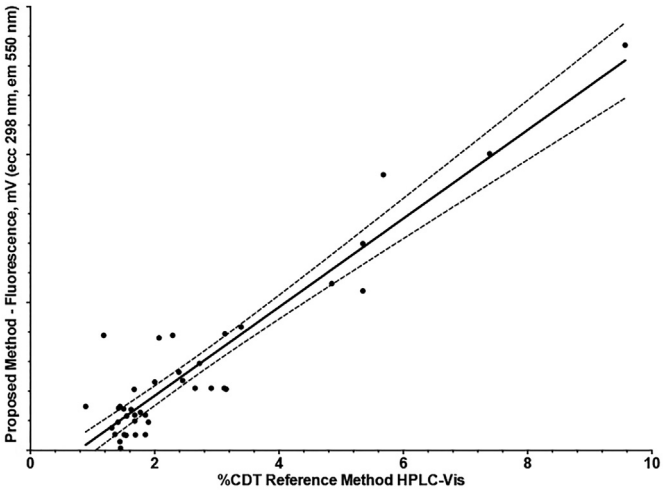

Fig. 2. Correlation between the results obtained using the proposed procedure and %CDT values calculated using HPLC-Vis. The analysis of 40 samples with a known %CDT (HPLC-Vis) was performed using the proposed method. In the graph: y-axis (proposed procedure) and x-axis (HPLC-Vis method).

fluorimeter was replaced with a non-professional detector based on the camera of a smartphone. Lacking, at the time of this experiment, a suitable monochromatic radiation source, the excitation was based on the xenon lamp of an old filter fluorimeter fitted with an optical filter at 300 nm (10 nm bandwidth). The emission radiation was filtered with a high band-pass filter (460 nm) in order to remove the natural fluorescence of proteins, occurring at wavelengths below 400 nm. This approach, although preliminary, proved suitable for recording and distinguishing the CDT-associated fluorescence of a “CDT positive” serum sample from that of a “CDT negative” one (Fig. 4). It is worth noting that the recorded signals could be processed by the image-processing software of the smartphone, which proved suitable for the analysis of the intensity of the signal. This perspective looks extremely interesting in view of data comparison.

4. Discussion

Notwithstanding CDT introduction in the diagnostics of alcohol abuses dates back to the ‘90s, the methods for its determination, after HPLC-Vis [9], capillary electrophoresis-UV [10], immunoassays with specific antibodies [11], isoelectric focusing [12] and disposable minicolumns [13] have shown little innovation except for the chip-based MALDI-TOF method [14]. In addition to the latter, in 2017, a new analytical principle, such as FRET has been proposed to increase both analytical sensitivity and specificity [6]. The energy transfer event is due to the selective chelation of terbium (III) by Tf in the binding pockets usually occupied by the ferric ion. This phenomenon, as described elsewhere [15–17], gives rise to a complex showing unique fluorescence characteristics. The use of FRET in an HPLC-FL method was reported to offer substantial advantages over current HPLC-Vis in terms of sensitivity and specificity, as discussed elsewhere [6].

Moreover, the high specificity of the terbium (III)-induced Tf fluorescence, which is minimally affected by other proteins and serum/plasma components seemed to offer a further possibility of CDT analysis without an expensive and instrumental chromatographic separation, which could become an ideal tool for screening purposes.

The developed procedure is based on a cut-off separation of the terbium (III) functionalized carbohydrate-deficient isotransferrins in serum by anion-exchange cartridges followed by their direct determination in the collected solution by fluorescence. The experimental parameters have been optimized in order to maximize the fluorescence signal and the specificity of the anion exchange isolation of the CDT-

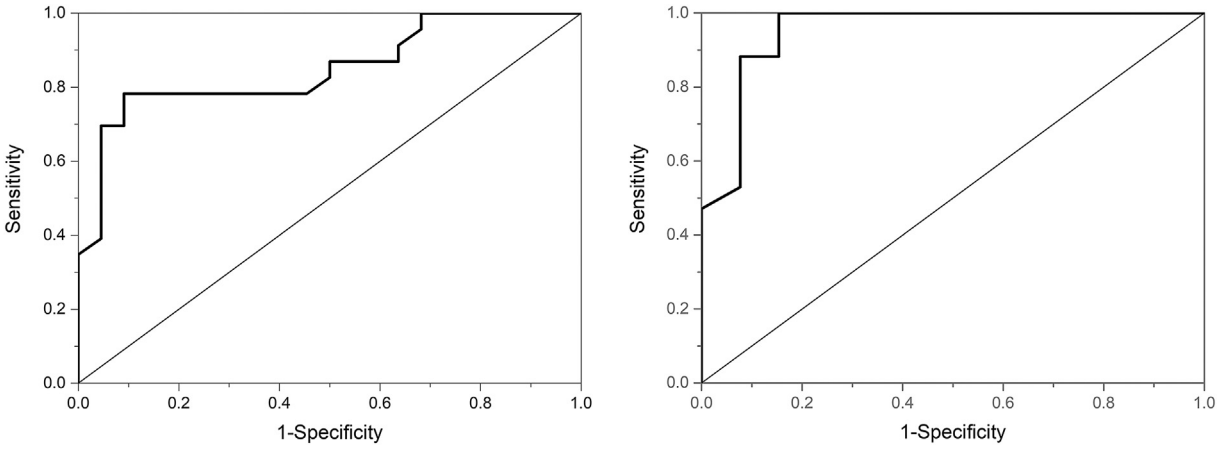

Fig. 3. Receiver operator curve (ROC) analysis. (A) ROC analysis of the analyzed samples by using the proposed method; (B) ROC analysis excluding the results in the grey zone.

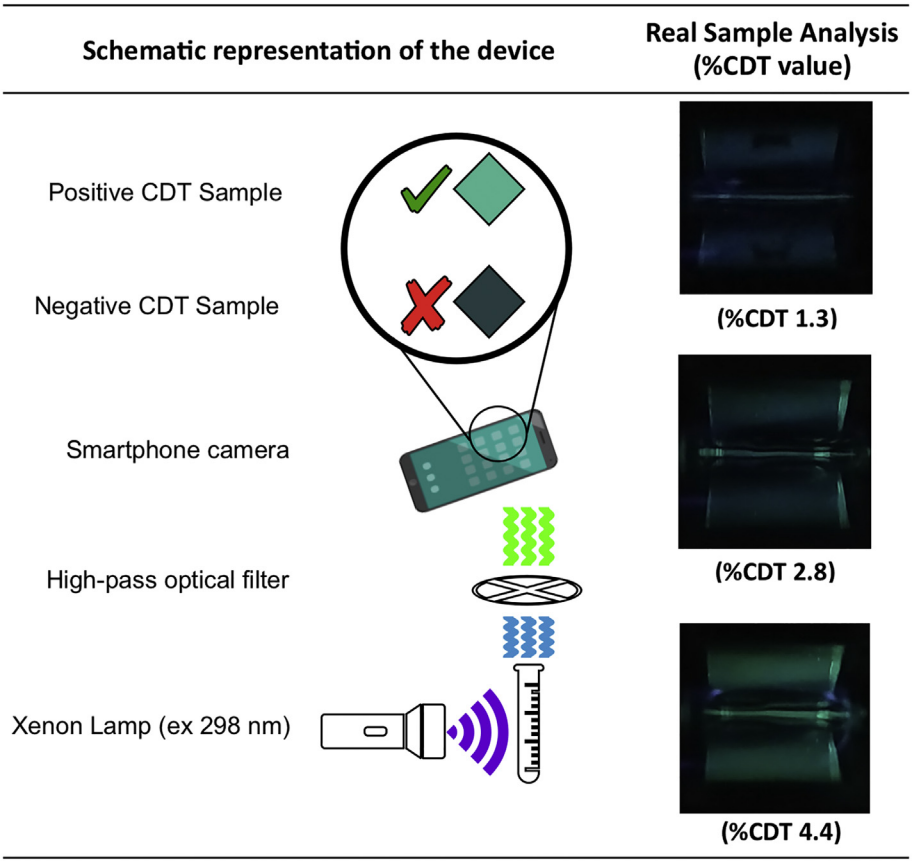

Fig. 4. Representation of the Point-of-Care device. Schematic representation of the proposed procedure modified in order to meet the requirements of a Point-of-Care device and images of the fluorescence emitted by three serum samples with different concentrations of %CDT.

related Tf glycoforms. The procedure is based on the use of a low-priced strong anion exchanger and a fluorimeter. The entire process requires < 10 min. The method, intended for qualitative analysis, has been tested in terms of precision and accuracy, and applied to real serum samples with known CDT concentration, showing a good correlation with a reference HPLC-Vis method.

For the isolation based on an anion exchange procedure, even if for the present study it has not been investigated, it has been widely demonstrated the incorrect determination of CDT due to rare transferrin isoforms, high trisialotrasferrin and high total transferrin [18]. In addition, other conditions have been reported as a possible cause of the alteration of the CDT value, e.g. severe hepatic failure, pregnancy, iron-deficiency, and combined pancreas and liver transplantation [18]. Although the latter are medical conditions which the doctor should know from the medical history of the patient, the risk of false-positive CDT results associated with genetic variants is a limit of the present procedure. However, considering its potential application for screening purposes in non-strictly regulated environments (e.g. clinical diagnosis), the evaluation of the method performances has been successful, in terms of ease of use, costs and reliability, precision and accuracy.

Eventually, the hypothesis of a non-instrumental version of this novel diagnostic method using as detector the camera of a smartphone has proved reasonably feasible.

5. Conclusions

In conclusion, the present method shows for the first time the applicability of FRET to develop a low-cost method for CDT analysis, which could be extensively be used in many clinical contexts. Operative simplicity and possibility of application without expensive instrumentation are the major pros of the new approach. On the other hand, its main limitation is the manual operation and the relative higher variability in comparison with instrumental methods. However, these weak points may not affect its practical usefulness considering that the results of this screening assay, must be confirmed with alternative, quantitative methods for any clinically relevant use.

Declaration of interest

Authors declare no conflicts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.06.003.

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