Biofluid infrared spectro-diagnostics: pre-analytical considerations for clinical applications

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Abstract

Several proof-of-concept studies on vibrational spectroscopy of biofluids have demonstrated that the methodology has promising potentials as a clinical diagnostic tool. However, these studies also show that there is lack of a standardised protocol in sample handling and preparation prior to spectroscopic analysis. One of the most important sources of analytical errors is the pre-analytical phase. For the technique to be translated into clinics, it is clear that a very strict protocol needs to be established for such biological samples. This study focuses on some of the aspects of the pre-analytical phase in the development of high-throughput **Fourier Transform Infrared (FTIR)** spectroscopy of some of the most common biofluids such as serum, plasma and bile. Pre-analytical considerations that can impact either the samples (solvents, anti-coagulants, freeze-thaw cycles...) and/or spectroscopic analysis (sample preparation such as drying, deposit methods, volumes, substrates, operators dependence...) and consequently on the quality and the reproducibility of spectral data will be discussed in this report.

Keywords: FTIR spectroscopy, biofluids, pre-analytical requirements, sample handling, standardisation, quality test, reproducibility

Introduction

During the last decade, a number of studies have highlighted the potential of vibrational spectroscopy applied to biofluids for screening, diagnostic and prognostic applications in the biomedical field [1-9]. These studies exploited the fact that biofluids exhibit spectral characteristics reflecting their biomolecular composition, which allows, through chemometric analysis, the identification of patterns reflecting sensitive and specific spectral biomarkers in various pathological conditions. Some biofluids such as serum and plasma appear as ideal media for routine clinical use as they are easily accessible, **collectable by minimally invasive method and repeatedly available** for monitoring disease progression or therapeutic response. Accordingly, most clinical studies have been performed using blood components and the quest for spectral blood biomarkers has been widely explored in patients with malignant and non-malignant diseases, as reviewed recently by Baker *et al* [10]. Other biofluids such as bile or cerebrospinal fluid are less accessible but of particular interest as their proximity with diseased tissue may facilitate the identification of the spectral signatures of the disease

[11,12]. Based on these data, biofluid vibrational spectroscopy appears as an innovative and promising facet of the concept of "liquid biopsy".

However, in spite of promising results, biofluid vibrational spectroscopy is still far from the routine clinical application. The great majority of studies are proof-of-concept studies based on a small number of subjects with and without the disease of interest. The results of these pilot studies should be analysed by cross validation methods and interpreted cautiously until validated in clinical trials performed in independent cohorts with a large number of patients and using golden standard diagnostic methods or complementary techniques. Most importantly, at the time where multicenter studies are being considered, there is a crucial need to standardise and validate the modalities of sample collection, storage and spectral acquisition. Standardisation of sample-related factors is the first step of pre-analytical validity of any technology intended to large scale and multicenter clinical applications. It is well-established that most errors in laboratory testing occur in the pre-analytical phase [13] and the need for standardisation of the procedures has been demonstrated in other high-throughput technologies such as proteomics [14,15], metabolomics [16,17] or genomics [18].

The work presented here will address the issue of pre-analytical variability in the field of high-throughput biofluid infrared transmission spectroscopy with a particular focus on blood components preparation. Factors that may affect the IR spectral quality and profile of biofluids will be discussed including biofluid dilution, volume and deposition modalities, repetition of freeze-thaw cycles, drying conditions, types of blood collection tubes used, intra- and inter-operator reproducibility.

Experimental

The studies were performed from a bank of serum and plasma samples stored at -80°C, originally taken for routine biochemical check-up at the Biochemistry Laboratory of Reims University Hospital. Serum was obtained by centrifugation of freshly clotted blood and plasma by centrifugation of blood collected in tubes containing ethylenediaminetetraacetic acid (EDTA) or lithium heparin as anticoagulants. Bile was obtained during endoscopic retrograde cholangiopancreatography after selective biliary cannulation and before any injection of contrast agent. After centrifugation, the cellular pellet was removed for routine cytological examination and the remaining supernatant was retrieved and stored at -80°C at the Pathology Department of Reims University Hospital. An informed consent was obtained

from all patients for performing a diagnostic and/or therapeutic endoscopic retrograde cholangiography.

1. Impact of biofluid volume and dilution on spectral quality test

The experimental procedures are detailed in figure 1. In brief, serum and plasma experiments were performed on pure and after 2-fold, 3-fold and 4-fold dilutions using normal saline (0.9% NaCl). As we have previously shown that bile can be analysed without dilution [12], only pure bile samples were used. Samples were deposited onto silicon plates, either 96-well plate with wells of 7 mm diameter or 384-well plate with wells of 3 mm diameter. Sample volumes of 5 and 10 μL were studied using the 96-well plate and 3 and 5 μL using the 384-well plate. A deposit of 1 μL without dilution was also tested with the 384-well plate. The deposits were spread drops onto the entire surface of the well or non-spread drops in the middle of the well. Ten replicates per sample were studied. After 1h drying at room temperature, the different plates were inserted into a high-throughput module (HTS-XT) coupled to an FTIR-spectrometer (Tensor 27, Bruker Optics GmbH, Ettlingen, Germany). Spectra were acquired in the transmission mode (OPUS v.6.5 software, Bruker Optics GmbH) in the 4000-400 cm^{-1} wavenumber range with a 4 cm^{-1} spectral resolution and 32 scans. A background reference spectrum was recorded before each sample analysis. The reference spectrum was automatically subtracted to obtain the final absorbance spectrum. A zero-filling factor of 2 and a Blackman-Harris-3-term function were applied for Fourier transformation.

The following quality tests (QT) were applied to each spectrum: 1) absorbance intensity: spectra were validated when the maximum absorbance (amide I band *c.a.* 1655 cm^{-1}) was below 1.8 arbitrary units; 2) signal-to-noise ratios were calculated according to the manufacturer's instructions for biological materials (Bruker Optics GmbH): the signals were calculated as the difference between the maximum and the minimum 1st derivative values in the range 1700-1600 cm^{-1} (signal 1 or S1) or as the difference between the maximum and the minimum 1st derivative values determined in the range 1260-1178 cm^{-1} (signal 2 or S2). Noise intensity (N) was calculated as the difference between the maximum and the minimum 1st derivative values in the range 2100-2000 cm^{-1} . Spectra met this quality test when ratios S1/N and S2/N were above 50 and 20 respectively; 3) signal-to-water ratio: water signal (W) was evaluated using the maximum 1st derivative values in the range 1842-1837 cm^{-1} . Ratio thresholds for spectral validation were S1/W and S2/W above 10 and 4 respectively.

Individual samples were considered as QT-validated for analysis when at least 8 replicate spectra out of 10 met the quality tests.

In order to analyse the impact of drop spreading, we performed experiments by successively using three-fold diluted serum and four-fold diluted plasma with spread and non-spread drops of 10 μL on 7 mm diameter wells. Spectra that passed the quality test were pre-processed on the wavenumber range from 1800 to 800 cm^{-1} . After baseline correction using a rubberband function, spectra were converted to second derivatives using the Savitsky-Golay algorithm, with 9-point smoothing to increase the spectral feature contrast. Then, their second derivatives were vector normalised. This spectral pre-processing was performed using the OPUS software (v6.5, Bruker Optics GmbH). Last, the processed spectra were classified using hierarchical cluster analysis (HCA) and principal component analysis (PCA) unsupervised methods, with spectra from spread and non-spread drops recorded in the same conditions. HCA consisted of grouping the spectra according to their degree of similarity. The method was based on Euclidean distance calculation between all the data sets by using the Ward's algorithm [19]. The merging process used the spectral information contained in the region between 1800–800 cm^{-1} . The result is displayed as a dendrogram showing the grouping of spectra in clusters according to a heterogeneity scale. In PCA data are reformulated as a linear combination of uncorrelated principal components (PCs) [20]. The first PC calculated accounts for the highest variance in the dataset. The second PC is uncorrelated with the first one *i.e.*, perpendicular to it and explains the next highest variance of the dataset, and so on. The PCs are also called loading vectors and their weights PC scores. PCA was performed in the same spectral range to analyse the spectra from spread and non-spread drops and to determine if the two sets of spectra are separated.

2. Impact of the dilution solvent: distilled water *versus* physiological water

In this set of experiments, the impact of the solvent used for dilution, distilled water or physiological water, was analysed. Three-fold diluted serum and plasma were prepared with each solvent. Then, 5 μL of samples were deposited onto a 384-well plate. After deposition, drops were air-dried at room temperature for 1h. Infrared spectra were acquired and pre-processed as described above. Finally, the pre-processed spectra were classified by HCA.

3. Impact of the type of anti-coagulant for plasma collection

Plasma samples were obtained by centrifugation of blood collected in tubes containing EDTA or lithium heparin as anticoagulants. After three-fold dilution, EDTA and lithium heparin plasma samples of 5 μL were deposited onto a 384-well plate and air-dried at room temperature for 1h. Then, spectra were acquired and subjected to quality test as described above. Spectra that passed the quality test were pre-processed and classified by HCA. A dendrogram was constructed to illustrate the level of similarity between spectra of plasma samples from EDTA and lithium heparin collection tubes. In order to evaluate the impact of EDTA (MW: 292 Da) on the spectral profile, FTIR spectra of plasma samples were also analysed after dialysis with a 10 kDa membrane to filter out EDTA molecules.

4. Spectral reproducibility

4.1 Impact of freeze-thaw cycles

The potential impact of repeated freeze-thaw cycles on quality test and reproducibility was studied. Fresh serum and serum samples after up to five consecutive freeze-thaw cycles (1h between each cycle) were studied. After thawing and three-fold dilution, serum samples of 5 μL were deposited onto a 384-well plate and dried at room temperature for 1h. Then, spectra were acquired and subjected to quality test as described above. Spectra that passed the quality test were pre-processed and classified by HCA to analyse the level of similarity between spectra according to the number of freeze-thaw cycles. In addition a PCA was performed and a PCA score plot was constructed to determine if separation of the different sets of spectra could be obtained.

4.2 Inter-operator spectral reproducibility

Inter-operator reproducibility was studied by comparing the spectra obtained by three different operators analysing the same sample under the same experimental conditions. Three-fold diluted serum was used for this purpose with 5 μL deposits onto a 384-well plate. After 1h drying at room temperature, spectra were acquired and those passing the quality test were pre-processed and analysed by HCA and PCA as described in the previous sections.

4.2 Intra- and inter-plate spectral reproducibility, day to day reproducibility

Reproducibility tests were performed using 5 μL of three-fold diluted serum spread by the same operator on 384-well plate. After 1h drying at room temperature, spectra were acquired and subjected to the QT as described above.

Intra-plate reproducibility was studied by depositing 360 replicates of the same sample under the same experimental conditions.

Inter-plate and day to day reproducibility was studied by comparing serum spectra obtained from three different patients using 3 different silicon plates on three different days.

Spectra that passed the quality test were pre-processed and classified by HCA to analyse the level of similarity between spectra.

5. Impact of drying modalities on spectral reproducibility

Since spectra from spread or non-spread drops are acquired after a drying period, the impact of drying duration on the QT and on the reproducibility was studied. Three-fold diluted serum and plasma as well as pure bile were used. Samples of 5 μL were deposited onto a 384-well plate. Then, they were dried for 45, 60, 120 min and 24h before FTIR measurements. The experiments were performed at 25°C in a close environment such as an incubator and at room temperature. Spectra were acquired and subjected to QT as described above. Spectra that passed the QT were pre-processed and classified by HCA and PCA as described above.

Results and discussion

Due to the development of high-throughput molecular technologies and associated bioinformatics, the number of studies dedicated to identification of new diagnostic biomarkers has increased exponentially over the last two decades. However, only a limited number of biomarkers have been validated for use in medical practice [21]. This is mainly explained by methodological flaws. Plebani and Carraro reported that most errors (68%) occurred in the pre-analytical phase and only 13% and 19% errors occurred in the intra-analytical and post-analytical phases respectively [22]. As for other high-throughput technologies, vibrational spectroscopic investigations are subject to the same problems although no statistics exist to date. The huge amount of data generated by spectroscopic analysis exposes this analysis to a significant risk of false positive findings. The risks should be minimised by rigorously controlling sample and patient related factors in the exploratory phase and by standardising

the conditions of spectral acquisition in the pre-analytical step. Thus, care should be taken to validate sample-related factors and patient-related factors. Standardisation of specimen collection and storage is crucial to reach experimental reproducibility not only in an individual laboratory but also between different laboratories. In addition, investigators should be aware of the risks of contamination during sample handling.

In the context of developing a high-throughput FTIR (HT-FTIR) transmission method for clinical diagnostics, we have investigated some important aspects involved in biofluid sample handling and preparation, which can impact on the spectral data and consequently on the outcome, and that we believe should be considered in the pre-analytical step prior to clinical applications. The study is focused on serum and plasma, and to a lesser extent bile, as they are the most encountered biofluids in clinical diagnostics.

1. Impact of biofluid volume and dilution on spectral quality test

Absorption FTIR spectroscopy is directly related to the Beer-Lambert law and the amount of analysed molecules has an impact on the signal quality.

Results are presented in Table 1. Dilution and volume of samples as well as size of the wells have a significant impact on the spectral QT. Spectra from pure serum and pure plasma using a volume ≥ 3 μL did not meet the QT because the amide I band is saturated with maximum absorbance intensity above 2 arbitrary units (Table 1). However, when the volume of pure serum was reduced to 1 μL , a majority of spectra was validated for non-spread deposits (Table 1). This is a point of great interest for clinical application as the deposit of a biofluid without dilution and without spreading would be the ideal approach for a high-throughput technology since it minimizes the number of operator's interventions and thus reduces risks of variability in the results. However, pipetting such low volumes can introduce additional errors and impact on the reproducibility of spectral data (ESI 1). The results deserve to be confirmed on a larger number of samples. Furthermore, the approach has not been generalised to plasma samples (Table 1). The reason why the great majority of spectra from a pure plasma sample of 1 μL did not meet the QT is an open question but is probably related to the presence of coagulation proteins in plasma and not in serum. When plasma and serum samples were diluted, the amide I band absorbance decreased to the acceptable range due to the lower concentration of proteins (ESI 2) in the sample and the proportion of QT-validated spectra increased. The two types of plates that are currently used in HT-FTIR spectroscopy are the

96-well and 384-well silicon substrates. Both types of plates have been tested in this study. Using the 96-well Si plate with 7 mm diameter wells, the optimal dilution for serum and plasma appears to be two-fold for spread drops and four-fold for non-spread drops (Table 1). Using the 384-well plate with 3 mm diameter wells, the optimal dilution was three-fold for serum and three- or four-fold for plasma depending on the volume (Table 1 and Figure 2). Overall, higher dilution and lower volume appear necessary to meet the QT when using the 3 mm diameter wells compared with the 7 mm diameter wells. This underlines that analyte amount in the deposit area is a limiting factor for obtaining QT validated spectra. As an example, in our experiments a serum volume of 10 μ L 2-fold diluted and deposited on a 7 mm diameter well resulted in a QT-validated spectrum as did a volume of 3 μ L 3-fold diluted and deposited on a 3 mm diameter well. However, given the specificity of each instrument, it should be underlined that each set up necessitates a proper pre-analytical validation.

From HCA and PCA analyses, it can be noted that pre-processed spectra from 2-, 3- and 4-fold dilution spread drops on 7 mm diameter wells are separated in three different groups (ESI 3) whereas pre-processed spectra from 3- and 4-fold dilution spread drops on 3 mm diameter wells are not differentiated (ESI 4). The above results indicate a higher heterogeneity with the 7 mm diameter wells compared to the 3 mm diameter wells although spectra were from the same sample and were normalised. It should be mentioned that a 5 μ L deposit onto a 3 mm diameter wells covers the whole area of the well, which avoids drop spreading and represents an advantage in terms of pre-analytical validity. In this context and depending on the dilution factor, a bigger volume is needed for the 7 mm diameter wells, which will induce a longer drying time. This is illustrated in ESI 5 where photographs of different volumes of serum dried on the two types of plates are shown. The impact of serum drop spreading on the spectral profile was studied on both 7 and 3 mm diameter wells and data were analysed by HCA and PCA. For the 7 mm diameter wells, the HCA plot showed two well-separated clusters of spectra corresponding to spread and non-spread drops indicating that the modalities of drop deposition on the surface of the plate had an influence on the spectral profile (ESI 6a). These results were confirmed by PCA analysis (ESI 6b). In addition, PC1 and PC2 loadings tend to indicate modifications in the protein spectral profile (ESI 6c). In contrast, for the 3 mm diameter wells, both HCA and PCA analyses showed no distinction between the spread and non-spread dried drops spectra (ESI 7a, b, c). This supports the above mentioned argument that the 5 μ L spread or non-spread deposit onto a 3 mm diameter wells covers the whole area of the well. Similar results were obtained for plasma samples (data not

shown). Thus, 5 μL of three-fold diluted serum or plasma appear as the ideal volume for high-throughput analysis. Figures 2a and 2b confirm these latter results respectively for serum and plasma *via* the scatter plots based on amide I absorbance by comparing the dilution factor (2-, 3- and 4-fold) as well as the spread and non-spread procedures.

Bile has been treated separately from serum and plasma because its composition is different. This is mainly due to lower concentrations of metabolites in bile compared to serum or plasma. Protein concentration in bile is about 5 g/L [23] as opposed to 60 to 80 g/L in serum. These specificities allow bile samples to be measured in their pure forms (ESI 8). The low absorption, in particular the amide I band, required the implementation of an adapted QT. Our results show that all pure bile spectra (except for 1 μL) were QT-validated for spread dried drop. All spectra from non-spread dried drops were rejected (Table 2).

2. Impact of the dilution solvent: distilled water *versus* physiological water

Distilled water and physiological water are often used as solvents in many analytical assays. Since biofluids contain high protein concentrations, we wanted to investigate whether these solvents could have an effect on spectral analysis. Results are displayed in Figure 3a where the dendrogram shows that spectra are clearly divided into two main clusters corresponding to each solvent used for dilution. This indicates that the solvent has a major impact on the spectral characteristics. It is known that distilled water induces protein precipitation (turbid solution), an effect which is not observed with physiological water (photograph insert, Figure 3b). If sample dilution is necessary, it is recommended using physiological water rather than distilled water.

3. Plasma collection: impact of the type of anti-coagulant

For biochemical assays used routinely in clinics, blood samples are collected using either lithium heparin or EDTA tubes depending on the targeted analyses. However, can these anti-coagulants have an effect on spectral data? To answer this question, spectra of plasmas from lithium heparin and EDTA tubes were acquired and compared in Figure 4a. It can be observed that plasma from EDTA tubes exhibit additional spectral features compared to plasma from lithium heparin tubes, specifically in the fingerprint region ($1800\text{-}800\text{ cm}^{-1}$). After a dialysis using a 10 kDa membrane, these artefactual peaks originating from small EDTA molecules

can be removed (Figure 4b). HCA classification displayed a complete separation between spectra from EDTA and lithium heparin plasma samples before dialysis. This clustering was no longer observed after dialysis (data not shown). Given these data, it appears more appropriate to use lithium heparin tubes for spectroscopic analysis of plasma. Unless there is a specific requirement for using plasma samples, serum appears as the sample of choice for biofluid spectroscopy. Use of plasma is important in specific diseases (e.g., hemophilia) where coagulating proteins are necessary for biochemical and hemostasis assays. In contrast with plasma, there is no need for using anticoagulants to collect serum rendering sample handling easier.

4. Spectral reproducibility

4.1 Impact of freeze-thaw cycles on spectral reproducibility

The impact of repeated freeze-thaw cycles before biofluid sample analysis is a matter of concern in biomedical technologies. Although they can be obtained non-invasively, serum and plasma are very valuable sample materials and they are stored at -80°C where they are supposed to be relatively stable. However, for clinical studies, repeated freeze-thaw cycles are unavoidable when the sample volume is limited. Thus, inadequate conditions for sample storage can have an impact on measured data as it has been reported in a recent metabolomics study [17]. Taking into account this finding, we have studied the effect of freeze-thaw cycles on spectral data. Fresh serum and serum samples after up to five consecutive freeze-thaw cycles were analysed and their spectral profiles compared. Figure 5a displays the HCA results obtained in the $1800\text{--}800\text{ cm}^{-1}$ spectral range and clearly demonstrates a clear-cut delineation between fresh and freeze-thawed serum samples even after 1 cycle.

The results were confirmed by PCA analysis showing the fresh samples as a completely separate group (Figure 5b). Both analyses show that all freeze-thawed data were mixed. Furthermore, the loadings of PC1 and PC2 tend to indicate that the most prominent modifications occur in the protein region (Figure 5c). When the fresh samples were removed, data analysis did not reveal any structures in the spectral datasets of the five freeze-thaw cycles (data not shown). Similar results have been obtained when fresh plasma was compared with plasma samples after up to five consecutive freeze-thaw cycles (data not shown). These findings suggest that FTIR spectroscopy is sensitive enough to distinguish fresh from freeze-thawed serum and plasma samples but cannot differentiate between the five freeze-thaw

cycles. On the other hand, metabolomics techniques being more sensitive have shown changes in metabolite ions [24] and macromolecules [25,26] after a few freeze-thaw cycles. Since sample freezing is unavoidable (large collection, multicentric studies, transportation...), it is therefore recommendable to avoid multiple freeze-thaw cycles of the same sample and favour multiple pure sample aliquots. However, our study has not taken into account the effect of freezing time on the spectral data, in particular for long term storage, and it would be interesting to investigate if FTIR spectroscopy could monitor any content deviations in metabolite ions and macromolecules like proteins, DNA and RNA.

4.2 Intra- and inter-plate spectral reproducibility, day to day reproducibility

In this study, intra- and inter-plate as well as day to day reproducibility has been investigated. For intra-plate reproducibility, 360 replicates of the same sample were deposited and measured by the same operator. The HCA analysis did not show any specific clustering and all spectra were completely mixed and the replicates were distributed over the whole cluster (data not shown).

Day to day reproducibility was studied by analysing serum from three patients on three consecutive days. The dendrogram obtained after HCA analysis (Figure 6) shows that the clustering is only based on patient information. This is an important finding because the patient to patient variation is greater than day to day variation. This experiment has been reproduced on two other plates and no significant inter-plate spectral variability has been observed (data not shown).

4.3 Inter-operator spectral reproducibility

The inter-operator reproducibility was investigated by three different operators using the same instrumentation to analyse the same sample on the same plate and on the same day. This is to mimic a normal working day procedure in a clinical setup where the instrument could be used by several users. HCA and PCA have been used to visualise any structure in the dataset. Both HCA and PCA results show that the spectral reproducibility is operator-independent (ESI 9). This is an important result for clinical implementation provided that the protocol is strictly followed.

The question is open whether this level of inter-operator reproducibility can be reached on different instruments of the same manufacturer or different manufacturers.

5. Impact of drying modalities on spectral reproducibility

High-throughput FTIR transmission spectroscopy of biofluids necessitates a drying phase for both spread and non-spread drops. This phase can impact on the QT and on the reproducibility of the spectral data. This effect has been studied on three-fold diluted serum and plasma as well as on pure bile. Five microlitre drops were dried at room temperature and at 25°C during 45, 60, 120 min and 24h before FTIR measurements. Table 3 summarises the results obtained for serum, plasma and bile samples and shows that a drying time of 45 min is not sufficient to meet the QT conditions (red dots). For all three biofluids, a minimum of one hour drying time is necessary at room temperature to pass the QT as opposed to two hours at 25°C. However, it is important to note that it would be more appropriate to dry for a longer duration at room temperature to make sure that the drying process is complete. The spectra obtained after 2h drying are differentiated by HCA from those recorded after 1h and 24h (ESI 10a). Additionally, PCA analysis shows a separation between 1h and 2h drying and between 2h and 24h drying at room temperature (ESI 10b, 10d). The PC loadings clearly show that water absorption contributes to the spectral variance (ESI 10c, 10e). The proximity of the clusters corresponding to data obtained after 1h and 24h drying suggests that samples rehydration may have occurred overnight. In a clinical application after the drying phase, it is therefore important to define the drying time and not to wait too long before recording spectra. When comparing spectra of drops dried at room temperature and at 25°C, we found that HCA shows no clustering according to these two conditions (data not shown).

This is an interesting aspect for the clinical use because sample drying can be performed at room temperature without the need of any additional laboratory equipment for controlling drying conditions.

Conclusion

The development of any laboratory analytical technique, including vibrational spectroscopy, that has the potential of being translated to clinical applications requires an evaluation of the potential factors of the pre-, intra- and post-analytical phases that can impact on data quality and consequently on the result outcome. It has been reported that in the procedures described for the laboratory analysis of a patient's sample, two-thirds of the issues originate from the pre-analytical procedures. In this context, we have investigated using HT-FTIR spectroscopy

some aspects of the pre-analytical procedures and their impact on the data quality and reproducibility. The results show that the type of biofluid to be analysed will condition the analytical procedures to be implemented. In a non-exhaustive manner, we have shown that the sample collection modality, the type of substrate, the dilution factor, the volume of sample deposited, the way the sample is deposited, the drying conditions, the inter-operator, the day-to-day variabilities are some of the aspects that need to be investigated for error tracking in the pre-analytical phase. Indeed, more work is needed to evaluate intra- and inter-centre variabilities as well as the effect of other factors involved in the intra- and post-analytical phases. The study has focused on the HT-FTIR methodology but it will be interesting to have similar studies on other vibrational spectroscopic modalities.

In order to stimulate discussion from the results of this study, the following points can be debated:

1. Serum is the optimum blood component for spectro-diagnostics of major diseases.
2. There is no requirement for advanced sample preparation.
3. No additional device is required for sample drying since this can be done at room temperature.
4. There is no issue with inter-operator/inter-day analysis.
5. Following the 1st freeze-thaw there is no issue with the a few freeze-thaws cycles but long term storage after several freeze-thaw cycles deserve to be investigated.
6. How do we assess and correct for instrument-to-instrument variation?
7. Development of an automated device for sample deposition and data acquisition can be more attractive to clinical laboratories.

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Tables and Figures Legends

Table 1: Results of quality test on FTIR serum and plasma spectra from spread and non-spread dried drops at various dilutions and volumes.

Table 2: Results of quality test on FTIR bile spectra from spread and non-spread dried drops at various volumes.

Table 3: Quality test results on FTIR raw spectra of 3-fold diluted spread drops (5 μ L) of serum (S), plasma (P) and pure spread bile (B) drops (5 μ L) left dried at room temperature (RT) or 25°C after 45, 60, 120 minutes and 24h.

Figure 1: Experimental protocol for studying the impact of dilution and volume on spectral quality test. S: spread dried drop, NS: non-spread dried drop, QT: quality test.

Figure 2: Scatter plots based on the Amide I absorbance value of serum (a) and plasma (b) replicate FTIR spectra from spread and non-spread dried drops (5 μ L deposits onto 3 mm diameter well) at various dilutions. Validated and discarded spectra after the quality test are represented by green and red dots respectively. The median absorbance value is represented by the black bar. S: spread dried drop, NS: non-spread dried drop.

Figure 3: (a) Hierarchical cluster analysis of FTIR pre-processed spectra from dried spread serum drops 3-fold diluted using either physiological or distilled water (deposits of 5 μ L onto 3 mm diameter wells). (b) Photograph of serum diluted with physiological water (left) and distilled water (right).

Figure 4: Comparison of FTIR pre-processed mean spectra of plasma collected in tubes containing lithium heparin (red) or EDTA (blue) as anticoagulants without dialysis (a) or after dialysis with a 10kDa membrane (b).

Figure 5: (a) Hierarchical cluster analysis of FTIR pre-processed spectra from fresh serum and after up to 5 freeze-thaw cycles (deposits of 5 μ L, 3-fold diluted onto 3 mm diameter wells). (b) PCA scatter plot of the spectral dataset from (a), (c) PC1 and PC2 loadings from (b). Fresh serum (dark blue), freeze-thaw cycle 1 (pink), 2 (light blue), 3 (orange), 4 (green), 5 (red).

Figure 6: Hierarchical cluster analysis of FTIR pre-processed spectra from serum of three patient samples deposited on a same silicon plate on three different days (deposits of 5 μ L, 3-fold diluted onto 3 mm diameter wells).

ESI 1: Scatter plot based on the Amide I absorbance value of serum replicate spectra from spread and non-spread pure dried drops (1 μ L deposits onto 3mm diameter well). Validated and discarded spectra after the quality test are represented by green and red dots respectively.

The median absorbance value is represented by the black bar. S: spread dried drop, NS: non-spread dried drop.

ESI 2: FTIR raw median spectra of serum samples (10 μ L deposits onto 7 mm diameter well) at different dilutions 2-fold (red), 3-fold (green), 4-fold (blue) dilutions and without dilution (pink).

ESI 3: (a) Hierarchical cluster analysis of FTIR pre-processed spectra from 2-fold (red), 3-fold (green) and 4-fold (blue) diluted serum (5 μ L) deposited onto 7 mm diameter wells. (b) PCA scatter plot of the spectral dataset from (a), (c) PC1 and PC2 loadings from (b).

ESI 4: (a) Hierarchical cluster analysis of FTIR pre-processed spectra from 3-fold (green) and 4-fold (blue) diluted serum (5 μ L) deposited onto 3 mm diameter wells. (b) PCA scatter plot of the spectral dataset from (a), (c) PC1 and PC2 loadings from (b).

ESI 5: Photographs of different volumes of dried serum drops on 7 mm diameter wells (a) and 3 mm diameter wells (b). S: spread drop, NS: non-spread drop.

ESI 6: (a) Hierarchical cluster analysis of FTIR pre-processed spectra from 3-fold diluted serum (5 μ L) spread (light blue) or non-spread (red) over the surface of 7 mm diameter wells. (b) PCA scatter plot of the spectral dataset from (a), (c) PC1 and PC2 loadings from (b).

ESI 7: (a) Hierarchical cluster analysis of FTIR pre-processed spectra from 3-fold diluted serum (5 μ L) spread (light blue) or non-spread (red) over the surface of 3 mm diameter wells. (b) PCA scatter plot of the spectral dataset from (a), (c) PC1 and PC2 loadings from (b).

ESI 8: FTIR raw median spectra of bile samples (5 μ L deposits onto 3 mm diameter well) without dilution.

ESI 9: (a) Hierarchical cluster analysis of FTIR pre-processed spectra from one 3-fold diluted serum sample spread (5 μ L) over the surface of 3 mm diameter wells by three different operators (red, light blue and purple). (b) PCA scatter plot of the spectral dataset from (a), (c) PC1 and PC2 loadings from (b).

ESI 10: (a) Hierarchical cluster analysis of FTIR spectra from 3-fold diluted serum (5 μ L) deposited onto 3 mm diameter wells and left dried at room temperature 1h (blue), 2h (green) or 24h (orange). All spectra were cut between 4000-800 cm^{-1} , rubberband baseline corrected and vector normalised. (b) PCA scatter plot of the spectra of serum dried at room temperature 1h (blue) and 2h (green). (c) PC1 and PC2 loadings from (b). (d) PCA scatter plot of the spectra of serum dried at room temperature 2h (green) and 24h (orange). (e) PC1 and PC2 loadings from (d).

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182x66mm (150 x 150 DPI)

177x112mm (150 x 150 DPI)

184x73mm (150 x 150 DPI)

187x182mm (150 x 150 DPI)

177x125mm (150 x 150 DPI)

Si plate	7 mm diameter well (96-well plate)								3 mm diameter well (384-well plate)								
Drop volume (μL)	5				10				1	3				5			
Dilution	0	1/2	1/3	1/4	0	1/2	1/3	1/4	0	0	1/2	1/3	1/4	0	1/2	1/3	1/4
Serum spread drops																	
Sample QT validation*																	
Validated spectra/sample**	0	9	10	0	0	10	10	9	7	0	5	10	10	0	1	10	10
Serum non-spread drops																	
Sample QT validation*																	
Validated spectra/sample**	1	2	9	10	0	0	2	10	8	0	4	10	10	0	1	10	10
Plasma spread drops																	
Sample QT validation*																	
Validated spectra/sample**	0	10	10	8	0	10	10	10	0	0	9	10	10	0	0	9	10
Plasma non-spread drops																	
Sample QT validation*																	
Validated spectra/sample**	0	1	6	10	0	0	0	10	2	0	0	10	10	0	0	5	10

* The sample was validated (green dot) when at least 8 replicates out of 10 met the quality test (QT) and discarded when less than 8 replicates met the QT (red dot).

** 10 replicates per sample were deposited.

186x90mm (150 x 150 DPI)

Silicon plate	7 mm diameter well (96-well plate)		3 mm diameter well (384-well plate)		
Drop volume (µL)	5	10	1	3	5
Dilution	0	0	0	0	0
Bile spread drops					
Sample QT validation*
Validated spectra/sample**	10	10	0	10	10
Bile non-spread drops					
Sample QT validation*
Validated spectra/sample**	6	6	0	6	7
* The sample was validated (green dot) when at least 8 replicates out of 10 met the quality test (QT) and discarded when less than 8 replicates met the QT (red dot).					
** 10 replicates per sample were deposited.					

185x69mm (150 x 150 DPI)

	45 min			60 min			120 min			24 h		
	S	P	B	S	P	B	S	P	B	S	P	B
Drying at RT												
<i>Sample QT validation*</i>
<i>Validated spectra/sample**</i>	3	0	1	10	10	10	10	9	9	9	10	9
Drying at 25°C												
<i>Sample QT validation*</i>
<i>Validated spectra/sample**</i>	0	0	0	0	0	0	10	10	10	10	10	10
<p>* The sample was validated (green dot) when at least 8 replicates out of 10 met the quality test (QT) and discarded when less than 8 replicates met the QT (red dot).</p> <p>** 10 replicates per sample were deposited.</p>												

154x65mm (150 x 150 DPI)

100x78mm (150 x 150 DPI)

125x99mm (117 x 108 DPI)

190x198mm (150 x 150 DPI)

191x193mm (150 x 150 DPI)

186x199mm (150 x 150 DPI)

189x197mm (150 x 150 DPI)

124x95mm (119 x 113 DPI)

189x178mm (150 x 150 DPI)