

Standardization of complex biologically-derived spectrochemical datasets

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Abstract

Spectroscopic techniques, such as Fourier-transform infrared (FTIR) spectroscopy, are used to study the interaction of light with biological materials. This interaction forms the basis of many analytical assays used in disease screening and diagnosis, microbiological studies, forensic and environmental investigations. Advantages of spectrochemical analysis are its low cost, minimal sample preparation, non-destructive nature and substantially accurate results. However, there is now an urgent need for repetition and validation of these methods in large-scale studies and across different research groups, which would bring the method closer to clinical and/or industrial implementation. In order for this to succeed, it is important to understand and reduce the effect of random spectral alterations caused by inter-individual, inter-instrument and/or inter-laboratory variations, such as variations in air humidity and CO₂ levels, and the aging of instrumental parts. Thus, it is evident that spectral standardization is crucial for the widespread adoption of these spectrochemical technologies. By using calibration transfer procedures, where the spectral response of a secondary instrument is standardized to resemble the spectral response of a primary instrument, different sources of variations can be normalized into a single model using computational-based methods, such as direct standardization (DS) and piecewise direct standardization (PDS); therefore, measurements performed under different conditions can generate the same result, eliminating the need for a full recalibration. In this paper, we have constructed a protocol for model standardization using different transfer technologies described for FTIR spectrochemical applications. This is a critical step towards the construction of a practical spectrochemical analysis model for daily routine analysis, where uncertain and random variations are present.

Introduction

Vibrational spectroscopy has shown great promise as an analytical tool for the investigation of numerous sample types with wide applications in diverse sectors, such as biomedicine, pharmaceuticals or environmental sciences¹⁻⁵. Fourier-transform infrared (FTIR) spectroscopy is one of the preferred techniques for identification of biomolecules through the study of their characteristic vibrational movements. Another commonly used approach is Raman spectroscopy, which provides complementary spectral information to IR. Raman spectroscopy exploits the inelastic scattering of light whereas IR studies light absorption. Both methods have their benefits and drawbacks. A limitation of IR, for instance, is that water generates undesired peaks at the region of interest, which can mask important biological information, and therefore extra sample preparation and/or spectral processing is necessary. On the contrary, Raman spectroscopy has an inherently weak signal and fluorescence interference, which can, however, be addressed by optimizing the experimental settings or by applying enhancement techniques to increase the Raman signal. For the purposes of this protocol we have used FTIR spectroscopy to demonstrate our standardization model.

Using chemometric approaches, the system is trained to recognize unique spectral features within a sample, so that when unknown samples are introduced an accurate classification is feasible. Alterations in the measurement parameters could interfere with the spectral signature and produce random variations. Therefore, a crucial step is spectral correction, or standardization, which would provide comparable results and allow system transferability. The idea is that non-biological variations, such as those arising from different users, locations or instruments, will no longer affect the classification result; therefore any collected data could be imported into a central database and handled for further exploration or diagnostic purposes. Several groups and companies

worldwide are developing spectrochemical approaches for diagnosis, discrimination and monitoring of diseases, as well as for other uses. Combination of multiple datasets would facilitate the conduction of large-scale studies which are still lacking in the field of bio-spectroscopy.

Sensor-based technologies

Sensor-based technologies are an integral part of daily life ranging from locating sensor-based technology, such as global positioning system (GPS)⁶, to image biosensors, such as X-rays⁷⁻¹⁰ and γ -rays¹¹⁻¹³, which are used extensively for medical applications. Other powerful approaches that make use of sensor-based technologies toward medical disease examination and diagnostics include circular dichroism (CD) spectroscopy¹⁴⁻¹⁷, ultraviolet (UV) or visible spectroscopy^{18,19}, fluorescence²⁰⁻²⁴, nuclear magnetic resonance (NMR) spectroscopy²⁵⁻²⁹ and ultrasound (US)^{7,30-33}.

Over the last two decades, optical biosensors employing vibrational spectroscopy, particularly IR spectroscopy, have seen tremendous progress in biomedical and biological research. A number of studies using the above-mentioned methods have focused on cancer investigation with malignancies such as brain³⁴⁻³⁷, breast³⁸⁻⁴⁰, oesophagus^{41,42}, skin⁴³⁻⁴⁷, colorectal⁴⁸⁻⁵⁰, lung⁵¹⁻⁵³, ovarian⁵⁴⁻⁵⁸, endometrial^{55,59,60}, cervical⁶¹⁻⁶⁴ and prostate⁶⁵⁻⁶⁸ cancer being some of them. Non-cancerous diseases have also been examined, namely neurodegenerative disorders⁶⁹⁻⁷², HIV/AIDS⁷³, diabetes⁷⁴⁻⁷⁶, rheumatoid arthritis^{77,78}, cardiovascular diseases^{79,80}, malaria⁸¹⁻⁸³, alkaptonuria⁸⁴, cystic fibrosis⁸⁵, thalassemia⁸⁶, prenatal disorders^{87,88}, macular degeneration^{89,90}, atherosclerosis^{80,91} and osteoarthritis⁹²⁻⁹⁴.

Limitations

Spectrochemical approaches are advantageous when compared with traditional molecular methods as they provide a holistic status of the sample under interrogation, thus generating typical spectral regions widely known as “fingerprint regions”. These methods have also been shown to be rapid, inexpensive and non-destructive while they also improve diagnostic performance and eliminate subjective diagnosis (*e.g.*, histopathological diagnosis), where inter- and intra-observer variability are present⁹⁵. However, like any other analytical method, vibrational spectroscopy also comes with some limitations. For instance, prior to FTIR studies, optimization of instrumental settings, sample preparation and operation mode also needs to be conducted in order to improve the spectral quality and molecular sensitivity^{4,96,97}. Overall, the above-mentioned barriers can be overcome after careful consideration of the experimental design.

A considerable limitation that is yet under-investigated in the field of spectrochemical techniques is associated with the difficulties entailed in data conformation and system standardization. Currently, there are multiple pilot studies showing promising results but an approach towards standardization for biological applications is lacking. Random variation between studies can originate from differences in instrumentation, operators, and environmental conditions, such as room temperature and humidity.

The main objective of this article is to present a protocol for model standardization which can be applied in FTIR spectrochemical techniques to rule out the chance of random spectral alterations. Inter-individual, inter-instrument, inter-sample and/or inter-laboratory variations can be a source of unwanted, non-biological alterations, thus leading to incorrect conclusions. However, for a method to become reliable and clinically translatable, it is important that measurements performed under different conditions generate comparable results. The aim of the

spectral standardization model presented here is to expedite multi-centre studies with large numbers of samples; this would bring these spectrochemical techniques closer to clinical implementation and facilitate life-changing decisions. We describe a protocol that has four main components: (i) sample preparation, (ii) spectral acquisition, (iii) data pre-processing and (iv) model standardization. The current protocol has an in-depth insight obtained from cross-laboratory collaborations with leading experts in the field. This article offers a step-by-step procedure, which can be implemented by a non-specialist in spectrochemical studies. For further information about instrumental and software options, spectral acquisition steps and data analysis for a range of different analytical systems the reader is directed towards additional protocols^{4,98-105}.

Applications

Spectrochemical approaches, in combination with computational analysis, have been proven to be effective for biomedical research through facilitating the diagnosis, classification, prognosis, treatment stratification and modulation or monitoring of a disease and treatment. However, these techniques are widely applicable to other fields as well, namely food industry¹⁰⁶⁻¹⁰⁹, toxicology^{2,110-112}, microbiology¹¹³⁻¹¹⁸, forensics¹¹⁹⁻¹²³, pharmacy^{2,3,124}, environmental and plant science¹²⁵⁻¹²⁷, as well as defence and security¹²⁸⁻¹³⁰. Applications of standardization algorithms vary according to the spectral technique and sample matrix studied, and have been mostly applied to Raman and Fourier-transform near-infrared (FT-NIR) spectroscopy. Table 1 summarizes some standardization applications.

1 **Table 1.** Examples of applications involving standardization techniques.

Sample matrix	Spectroscopic technique	Aim	Ref.
Tissue	Raman	Standardization of various perturbations on Raman spectra for diagnosis of breast cancer based on snap frozen tissues	131
	Raman	Standardization of spectra acquired in 3 different sites for analysing oesophageal samples based on snap frozen tissues	132
Cells	Raman	Standardization of spectra acquired with 4 different instruments for classification of three different cultured spore species	133
Biofluids	FT-NIR	Standardization of spectra acquired with 3 different instruments for measuring haematocrit in the blood of grazing cattle	134
	LC-MS	Standardization of spectra acquired with 2 different instruments for mapping retention times and matching metabolite features of subjects diagnosed with small cell lung cancer based on blood serum and plasma samples analysis	135
Pharmaceutical materials	Raman	Standardization of spectra acquired with 5 different instruments for analysing various pharmaceutical excipients, active pharmaceutical ingredients (APIs) and common contaminants	136
	FT-NIR	Standardization of spectra acquired with 2 different instruments for simultaneous determination of rifampicin and isoniazid in pharmaceutical formulations	137
	FT-NIR	Standardization of spectra acquired with 2 different instruments for predicting content of 654 pharmaceutical tablets	138
Food	FT-NIR	Standardization of spectra acquired with 3 different instruments for predicting parameters in corn samples	138
	FT-NIR	Standardization of spectra acquired with 2 different instruments for predicting vitamin C in navel orange	139
	FT-NIR	Standardization of spectra recorded in 4 different labs for determining moisture, proteins and oil content in soy seeds	140
	FT-NIR	Standardization of spectra acquired by a benchtop and portable instrument for determining total soluble solid contents in single grape berry	141
	FT-NIR	Standardization of spectra acquired by a benchtop and portable instrument for determining total soluble solid contents in single grape berry	142
Plant	UV-Vis	Standardization of visible spectra acquired with 3 different instruments for measuring pH of Sala mango	143
	FT-NIR	Standardization of spectra acquired with 2 different instruments for predicting baicalin contents in radix scutellariae samples	139
	FT-NIR	Standardization of spectra acquired by 2 different instruments and in three physical states (powder, filament and intact leaf) for determining total sugars, reducing sugars and nicotine in tobacco leaf samples	144
Cosmetic	NMR	Standardization of spectra acquired with 3 different instruments for authenticity control of sunflower lecithin	145
	CD spectroscopy	Standardization of spectra acquired between standard and real-world samples for determining Pb ²⁺ in cosmetic samples	146
Inorganic substances	FT-IR	Standardization of interferogram spectra acquired with 2 instruments for classifying acetone and SF ₆ samples	147
Fuel	FT-IR	Standardization of spectra acquired with 2 different instruments for predicting density of crude oil samples	148

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3 Model transferability

4 Transferability models have been previously developed, however this is still an under-
5 investigated field, especially for biomedical applications. These models use computer-based
6 methods to standardize spectral data generated across different experimental settings (*e.g.*,
7 different instruments, operators or laboratories). An inclusive standardization protocol that
8 could be implemented in a range of different spectrochemical approaches is of great need.
9 Differences are present even between identical instruments; for instance, changes in signal
10 intensity caused by replacement, alignment or ageing of optical and spectrometer components,
11 natural variations in optics and detectors construction, changes in measurement conditions
12 (temperature and humidity), changes in physical constitution of the sample (particle size and
13 surface texture) and operator discrepancies could all lead to wavenumber shifts and artefacts
14 in the spectra. In all of these cases, prediction errors of the estimated group categories (*e.g.*,
15 whether the sample is classified as healthy or cancerous) can become very large, especially
16 when the whole spectrum is used in the model. Standardization techniques aim to generate a
17 uniform spectral response under differing conditions, ensuring the interchangeability of results
18 obtained in different situations, without having to perform a full calibration for each situation.

19 Previous standardization methods include the use of simple slope and bias
20 correction^{149,150}, direct standardization (DS)¹⁵¹⁻¹⁵⁵, piecewise direct standardization
21 (PDS)^{149,156-158}, piecewise linear discriminant analysis (PLDA)¹⁴⁷, guided model
22 reoptimization (GMR)¹⁵⁸, back-propagation neural network (BNN)¹⁴⁷, generalized least
23 squares weighting (GLSW)¹⁵⁹, model updating (MU)^{160,161}, orthogonal signal correction
24 (OSC)^{162,163}, orthogonal projections to latent structures (OPLS)¹⁴⁸, wavelet hybrid direct
25 standardization (WHDS)¹⁵⁷, maximum likelihood PCA (MLPCA)¹⁶⁴, Shenk and Westerhaus
26 method (SW)^{165,166}, positive matrix factorization (PMF)^{167,168}, artificial neural networks (ANN)
27 drift correction¹⁶⁹, transfer *via* extreme learning machine auto-encoder method (TEAM)¹⁷⁰,

28 calibration transfer based on the maximum margin criterion (CTMMC)¹⁷¹, calibration transfer
 29 based on canonical correlation analysis (CTCCA)¹⁷² and calibration methods, such as
 30 wavenumber offset correction, instrument response correction and baseline correction¹³². In
 31 this protocol, we use direct standardization (DS) and piecewise direct standardization (PDS),
 32 because they are the most common methods for spectral standardization.

33 **Direct standardization.** DS is one of the most used methods for data standardization. It was
 34 initially proposed to correct relatively large spectral differences between data collected from
 35 the same sample measured by two different instruments¹⁴⁹. In DS, the entire spectrum from a
 36 new secondary response (*e.g.*, a different instrument) is transformed to resemble the spectrum
 37 from the primary source (*e.g.*, original instrument)¹⁵¹. This is performed based on a linear
 38 relationship between the data acquired under different circumstances¹⁶⁰:

$$39 \quad \mathbf{S}_1 = \mathbf{S}_2 \mathbf{F} \quad (01)$$

40 where \mathbf{S}_1 represents the data acquired for the primary response; \mathbf{S}_2 represents the data acquired
 41 for the secondary response; and \mathbf{F} is the transformation matrix that maintains the relationship
 42 between \mathbf{S}_1 and \mathbf{S}_2 .

43 The transformation matrix \mathbf{F} is estimated in a least-squares sense by¹⁷³:

$$44 \quad \mathbf{F} = \mathbf{S}_2^+ \mathbf{S}_1 \quad (02)$$

45 where \mathbf{S}_2^+ is the pseudo-inverse of \mathbf{S}_2 , calculated by:

$$46 \quad \mathbf{S}_2^+ = (\mathbf{S}_2^T \mathbf{S}_2)^{-1} \mathbf{S}_2^T \quad (03)$$

47 in which T stands for the matrix transpose operation.

48 Then, when samples are measured under the secondary system, the signals generated \mathbf{X}
 49 are transformed to resemble the primary system response by¹⁶⁰:

50 $\hat{\mathbf{X}}^T = \mathbf{X}^T \mathbf{F}$ (04)

51 where $\hat{\mathbf{X}}$ is the standardized response for \mathbf{X} .

52 Problems related to different background information between instruments can affect
 53 the standardization procedure. To correct for this, the standardization process is usually adapted
 54 with the background correction method¹⁷³, in which the transformation matrix described in Eq.
 55 02 is calculated with a background correction factor (\mathbf{F}_b) and an additive background correction
 56 vector \mathbf{b}_s as follows:

57 $\mathbf{S}_1 = \mathbf{S}_2 \mathbf{F}_b + \mathbf{1} \mathbf{b}_s^T$ (05)

58 where $\mathbf{1}$ is an all-ones vector and \mathbf{b}_s is obtained by:

59 $\mathbf{b}_s = \mathbf{s}_{1m} - \mathbf{F}_b^T \mathbf{s}_{2m}$ (06)

60 in which \mathbf{s}_{1m} is the mean vector of \mathbf{S}_1 and \mathbf{s}_{2m} is the mean vector of \mathbf{S}_2 .

61 One of the key steps for DS is the selection of the number of samples to transfer (called
 62 “transfer samples”). These are samples’ spectra from the primary system (\mathbf{S}_1) that will be used
 63 to transform the signal obtained using the secondary system (\mathbf{S}_2). The transfer samples are
 64 obtained from a same cohort of samples (*e.g.*, plasma samples) measured in the two instruments
 65 (primary and secondary systems). Usually, the procedure for selecting transfer samples is based
 66 on sample selection techniques, such as Kennard-Stone (KS) algorithm¹⁷⁴ or leverage¹⁴⁹.
 67 Subsequently, the number of transfer samples is evaluated using a validation set through an
 68 arbitrary cost function. For quantification applications, a common cost function is the root-
 69 mean-square error of prediction, while for classification one can use the misclassification rate.

70 A disadvantage of DS is that each transformed variable is calculated using the whole
 71 spectrum, which carries a high risk of overfitting. The estimation of \mathbf{F} in Eq. (02) is an ill-

72 conditioned problem, because the number of variables (*e.g.*, wavenumber) may be much larger
73 than the number of standard samples.

74 **Piecewise direct standardization.** PDS is another standardization procedure commonly
75 employed for system transferability. It is based on DS, however it uses windows (*e.g.*,
76 wavenumber portions) to make the standardization process more suitable for smaller regions
77 of the data. When compared to DS, PDS is calculated by using the transformation matrix **F**
78 with most of its off-diagonal elements set to zero¹⁴⁹. With this, PDS fits minor spectral
79 modifications not covered by DS. PDS is the technique of preference for correcting smaller
80 spectral variations, such as small wavelengths shift, intensity variations, and bands enlargement
81 and reduction¹⁴⁹. In addition, an advantage of PDS compared to DS is that the local rank of
82 each window will be smaller than the rank of the whole data matrix, which means that the
83 number of standard samples can be smaller, and indeed good results have been obtained with
84 very few samples.

85 One disadvantage of PDS is the need of an additional optimization process, because in
86 addition to the number of transfer samples, PDS also needs a window size optimization, which
87 might lead to a risk of overfitting. In this protocol, window size optimization is made using a
88 cost function expressed as the misclassification rate calculated for each window size tested,
89 being evaluated using a validation set where the window with smaller misclassification is
90 selected for final model construction.

91 Experimental Design

92 Any study using vibrational spectroscopy, follows these general steps: careful
93 experimental design, protocol optimisation and development of experimental procedure
94 document, sample collection and preparation, spectral collection, pre-processing of the derived
95 information and lastly the use of chemometrics for exploratory, classification and

96 standardization purposes. FTIR spectroscopy is described in more detail in this study, however,
 97 the standardization protocol described here can be adapted to a range of techniques, including
 98 attenuated total reflection (ATR-FTIR), transmission and transflection FTIR, near-IR (NIR),
 99 UV-visible, NMR spectroscopy and mass spectrometry (MS). Nevertheless, intrinsic features
 100 of each technique should be taken into consideration before standardization and the protocol
 101 may change depending on the application of interest.

102 A number of biological samples can be analyzed with the above-mentioned analytical
 103 methods such as tissues, cytological materials or biological fluids. Sample type and preparation
 104 may differ depending on the technique that is employed each time. For instance, IR
 105 spectroscopy is limited by water interference at the fingerprint region that can mask the signal
 106 of the analyte close to the water peak. This could be addressed with an extra step of sample
 107 drying, in contrast to Raman spectroscopy, for example, where water does not generate signal
 108 in this region.

109 Typical steps for sample preparation, acquisition of spectra and data pre-processing are
 110 briefly presented here. However, the main focus of this protocol is placed on the calibration
 111 transfer and standardization procedures. Readers are directed to additional literature for more
 112 detailed information regarding sample format and preparation^{4,98-100,105,175-177}, suitability of
 113 substrates^{4,99}, instrumentation settings^{4,98,99,105,175,177,178} or available software packages (Table
 114 2) and manufacturers^{4,99}.

115 **Table 2.** Software packages for data standardization.

Software	Website	Description	Availability
PLS_Toolbox	http://www.eigenvector.com/	MATLAB toolbox for chemometric analysis. Contains standardization routines using DS, PDS, double window PDS, spectral subspace transformation, GLSW, OSC, and alignment of matrices.	Commercial
Unscrambler® X	http://www.camo.com/	Software for multivariate data analysis and design of experiments.	Commercial

OPUS	https://www.bruker.com/	Contains standardization routines using interpolation, bias and slope correction, and PDS. Spectral acquisition software with data processing features. Contains a standardization routine using PDS.	Commercial
Pirouette®	https://infometrix.com/	Chemometrics modelling software. Contains standardization routines using DS and PDS.	Commercial

116

117 Experimental design: sampling

118 **Sample preparation.** Biological samples have been studied extensively with spectrochemical
119 techniques for disease research. Tissue specimens can be analysed fresh, snap-frozen or
120 formalin-fixed, paraffin-embedded (FFPE). Fresh or snap-frozen histology sections are
121 preferable as they are devoid of contaminants whereas FFPE treatment contributes to
122 characteristic peaks, hindering the biological information. FFPE tissues can be deparaffinized
123 either by chemical methods (*e.g.*, incubation in xylene, hexane or Histo-Clear solutions)⁴,
124 which can alter tissue structures and be inefficient for the complete wax removal¹⁷⁹, or by
125 applying chemometrics (*e.g.*, digital dewaxing)^{180,181}, which keeps the tissue intact but might
126 introduce artefacts due to over- or under-estimation of the wax contribution¹⁷⁹.

127 Fixatives, such as ethanol, methanol or formalin, are often used for the preservation of
128 cytological material, also generating strong peaks and interfering with the spectra; thus, a
129 washing step is crucial before spectroscopic interrogation. Fixation in tissue or cells for
130 preservation purposes generates protein cross-linking which can cause changes in the spectra,
131 especially on the Amide I peak¹⁸². Alternatively, cells can be studied live after washing from
132 residual medium.

133 Preparation and pre-treatment of biological fluids depend on the sample type. Some of
134 the biofluids that have been previously used in spectroscopic studies include blood (whole
135 blood, plasma or serum), urine, sputum, saliva, tears, cerebrospinal fluid (CSF), synovial fluid,
136 ascitic fluid or amniotic fluid¹⁸³⁻¹⁸⁵. An initial centrifugation step should precede analysis in

137 cases where the cells present in these fluids are not the focus of the study; the supernatant could
138 then be kept for further analysis. In blood-based studies, the user should also consider the
139 anticoagulant of preference (*e.g.*, EDTA, citrate or heparin) as it could generate unwanted
140 spectral peaks¹⁸⁶⁻¹⁸⁸. Careful planning of experiments as well as consistence throughout a study
141 are of great importance for the generation of robust results. Care should be taken to generate
142 samples that are stable, since the spectral differences between the data collected under different
143 situations (*e.g.*, different instruments or temperature) should be directly related to the difference
144 between the systems and not a change caused by chemical or physical degradation of the
145 samples. Optimal sample thickness, suitability of substrates and sample formats can differ from
146 one analytical technique to another and thus the user should decide and tailor these according
147 to the study's objective (a list with appropriate substrates is given in the Materials-Equipment
148 section). Another consideration is the number of freeze-thaw cycles and long-term storage as
149 these could compromise the integrity of the samples^{186,189}. Preferably, FFPE tissue samples
150 should be analysed after thorough dewaxing and freeze-thaw cycles or long-term storage
151 avoided since these could result in many confounding factors for analysis.

152 **Spectral acquisition.** Depending on the study's objective, FTIR spectral information can be
153 collected using either point spectra or imaging.

154 FTIR spectra can be collected in different operational modes, namely ATR-FTIR,
155 transmission or transflection. Instrument parameters such as resolution, aperture size,
156 interferometer mirror velocity and co-additions have to be optimised before acquisition of
157 spectra to achieve high SNR^{4,98}. Metal surfaces can also be used to increase the IR signal in a
158 technique known as surface-enhanced IR absorption (SEIRA)^{190,191}. As water interference can
159 mask biological information in IR spectra, the user can purge the spectrometer with dry air or
160 nitrogen gas to reduce the internal humidity of the instrument, or use computational analysis to
161 remove the water signature. In addition, samples should be dried until all water content

162 evaporates; however, drying of a sample is not without consequences, since chemical changes
163 may occur such as loss of volatile compounds. A background sample is collected regularly to
164 account for any changes in the atmospheric or instrument conditions.

165 For analysing homogenous samples (*e.g.*, biofluids), measurements can be performed
166 by acquiring spectra on different regions of the centre of a drop and across its borders. In
167 transmission measurements, the sample can be measured raw or diluted. Usually, 10 spectra
168 are collected per sample. A higher number of spectral replicas can be performed to decrease
169 the standard-deviation (SD) between measurements, since the SD is proportion to $1/\sqrt{n}$, where
170 n is the number of replicas. For heterogeneously distributed samples (*e.g.*, tissues), spectra
171 should be acquired covering the sample surface as uniformly as possible, to ensure that all
172 sources of variation in the samples are stored in the spectral data. Samples replicas are also
173 recommended at least as triplicates. For precision estimation, at least six replicates at three
174 levels should be performed. The minimum number of samples for analysis can be estimated
175 using a power test at an 80% power¹⁹². Further details regarding sampling methodologies for
176 analysing biological materials using FT-IR spectroscopy can be found in our previous
177 protocols^{4,98}.

178 Experimental design: data quality evaluation

179 Before processing, the data can be assessed to identify presence of anomalous
180 behaviours or biased patterns. This can be made initially by visual inspection (*e.g.*,
181 identification of very anomalous spectra) followed by Hotelling T^2 *versus* Q residuals charts
182 using only the mean-centred spectra. PCA residuals¹⁹³ can be explored to identify biased
183 patterns, in which heteroscedastic distributions are signs of biased experimental measurements;
184 while homoscedastic distributions are associated with good sampling. SNR can be estimated
185 by dividing the power (P) of signal by the power of noise, that is $SNR = P_{signal}/P_{noise} =$

186 $(A_{signal}/A_{noise})^2$, where A is the amplitude; or by the inverse of the coefficient of variation,
187 when only non-negative variables are measured. Collinearity can be evaluated by calculation
188 of the condition number, which is a matrix calculation that measures how sensitive the result
189 is to perturbations in the input data (*i.e.*, spectra) and to roundoff errors made during the
190 solution process. This value is naturally high for spectral data (high collinearity).

191 Experimental design: pre-processing

192 Data pre-processing is used to maximise the SNR. This process is fundamental for
193 correcting physical interferences, such as light scattering, different sample thickness, different
194 optical paths and instrumental noise. Therefore, the pre-processing step has fundamental
195 importance to highlight the signal of interest, reduce interferences and possibly correct
196 anomalous samples.

197 For standardization applications, the pre-processing step is also important for reducing
198 differences between the different systems that are used. Before any additional pre-processing,
199 the spectrum should be truncated to the biofingerprint region (*e.g.*, 900-1800 cm^{-1}) before
200 analysis. This region contains the main absorptions from biochemical compounds and it suffers
201 only minor effects of environmental variability, such as air humidity (free $\nu\text{O-H} = 3650\text{--}3600$
202 cm^{-1} , hydrogen-bonded $\nu\text{O-H} = 3400 - 3300 \text{ cm}^{-1}$) and air CO_2 ($\nu_s\text{CO}_2 = 2350 \text{ cm}^{-1}$)¹⁹⁴. Table
203 **3** summarizes the main pre-processing techniques for correcting noise in biologically-derived
204 datasets.

205 **Table 3.** Main pre-processing used for biologically-derived datasets.

Pre-processing	Interfering	Technique	Advantage	Disadvantage	Optimization
Savitzky-Golay smoothing ¹⁹⁵	Instrumental noise	ATR-FTIR, FTIR, NIR, Raman, NMR, UV-Vis	Corrects spectral noise without changing the shape of data significantly	The polynomial order and window size for polynomial fit affects the result	The polynomial function should have an order similar to the spectral data (<i>e.g.</i> , 2 nd order polynomial function for IR data) and the window size should be an odd number and not too small (keeping the noise) or too large (changing the spectral shape)
Multiplicative scatter correction (MSC) ¹⁹⁶	Light scattering (Mie scattering), different pressure over the sample when using ATR or probe, different lengths of optical path	ATR-FTIR, FTIR, NIR, Raman, NMR, UV-Vis	Corrects light scattering maintaining the same spectral shape and signal scale	Need of a reference spectrum representative of all measurements	The reference spectrum is regularly set as the average spectrum across all training samples
Standard normal variate (SNV) ¹⁹⁷	Light scattering (Mie scattering), different pressure over the sample when using ATR or probe, different lengths of optical path	ATR-FTIR, FTIR, NIR, Raman, NMR, UV-Vis	Corrects light scattering maintaining the same spectral shape	Creates negative signals since the data are centralized to zero (y-scale)	--
Spectral differentiation ¹⁹⁵	Light scattering (Mie scattering), different pressure over the sample when using ATR or probe, different lengths of optical path, background absorption interfering	ATR-FTIR, FTIR, NIR, Raman, NMR, UV-Vis	Corrects light scattering and baseline problems; highlights smaller spectral differences	Changes the signal scale, shifts the data and increases noise	The order of the derivative function should be used carefully to avoid increased noise (usually 1 st or 2 nd order differentiation is preferred). The differentiation can be coupled to Savitzky-Golay smoothing
Baseline correction ¹⁹⁸	Background absorption interfering	ATR-FTIR, FTIR, NIR, Raman, NMR, UV-Vis, MS	Corrects the baseline maintaining the same spectral shape	--	There are many methods for baseline correction (<i>e.g.</i> , rubber band, automatic weighted least squares, Whittaker filter). The method chosen should be maintained consistent for all systems used

Normalization ⁹⁵	Different sample thickness and concentration	ATR-FTIR, FTIR, Raman	Avoids influence of non-desired signals among the samples	The normalization might hide signal differences between samples at important bands, such as Amide I and Amide II; and also may introduce non-linearities	--
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207 Figure 1 shows the effect of a pre-processing approach employed for a blood plasma
208 dataset acquired under different experimental conditions (*i.e.*, different systems and operators).
209 In this Figure, the reduction of the spectral differences between the systems is evident after
210 data pre-processing (Savitzky-Golay smoothing, MSC, baseline correction and normalization).

211 After pre-processing (Table 3), a scaling step should be done, because most
212 classification methods require all the variables (*e.g.*, wavenumbers) in the dataset to be at the
213 same scale in order to work properly.

214 For spectral data, mean-centring (also referred as “standardization” by Hastie et al.¹⁹⁹)
215 is a very reasonable approach, after which all variables in the dataset will have zero mean.
216 When data contain values represented by different scales (*e.g.*, after data fusion using both IR
217 and Raman spectra), block-scaling should be used, where each block of data (*i.e.*, data from
218 each instrumental technique) would have the same sum-of-squares (normally after mean-
219 centring).

220 Another important aspect of pre-processing is the order in which each step is applied.
221 Pre-processing should be employed in a logical order so that the next pre-processing step is not
222 affected by the previous one. For example, pure spectral differentiation cannot be employed
223 before smoothing, since the spectral differentiation will increase the original noise. Therefore,
224 smoothing should be applied before differentiation. Albeit, Savitzky-Golay routine
225 incorporates smoothing and spectral differentiation so, in practical terms, these can be
226 performed together. To summarise, the suggested order of pre-processing is as follows:

- 227 1. Spectral Truncation
- 228 2. Smoothing
- 229 3. Light scattering correction
- 230 4. Baseline correction

231 5. Normalization

232 6. Scaling

233 Further details about these pre-processing steps are provided in “Procedure: Data pre-
234 processing” section. When using different instruments but same type of sample, the pre-
235 processing steps should be the same for the data acquired under different circumstances.

236 Experimental design: data analysis

237 **Sample splitting.** Sample splitting is fundamental for constructing a predictive chemometric
238 model. It consists of a data analysis step performed before construction of a chemometric
239 model, in which a portion of the samples are assigned to a training set, while the remaining
240 samples are assigned to a validation and/or test set. The training set is used for model
241 construction, the validation set for model optimization, and the test set for final model
242 evaluation. The process of dividing the samples in three sets can be performed manually or by
243 computer-based methodologies. Manual splitting can generate biased results, therefore we
244 recommend a computational-based split instead. Some examples of these include random
245 selection, leverage¹⁴⁹ or the KS algorithm¹⁷⁴. KS works based on Euclidian distance calculation
246 by firstly assigning the sample with the maximum distance to all other samples to the
247 calibration set, and then by selecting the samples which are as far away as possible from the
248 selected samples to this set, until the designed number of selected samples is reached. This
249 ensures that the calibration model will contain samples that uniformly cover the complete
250 sample space, where no or minimal extrapolation of the remaining samples are necessary;
251 avoiding problems of manual or random selection, such as non-reproducibility and non-
252 representative selection. Usually, the dataset is split with 70% of the samples assigned for
253 training, 15% for validation and 15% for test. In this case, the test set is dependent on the initial
254 group of samples measured, and it is not a regular independent test set where a new set of
255 similar samples are measured.

256 **Exploratory analysis.** Exploratory analysis is an important tool to provide an initial
257 assessment of the data. Using exploratory analysis, the analyst can see the clustering patterns
258 and then draw conclusions related to the nature of samples, outliers and experimental errors.
259 One of the most common techniques for exploratory analysis is principal component analysis
260 (PCA), in which the original data are decomposed into a few principal components (PCs)
261 responsible for most of the variance within the original dataset. The PCs are orthogonal to each
262 other and are generated in a decreasing order of explained variance, so that the first PC
263 represents most of the original data variance, followed by the second PC and so on²⁰⁰.
264 Mathematically the decomposition takes the form:

$$265 \quad \mathbf{X} = \mathbf{TP}^T + \mathbf{E} \quad (07)$$

266 where \mathbf{X} represents the pre-processed data (*e.g.*, pre-processed samples' spectra); \mathbf{T} are the
267 scores; \mathbf{P} are the loadings; and \mathbf{E} are the residuals.

268 The PCA scores represent the variance in the sample direction and they are used to
269 assess similarities/dissimilarities among the samples, thus detecting clustering patterns. The
270 PCA loadings represent the variance in the variable (*e.g.*, wavenumber) direction and they are
271 used to detect which variables show the highest importance for the pattern observed on the
272 scores. The PCA loadings are commonly employed as a tool for searching spectral markers
273 that distinguish different biological classes²⁰¹. The PCA residuals represent the difference
274 between the decomposed and original data and can be used to identify experimental errors.
275 Ideally, the PCA residuals should be random and close to zero, representing a heteroscedastic
276 distribution. Otherwise, they can indicate experimental bias according to a homoscedastic
277 distribution.

278 For standardization applications, PCA is a fast, intuitive and reliable tool to observe if
279 there are differences between the spectra acquired by different systems. Ideally, if the same

280 sample is measured under different conditions (different laboratories, instrument
281 manufacturers or user operators) their PCA scores should be random and completely
282 superposed. If a discrimination pattern is observed on the PCA scores, then it is indicative that
283 the data need standardization. Figure 2 illustrates a PCA scores plot from the same samples
284 (blood plasma of healthy controls) measured using three IR instruments before (Fig. 2a) and
285 after (Fig. 2b) PDS. Even though the samples in Fig. 2a are pre-processed, three different
286 clusters are still evident. After PDS the samples measured using different systems are
287 normalized into a single cluster.

288 **Outlier detection.** Outlier detection is important to prevent samples, which differ from the
289 original dataset, from affecting the results using predictive models. Outliers can be attributed
290 to experimental errors, such as inconsistent sample preparation or spectral acquisition, or to
291 larger experimental noise, such as Johnson noise, shot noise, flicker noise and environmental
292 noise. These samples can have large leverage for classification, masking the real signal from
293 the samples of interest; therefore, it is advised that they be removed from the dataset used to
294 train the predictive model.

295 To detect outliers, techniques such as Jack-knife²⁰², Z-score²⁰³ or K-modes clustering²⁰⁴
296 can be utilised among others²⁰⁵. One of the most popular and visually intuitive technique for
297 detecting outliers is the Hotelling T^2 vs Q residual test²⁰⁶. In this test, a chart is created using
298 the Hotelling T^2 values in x -axis and the Q residuals in the y -axis, generating a scatter plot. The
299 Hotelling T^2 represents the sum of the normalized squared scores, which is the distance from
300 the multivariate mean to the projection of the sample onto the PCs²⁰⁷. The Q residuals represent
301 the sum of squares of each sample in the error matrix, thus measuring the residues between a
302 sample and its projection onto the PCs²⁰⁷. All samples far from the origin of this graph are
303 considered outliers and should be removed one at a time, as the PCA is highly influenced by
304 the samples that are included in the model. Samples with high values in both Hotelling T^2 and

305 Q residuals are the worst outliers; while samples with high values in only one of these axis are
 306 the second worst outliers. Supplementary Method 1 illustrates an example for outlier detection.
 307 Squared confidence limits can be draw based on this graph; however, this can hinder outlier
 308 detection. For example, if the confidence limits is set at a 95% level, certain amount of data-
 309 points (5%) should be statistically outside these boundaries.

310 **Classification.** Classification techniques are employed for sample discrimination. Using
 311 chemometric analysis, one can distinguish classes of samples based on their spectral features
 312 and then make further predictions based on these. The prediction capability of a classification
 313 model should be evaluated with external samples (unknown samples) through the calculation
 314 of figures of merit, including accuracy (proportion of samples correctly classified considering
 315 true positives and true negatives), sensitivity (proportion of positives that are correctly
 316 identified) and specificity (proportion of negatives that are correctly identified)²⁰⁸.

317 There are many types of classification techniques for spectral data. Table 4 summarizes
 318 the main classification techniques employed for bio-spectroscopy applications, along with their
 319 advantages and disadvantages.

320 **Table 4.** Classification techniques.

Classification Technique	Advantage	Disadvantage
Linear discriminant analysis (LDA) ²⁰⁹	Simplicity, fast calculation	Needs data reduction, does not account for classes having different variance structures, greatly affected by classes having different sizes
Quadratic discriminant analysis (QDA) ²⁰⁹	Fast calculation, accounts for classes having different variance structures, not much affected by classes having different sizes	Needs data reduction, higher risk of overfitting
Partial least squares discriminant analysis (PLS-DA) ²¹⁰	Fast calculation, high accuracy	Greatly affected by classes having different sizes, needs optimization of the number of latent variables (LVs)
K-Nearest Neighbours (KNN) ²¹¹	Simplicity, non-parametric, suitable for large datasets	Time consuming, needs optimization of the distance calculation method and <i>k</i> value, highly sensitive to the “curse of dimensionality” ¹⁹⁹
Support vector machines (SVM) ²¹²	Non-linear classification nature, high accuracy	High complexity, high risk of overfitting, needs optimization of kernel function and SVM parameters, time consuming

Artificial neural networks (ANN) ²¹³	Non-linear classification nature, ability to work with incomplete knowledge, high accuracy	High computational cost, needs optimization of the number of neurons and layers, no interpretability (“black box” model)
Random forests ²¹⁴	Non-linear classification nature, high accuracy, relatively low computational cost	High risk of overfitting, needs optimization of the number of trees, no interpretability (“black box” model)
Deep learning approaches ²¹⁵	Non-linear classification nature, native feature extraction (e.g., in convolutional neural networks (CNN)), local spatial coherence (CNN), high accuracy	High computational cost, needs hyperparameter optimization, needs large datasets, time consuming, no interpretability (“black box” model)

321

322 When employing classification techniques, one must follow a parsimony order²¹⁶,
323 where the simplest algorithms should be used first, reducing the need for more complex
324 algorithms which would require more optimization steps. An order for using these
325 classification algorithms is: LDA>PLS-DA>QDA>KNN>SVM>ANN>Random forests>Deep
326 learning approaches, from the simplest to the most complex.

327 Classification algorithms can be coupled to feature extraction and feature selection
328 techniques in order to reduce data collinearity/redundancy, thus reducing the risk of overfitting
329 in the classifier training, and speeding up such training, as there are less variables involved.
330 An additional benefit of such a feature extraction/selection step is to provide spectral markers
331 identification as a “side-effect” (depending on the feature extraction/selection method applied).
332 For feature extraction, the most popular technique is PCA. In this case, a PCA is firstly applied
333 to the data, and then the PCA scores are used as the input variables (instead of the wavenumbers
334 data points) for the classification techniques mentioned above²¹⁷. PLS-DA is also a feature
335 extraction technique²¹⁰, and normally it performs better than a PCA followed by LDA, as the
336 scores from a PCA does not necessarily describe the difference between the samples, but rather
337 the variance in the data. In PLS-DA, a partial least squares (PLS) model is applied to the data
338 in an interactive process reducing the original variables to a few number of LVs, where a LDA
339 is used for classifying the groups²¹⁸. Other discriminant classifiers, in particular QDA, also
340 could be used in this classification step to circumvent problems observed with LDA. For feature

341 selection, there are many techniques commonly employed in biological datasets, including
342 genetic algorithm (GA)²¹⁹ and successive projections algorithm (SPA)²²⁰. The variables (*e.g.*,
343 wavenumbers) selected by these techniques are used as input variables for the classification
344 models described in Table 2. An important advantage of GA is its relatively low-computational
345 cost compared to SPA and reduction of data collinearity. Furthermore, GA-based techniques
346 are intuitive and simple to understand in the algorithmic sense but they also have a non-
347 deterministic nature and require optimization of many parameters. SPA's advantage relies on
348 its deterministic nature, minor parameter optimization and reduction of data collinearity,
349 however, it is very time consuming. For hyperspectral imaging, feature selection can also be
350 performed by Minimum Redundancy Maximum Relevance (mRMR) algorithm²²¹, where the
351 selection process is based on maximizing the relevance of extracted features and
352 simultaneously minimize redundancy between them.

353 **Standardization.** Data standardization should be employed when a primary classification
354 model is built and new data comes to be predicted from a secondary system (different
355 laboratory or instrument manufacturers), or when there is a change in instrument components
356 (*e.g.*, laser, gratings, etc.) or when the data of the chemometric model are acquired under
357 different circumstances (different analysts, days, instrumental settings, etc.). As previously
358 mentioned, the most common and reliable methods for data standardization are the DS and
359 PDS algorithms. These methods can be found in a few software packages (described in Table
360 3).

361 Figure 3 summarises the standardization protocol using DS applied to spectra acquired
362 under different conditions. The first step consists of applying KS algorithm for selecting the
363 number of transfer samples from the primary system as well as the number of training samples
364 for the secondary systems, which is ideally 70% of the dataset. Thereafter, the DS transform
365 generation algorithm is employed to estimate the transform matrix. The validation set of the

366 secondary system is then used with the classification model of the primary system to evaluate
367 the optimum number of transfer samples. This optimization step is repeated depending on the
368 number of transfer samples from the primary system. After this number is defined, the
369 validation set of the secondary system is finally standardized and the final classification model
370 is subsequently applied. This procedure is realized with a certain number of samples measured
371 in all instruments being standardized. This procedure should be realized in as similar manner
372 as possible to reduce spectral differences. After the model is standardized and proper validated,
373 new external samples can be measured in any of the instruments and predicted by the
374 standardized classification model.

375 For PDS, an extra step is added after defining the number of transfer samples to estimate
376 the optimum window size. The dashed region in Fig. 3 is repeated according to the window
377 size.

378 For multi-laboratory studies the flowchart depicted in Fig. 4 illustrates how the
379 standardization protocol should be employed.

380 In Fig. 4, spectra acquired under different experimental conditions are used for a global
381 standardization model. A primary system should be designated and then all spectra from
382 secondary systems are equally pre-processed, followed by an exploratory analysis to assess
383 samples' similarities/dissimilarities, outlier detection, standardization by the method outlined
384 in Figure 3; the final model construction follows last. With this, all sources of variations present
385 in different systems can be included into a general chemometric model.

386

387 MATERIALS

388 REAGENTS

- 389 • Biological samples (tissue, cells, biofluids)(see Reagent Setup).

390 ▲ **CRITICAL** Human samples should be collected with appropriate local institutional
391 review board for ethical approval and adhere to the Declaration of Helsinki principles.
392 Similarly, for studies involving animals, all experiments should be performed in
393 accordance with relevant guidelines and regulations. Ethical approval has to be obtained
394 before any sample collection.

- 395 • Optimal cutting temperature (OCT) compound (Agar Scientific, cat. no. AGR1180)
- 396 • Liquid nitrogen (BOC, CAS no. 7727-37-9) ! **CAUTION** Asphyxiation hazard; make sure
397 room is well ventilated. Causes burns; wear face shield, gloves and protective clothing.
- 398 • Paraplast Plus paraffin wax (Thermo Fisher Scientific, cat. no. SKU502004)
- 399 • Isopentane (Fisher Scientific, cat. no. P/1030/08) ! **CAUTION** Extremely flammable,
400 irritant, aspiration hazard and toxic; use in a fume hood.
- 401 • Distilled water
- 402 • PBS (10×; MP Biomedicals, cat. no. 0919610)
- 403 • Virkon (Antec, DuPont, cat. no. A00960632)
- 404 • Trypsin–EDTA (0.05%, Sigma-Aldrich, Thermo Fisher Scientific cat. no. 25300054)

405

406 **Anticoagulants**

- 407 • EDTA (Thermo Fisher Scientific, BD Vacutainer, cat. no. 02-687-107)
- 408 • Sodium citrate (Thermo Fisher Scientific, BD Vacutainer)
- 409 • Lithium/sodium heparin (Thermo Fisher Scientific, BD Vacutainer)

410

411 **Fixative and preservative agents**

- 412 • Formalin, 10% (vol/vol; Sigma-Aldrich, cat. no. HT501128) ! **CAUTION** Potential
413 carcinogen, irritant and allergenic; use in a fume hood.
- 414 • Ethanol (Fisher Scientific, cat. no. E/0600DF/17)
- 415 • Methanol (Fisher Scientific, cat. no. A456-212) ! **CAUTION** Toxic vapours; use in a fume
416 hood.
- 417 • Acetone (Fisher Scientific, cat. no. A19-1) ! **CAUTION** Acetone vapors may cause
418 dizziness; use in a fume hood.
- 419 • ThinPrep (PreservCyt Solution, Cytoc Corp)
- 420 • SurePath (Becton Dickinson Diagnostics)

421

422 **Dewaxing agents**

- 423 • Xylene (Sigma-Aldrich, cat. no. 534056) ! **CAUTION** Potential carcinogen, irritant and
424 allergenic; use in a fume hood.
- 425 • Histo-Clear (Fisher Scientific, cat. no. HIS-010-010S) ! **CAUTION** It is an irritant.
- 426 • Hexane (Fisher Scientific, cat. no. 10764371) ! **CAUTION** Extremely flammable liquid,
427 can cause skin irritation; use protective equipment as required; use in a fume hood.

428

429 **EQUIPMENT**

- 430 • Microtome (Thermo Fisher Scientific, cat. no. 902100A; or cat. no. 956651)
- 431 • Wax dispenser (Electrothermal, cat. no. MH8523B)
- 432 • Sectioning bath (Electrothermal, cat. no. MH8517)
- 433 • Centrifuge (Thermo Fisher Scientific, cat. no. 75002410)
- 434 • Desiccator (Thermo Fisher Scientific, cat. no. 5311-0250)
- 435 • Desiccant (Sigma-Aldrich, cat. no. 13767)
- 436 • Laser power meter (Coherent, cat. no. 1098293)

- 437 • Spectrometer
- 438 • Computer system

439

440 **Substrates**

441 ▲ **CRITICAL** Substrate should be carefully chosen depending on the spectrochemical
442 approach and the experimental mode that will be used. For more details about the choice
443 of substrate see ref ^{4,99}.

- 444 • Low-E slides (Kevley Technologies, CFR)
- 445 • BaF₂ slides (Photox Optical Systems)
- 446 • CaF₂ slides (Crystran, cat. no. CAF10-10-1)
- 447 • Silicon multi-well plate (Bruker Optics)
- 448 • Glass slides (Fisher Scientific, cat. no. 12657956)
- 449 • Quartz slides (UQG Optics, cat. no. FQM-2521)
- 450 • Aluminum-coated slides (EMF, cat. no. AL134)
- 451 • Mirrored stainless steel (Renishaw, cat. no. A-9859-1825-01)

452

453 **REAGENT SETUP**

454 **Tissue** For FFPE tissue, the excised specimen is immersed in fixative (*e.g.*, formalin),
455 dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. Specimens can then
456 be stored indefinitely at room temperature. For snap-frozen tissue, the specimen is immersed
457 in OCT, followed by cooling of isopentane with liquid N₂.

458 ▲ **CRITICAL** Snap-frozen tissue should be thawed before analysis. Spectroscopic analysis
459 should be performed directly after excision in case of fresh tissue to avoid sample degradation.

460 **Cells** Cells can be treated with a suitable fixative or preservative solution or studied alive.

461 ▲ **CRITICAL** In case cells are fixed or stored in a preservative solution, a number of washing
462 steps using centrifugation should be followed prior to spectroscopic analysis to remove
463 unwanted signature. If cells are studied alive, optimum living conditions (*e.g.*, growth medium,
464 temperature and pH) should be maintained; washing of live cells from medium is also
465 necessary.

466 **Biofluids** Biofluids can be collected in designated, sterile tubes using standard operating
467 procedures to achieve uniformity of performance. Preparation of biofluids depends on the
468 sample type and the experiment's objective. If cellular material is not directly studied, it should
469 be removed from the biofluid before storage. Biofluids can be analysed right after their
470 collection or stored at a -80°C freezer.

471 ▲ **CRITICAL** If biofluids have been stored in a freezer, it is essential that they are fully
472 thawed before acquiring aliquots for spectroscopic analysis.

473 ▲ **CRITICAL** Users are advised to store biofluids in smaller, single-use aliquots at -80°C to
474 avoid repeated freeze-thaw cycles.

475

476 EQUIPMENT SETUP

477 The user can choose from a range of different instrumental setups and spectral acquisition
478 modes. General information about FTIR systems is provided below. For more details about
479 equipment setup see refs.^{4,98,99}.

480 The FTIR spectrometer can be left on for long periods of time. Before spectral acquisition, the
481 user should check the interferogram signal for amplitude and position and keep a record of the
482 measurements.

483 ▲ **CRITICAL** For detectors that require a prior cooling step using liquid nitrogen (*e.g.*,
484 mercury cadmium telluride (MCT) detectors), the signal should be allowed to stabilize for
485 approximately 10 min before data collection.

486 ▲ **CRITICAL** In case that the interferogram signal deviates from the last measurement, re-
487 alignment or part replacement may be required.

488 **Software:** Software for spectral acquisition is typically provided by the manufacturer. Software
489 packages for spectral analysis and data standardization are provided in Table 3.

490 PROCEDURE

491 Sample preparation

492 1| Prepare the biological samples for spectrochemical analysis using the following steps: option
493 A for FFPE tissue samples, option B for snap-frozen or fresh tissue samples, option C for cells
494 and option D for biofluids.

495 ▲ **CRITICAL** Sample preparation is briefly presented in this protocol. More details about
496 sample preparation can be found in refs.^{4,98,99}.

497 (A) Tissue (FFPE) • TIMING 1-1.5 h

498 (i) Obtain FFPE tissue blocks.

499 (ii) Section the whole tissue block using a microtome to obtain tissue sections at desired
500 thickness (2-10 μm).

501 ▲ **CRITICAL STEP** Cooling of the tissue on an ice block for 10 min prior to sectioning,
502 hardens the wax and allows easier cutting.

503 (iii) Float the tissue ribbons in a warm H₂O bath (40-44°C) and then deposit onto the
504 substrate of choice.

505 (iv) Allow the tissue sections to dry either at room temperature (30 min) or in a 60°C
506 oven (10 min).

507 ▲ **CRITICAL STEP** The tissue slide may be dried in the oven for longer periods of time,
508 depending on the type of tissue, to ensure optimal, initial melting of the wax.

509 (v) Dewax the samples by performing three sequential immersions in a dewaxing
510 reagent such as fresh xylene, Histo-Clear solution or hexane (each immersion should last at
511 least 5 min).

512 ▲ **CRITICAL STEP** Thorough dewaxing is important for eliminating all spectral peaks
513 attributed to paraffin.

514 (vi) Immerse the tissue slide in acetone or ethanol (5 min) to remove the xylene and
515 then left to air-dry.

516 ■ **PAUSE POINT** Slides can be stored in a desiccator at room temperature for at least 1 year.

517 **(B) Tissue (Snap-frozen or fresh) • TIMING 2 h + drying time (3 h for FTIR only)**

518 ▲ **CRITICAL** Snap-frozen tissue can be stored at -80°C for several months.

519 ▲ **CRITICAL** For fresh tissue, proceed to step 1B(ii).

520 (i) Acquire snap-frozen tissue from freezer and place onto a cryostat (30 min) to allow
521 the tissue to reach the cryostat's temperature (-20°C).

522 (ii) Use a cryostat to obtain tissue sections at desired thickness (8-10 µm).

523 (iii) Deposit the tissue sections onto an appropriate substrate before spectra are
524 collected (see a list of substrates in the Materials-Equipment section).

525 ▲ **CRITICAL** For FTIR studies the tissue sections need to dry for at least 3 h to remove the
526 H₂O interference from the IR spectra.

527 ▲ **CRITICAL** Exposure to light should be minimised to prevent sample degradation due to
528 oxidation.

529 **(C) Cells (fixed or live) ● TIMING 30 min + desiccation time (3 h for FTIR only)**

530 ▲ **CRITICAL** If you are working with fixed cells, do step 1C(i) and then proceed to step
531 1C(iii). If you are working with live cells, proceed to step 1C(ii)

532 (i) Wash fixed cells to remove the fixative or preservative solution as these chemicals
533 cause spectral interference in the fingerprint region. Three sequential washes with distilled H₂O
534 or PBS have been shown to remove unwanted peaks.

535 (ii) Detach cultured cells from the growth substrate adding 2-3 mL of fresh warm
536 trypsin/EDTA solution to the side wall of the flask; gently swirl the contents to cover the cell
537 layer. Wash with warmed sterile PBS to remove the medium and trypsin (×3 times; gentle
538 centrifuge at 300 g for 7 min).

539 ▲ **CRITICAL STEP** All reagents should be warmed to 37°C to reduce the shock to cells and
540 maintain morphology.

541 (iii) After the final wash, resuspend the remaining cell pellet in distilled H₂O (~50-100
542 µL) and mount onto a substrate of choice; allow sample to dry before analysis.

543 ▲ **CRITICAL STEP** The final suspension of cells (~50-100 µL) should be evenly deposited
544 on the slide either by cytospinning or by micro-pipetting. For cytospinning, take a maximum
545 volume of 200 µL of cells in suspension (spin-fixed cells at 800 g for 5 min). After spinning,
546 leave the slide to air-dry.

547 ▲ **CRITICAL** For FTIR studies the sample needs to dry for at least 3 h.

548 **(D) Biofluids (frozen or fresh) • TIMING 5 min + thawing (20 min) + drying (1-1.5 h)**

549 **▲ CRITICAL** If biofluids are analysed fresh, immediately after collection, continue to step
550 1D(ii).

551 (i) Acquire biofluids from the -80°C freezer and allow them to fully thaw.

552 (ii) Mix or gently vortex the sample before obtaining the desired volume for analysis.

553 **▲ CRITICAL STEP** Only a small amount of the biofluid is typically required for
554 spectroscopic studies (1-100 µL). However, this depends and should be tailored according to
555 the study and experimental design. For instance, in case a substrate is used for experiments in
556 the ATR mode, a larger volume is preferred as it allows spectral acquisition from multiple
557 locations of the blood spot. On the contrary, if no substrate is used, such as in the case of the
558 direct deposition of the sample on the ATR crystal, smaller volumes can also be used.

559 (iii) Deposit the biological fluid onto an appropriate substrate.

560 **▲ CRITICAL STEP** For ATR-FTIR spectroscopic studies, an alternative option is to deposit
561 the sample directly on the ATR crystal instead of a substrate if the instrumentation setting
562 allows (*i.e.*, if crystal is facing upwards). However, if the sample is sufficiently thick (>2-3 µm)
563 to avoid substrate interference, then the use of a holding substrate is advantageous as it allows
564 measurements from multiple locations as well as longer storage.

565 **▲ CRITICAL STEP** For FTIR studies the sample needs to dry adequately before
566 spectroscopic analysis (50 µL dry within approximately 1 h at room temperature). Drying can
567 be sped up by using a gentle stream of air over the sample at a specific flow rate (in a sterile
568 laminal flow hood).

569

570 Spectral acquisition for **FTIR spectroscopy** • **TIMING 2 - 5 min per spectrum**

571 ▲ **CRITICAL** Spectrochemical information can be collected as follows for FTIR
572 spectroscopy.

573 ▲ **CRITICAL** Spectral acquisition is briefly presented in this protocol. More details can be
574 found in refs.^{4,98,99}.

575
576 2 | Optimise the settings before each new study to increase the SNR (see ‘Experimental
577 design: spectral acquisition’).

578 ▲ **CRITICAL STEP** Some of the parameters that need to be adjusted include the
579 resolution, spectral range, co-additions, aperture size, interferometer mirror velocity,
580 and interferogram zero-filling.

581 ▲ **CRITICAL STEP** To improve reproducibility and decrease differences between
582 the data collected by different operators, the spectral resolution should be set constant,
583 since it can cause major differences between data collected across different
584 experimental setups.

585 ▲ **CRITICAL STEP** The pressure applied on the sample in the ATR mode affects the
586 signal intensity (*i.e.*, absorbance) between data collected by different instruments and
587 operators. Thus, the pressure applied on the sample should be as similar as possible
588 across different experimental setups to reduce differences between the spectra
589 collected. Depending on the sampling mode that has been chosen (ATR-FTIR,
590 transmission or transflection), deposit the sample onto the appropriate holding
591 substrate.

592 3 | Acquire a background spectrum to account for atmospheric changes.

593 ▲ **CRITICAL STEP** This should be done before every sample.

594 4 | Load the sample and visualise the region of interest; information can then be acquired
595 either as point map or as image maps.

596 ▲ **CRITICAL** Typically, 5-25 point spectra are collected per sample while for image
597 maps the step size should be the same or smaller than the selected aperture size divided
598 by two. Sampling can be performed with 6 replicates in 3 levels.

599

600 ■ **PAUSE POINT** Save the acquired data in a database until further analysis.

601 Data quality evaluation ● **TIMING 15 min – 4 h (depending on the size of the dataset)**

602 5 | Evaluate the raw data using quality tests to identify anomalous spectra or biased
603 patterns before applying pre-processing. This can be made by visual inspection of the
604 collected spectra followed by Hotelling T^2 versus Q residuals charts (see Supplementary
605 Method 1) using only the mean-centred data, and analysis of PCA residuals. Samples
606 far from the origin of the Hotelling T^2 versus Q residuals chart should be removed, and
607 PCA residuals should be random and close to zero. Further instructions about data
608 quality evaluation can be found at “Experimental Design: data quality evaluation”
609 section.

610 Data pre-processing ● **TIMING 15 min – 4 h (depending on the size of the dataset)**

611 ▲ **CRITICAL** Steps 6-11 below can be modified depending on the nature of the dataset. Table
612 1 provides more details about these pre-processing steps. In case of an ATR-FTIR dataset
613 where samples were acquired and analysed under different experimental conditions, the pre-
614 processing method should follow this order:

615 6 | **Cutting at biofingerprint region (900-1800 cm^{-1}).** Truncate the spectra to the
616 biofingerprint region, to eliminate atmospheric interference present in other regions of
617 the spectra.

- 618 7 | **Savitzky-Golay smoothing for removing spectral-noise.** Window size varies
619 according to the size of the spectra dataset (*e.g.*, wavenumber). The window size should
620 be an odd number, since a central data point is required for the smoothing process. Try
621 different window sizes from 3 to 21 and observe how the spectra change (in shape) and
622 how the noise is reduced. Use the smallest window that removes the noise considerably
623 whilst maintaining the original spectral shape. Using a spectral resolution of 4 cm^{-1} , the
624 biofingerprint region ($900\text{-}1800\text{ cm}^{-1}$) usually contains 235 wavenumbers. In that case,
625 a window size of 5 points should be used. The polynomial order for Savitzky-Golay
626 fitting should be 2nd order for IR spectroscopy due to the band shape.
- 627 8 | **Light scattering correction using either multiplicative scatter correction (MSC),**
628 **SNV or 2nd derivative.** First try using MSC or SNV, as MSC maintains the spectral
629 scale and both methods maintain the original spectral shape. If the results are not
630 satisfactory (*e.g.*, classification accuracy $< 75\%$), try using the 2nd derivative spectra.
- 631 9 | **Perform baseline correction using automatic weighted least squares or rubber**
632 **band baseline correction.** If spectral differentiation is applied as light scattering
633 correction method, baseline correction is not necessary.
- 634 10 | **Normalization** Normalize the spectrum to the amide I peak or amide II peak, or
635 perform a vector normalization (2-Norm, length = 1) to correct different scales across
636 spectra (*e.g.*, due to different sample thicknesses when using FTIR in transmission
637 mode).
- 638 11 | **Scaling** Mean-centre the data for each variable, and divide this value by the variable
639 standard deviation. In case of data fusion, block-scaling should be used.

640 Data analysis

641 **Exploratory analysis. • TIMING 1h – 4 d (depending on the data size)**

642 12 |Determine whether a standardisation procedure is necessary by performing PCA. The
643 PCA scores plot (PC1 vs PC2) should generate a unique clustering pattern for the same
644 type of sample. If two or more clusters are observed for the same type of sample
645 measured under different experimental conditions, then a standardisation procedure is
646 necessary (see Figure 2).

647 **Outlier detection. • TIMING 1h – 1 d (depending on the data size)**

648 13 |Apply PCA to the dataset and then estimate the Q residuals and Hotelling T^2 values.
649 Use the chart of Q residuals *versus* Hotelling T^2 to identify outliers. The outliers (*e.g.*,
650 cosmic rays, artefacts, low signal spectra and substrate only (non-tissue) spectra) should
651 be removed from the data set before proceeding to the next steps.

652 **Sample split. • TIMING 1 – 4 h (depending on the data size)**

653 14 |Separate the samples that will be used for the training and the test sets. Sample split
654 should be performed before construction of standardization of multivariate
655 classification models. The samples can be split into training (70%) and test (30%) sets,
656 using a cross-validated model; or split into training (70%), validation (15%) and test
657 (15%) sets without using cross-validation. To maintain consistency and account for a
658 well-balanced training model, KS algorithm should be employed to separate the
659 samples into each set. KS algorithm is freely available at
660 <https://doi.org/10.6084/m9.figshare.7607420.v1>.

661 **Standardization. • TIMING 1h – 4 d (depending on the data size)**

662 ▲ **CRITICAL** Standardization methods should be employed in the following order:
663 DS > PDS (DS should be done before PDS), since the latter is more complex and

664 requires an additional optimization step (window size optimization). The data from the
665 secondary response should be separated into training (70%), validation (15%) and test
666 (15%) sets using KS algorithm. The number of transfer samples should be firstly
667 optimized using the validation set from the secondary response. Then, when employing
668 PDS, the window size should be optimized according to the size of the dataset.

669 15 |Use DS to vary the number of transfer samples from 10-100% of the training set from
670 the primary system. Use the validation set from the secondary instrument to find the
671 optimum number of transfer samples using the misclassification rate as cost function.

672 16 |Perform PDS using the optimum number of samples found with DS. Test different
673 window sizes using the validation set from the secondary system with the
674 misclassification rate as cost function. The window size should vary from 3-29 for a
675 spectral set with resolution of 4 cm^{-1} in the biofingerprint region (235 variables).

676 **Model construction. • TIMING 1h – 4 d (depending on the data size)**

677 ▲ **CRITICAL** Feature extraction (*e.g.*, by means of PCA) or feature selection (*e.g.*,
678 by means of GA or SPA) should be employed to reduce data collinearity and speed up
679 data processing and analysis time. PLS-DA is already a feature extraction method, thus
680 the performance of prior feature extraction is not necessary in this case. The
681 classification technique employed must follow a parsimony order: LDA>PLS-
682 DA>QDA>KNN>SVM>ANN>Random forests>Deep learning approaches.

683 17 |Apply the feature extraction or selection technique. The optimization of the number of
684 PCs during PCA can be performed using an external validation set (15% of the original
685 dataset) or using cross-validation (leave-one-out for small dataset [ppl samples] or
686 venetian blinds [sample splitting: 10] for large datasets [>20 samples]). GA should be
687 realized three-times starting from different initial populations and the best result using
688 an external validation set (15% of the original dataset) should be used. Cross-over

689 probability should be set for 40% and mutation probability should be set for 1-10%
690 according to the size of the dataset.

691 18 |The classification method should be employed using optimization with an external
692 validation set or cross-validation, especially for selecting the number of latent variables
693 of PLS-DA and the kernel parameters for SVM. The kernel function for SVM should
694 be RBF kernel, due to its adaptation to different data distributions. To avoid overfitting,
695 cross-validation should be always performed during model construction to estimate the
696 best RBF parameters.

697 ? TROUBLESHOOTING

698 **Spectral acquisition:** Spectral resolution, spectral range, SNR and signal aperture should be
699 optimized during experimental setup. Operators using different systems should try to keep
700 these parameters constant to reduce spectral differences.

701 **Data pre-processing:** To reduce spectral differences, the same data pre-processing should be
702 applied for spectra acquired in different systems.

703 **Standardization:** To improve the prediction capability of the classification model, the primary
704 system used should be the one with highest spectral resolution and smallest noise, since all data
705 from the secondary systems will be standardized to this pattern.

706 • TIMING

707 **Sample preparation:**

708 **Step 1(A)** Tissue (FFPE): 1-1.5 h

709 **1(B)** Tissue (Snap-frozen or fresh): 2 h + drying time (3 h)

710 **1(C)** Cells (fixed or live): 30 min + desiccation time (3 h)

711 **1(D)** Biofluids (frozen or fresh): 5 min + thawing (20 min) + drying (1-1.5 h)

712 **Steps 2-4, Spectral acquisition:** 1 s – 5 min per spectrum (depending on the instrument and
713 spectral acquisition configurations)

714 Step 5, Data quality evaluation: **15 min – 4 h (depending on the size of the dataset)**

715 **Steps 6-11, Data pre-processing:** 15 min – 4 h

716 **Data analysis:**

717 **Step 12,** Exploratory analysis: 1 h – 4 d

718 **Step 13,** Outlier detection: 1 h – 1 d

719 **Step 14, Sample split: 1- 4h (depending on sample size)**

720 Step 15-16, Standardization: 1 h – 4 d

721 **Step 17-18,** Model construction: 1 h – 4 d

722 ANTICIPATED RESULTS

723 To illustrate how this protocol can be used in practice, we conducted a pilot study to
724 evaluate the effect of different instrument manufacturers and operators towards spectral
725 acquisition of healthy controls and ovarian cancer samples based on blood plasma (5 healthy
726 controls with 10 spectra per sample; 5 ovarian cancers with 10 spectra per sample) for a binary
727 classification model using ATR-FTIR spectroscopy. All specimens were collected with ethical
728 approval obtained at Royal Preston Hospital UK (16/EE/0010). Table 4 summarizes the
729 experimental conditions in which the experiments were performed.

730 **Table 4.** Experimental conditions for pilot study.

Instrument	Operator	Spectral range	Number of co-additions	Spectral resolution	Room temperature	Air humidity
A	1	4000-400 cm ⁻¹	32	4 cm ⁻¹	23.0°C	23%
	2	4000-400 cm ⁻¹	32	4 cm ⁻¹	23.4°C	26%
B	1	4000-400 cm ⁻¹	32	4 cm ⁻¹	24.0°C	26%
	2	4000-400 cm ⁻¹	32	4 cm ⁻¹	24.9°C	24%
C	1	4000-400 cm ⁻¹	48	4 cm ⁻¹	22.5°C	28%
	2	4000-400 cm ⁻¹	48	1 cm ⁻¹	22.8°C	26%

731

732 Instrument A and B were Bruker Tensor 27 with an HELIOS ATR attachment while
733 instrument C was an ATR-FTIR Thermo Scientific Nicolet iS10. The spectra were collected
734 for the same types of samples within three different days (operator 1: instrument A in day 1,
735 instrument B in day 3, and instrument C in day 2; operator 2: instrument A in day 2, instrument
736 B in day 1, and instrument C in day 3) and across two different laboratories (instrument A and
737 B in laboratory 1 and instrument C in laboratory 2). Each operator prepared the samples
738 individually from the same bulk, and measured them individually. Spectral acquisition times
739 were around 30 s for instruments A and B, and 40 s for instrument C.

740 **Effect of different instruments**

741 Three different ATR-FTIR spectrometers were used to analyse the samples. Data were
742 pre-processed by truncating at the biological fingerprint region ($900\text{-}1800\text{ cm}^{-1}$), followed by
743 Savitzky-Golay smoothing (window of 15 points, 2nd order polynomial function), MSC,
744 baseline correction using automatic weighted least squares and vector normalization (2-Norm,
745 length = 1). Each data set (A, B and C) was pre-processed individually. The raw and pre-
746 processed spectra for healthy controls and ovarian cancer samples are depicted in
747 Supplementary Figure 1. All spectra collected by the three instrument maintained the same
748 spectral shape, indicating that the chemical information stayed the same; however, large
749 differences between the absorbance intensity were observed between instrument C and the
750 others (A, B), being caused due to different pressures applied on the sample in the ATR module.
751 The pressure applied to keep the sample in contact with the ATR crystal directly affects the
752 spectral signal intensity, which for instrument A and B (same manufactures) were somewhere
753 controlled by a contra weight, while for instrument C the pressure was set based on a
754 mechanical screw on the device, thus being biased by the operator usage. The absorbance
755 intensity variation between A and B is observed for this same reason, but in a minor scale.

756 Outlier detection was performed using a Hotelling T^2 versus Q residual test (Supplementary
757 Figure 2).

758 **(i) Classification.** Classification was performed using PCA-LDA (10 PCs, explained
759 variance of 99.21%). Fig. 5a depicts the discriminant function (DF) score plot for PCA-LDA
760 using only the primary system (ATR-FTIR A). As observed, there is an almost perfect
761 separation between the samples from the two classes (accuracy = 100%, sensitivity = 100%,
762 specificity = 100%). However, when the spectra acquired using instruments B and C are
763 predicted using the model for A, the results decreased significantly (accuracy = 66.7%,
764 sensitivity = 83.2%, specificity = 48.9%) (Fig. 5b), necessitating the use of a standardization
765 procedure.

766 **(ii) Standardization.** Standardization was employed using both DS and PDS in order
767 to compare the two methods. The number of transfer samples for DS was optimized according
768 to the misclassification rate obtained for the validation set using the secondary system (Fig.
769 6a). An optimum number corresponding to 80% of the samples in the training set of the primary
770 system (55 transfer samples) was obtained, resulting to a misclassification rate of 22.2% in the
771 validation set of the secondary system. This improved the accuracy (77.8%) and specificity
772 (80.0%). Sensitivity decreased to 75.0%, which is an acceptable value. The results after DS are
773 better balanced than without standardization. Fig. 6b shows the DF plot for the PCA-LDA
774 model using the training of the primary system and prediction with the secondary system after
775 DS.

776 PDS was also applied. The number of transfer samples was maintained as 55 (80% of
777 the primary training set) and the window size was optimized by using the validation set of the
778 secondary system. An optimum window size of 23 wavenumbers was selected with a
779 misclassification rate of 25.9% (Fig. 6c). The accuracy, sensitivity and specificity using PDS

780 were 74.1%, 71.4% and 75.0%, respectively. The DS presented a slightly higher performance
781 than PDS for this dataset. However, DS generated some outliers not observed before, while
782 PDS did not. Thus, in general, PDS provided a better standardization of the data. The PCA-
783 LDA DF plot after PDS is depicted in Fig. 6d.

784

785 **Effect of different operators**

786 The effect of different user operators acquiring spectra from the same samples using
787 the same instruments was also evaluated. Similarly to before, data were pre-processed by
788 cutting the biological fingerprint region (900-1800 cm^{-1}), followed by Savitzky-Golay
789 smoothing (window of 15 points, 2nd order polynomial function), MSC, baseline correction
790 using automatic weighted least squares and vector normalization (2-Norm, length = 1). Each
791 dataset was pre-processed individually. All raw and pre-processed spectra varying operators
792 are depicted in Supplementary Figures 4 and 5. Outlier detection was performed using a
793 Hotelling T^2 versus Q residual test (Supplementary Figure 7). The PCA scores plots for the
794 pre-processed spectra are depicted in Supplementary Figure 6. The main difference between
795 the operators was observed for instrument C Supplementary Figure 5, since the spectral
796 resolutions used by them were different, which can cause major data distortion.

797 **(i) Classification.** Classification was performed using PCA-LDA (10 PCs, explained
798 variance of 98.62%). Fig. 7a depicts the DF score plot for PCA-LDA using only the primary
799 system (Operator 1). There is a significant separation between the samples from the two classes
800 (accuracy = 88.4%, sensitivity = 77.3%, specificity = 100%). When the spectra acquired by
801 Operator 2 are predicted using the model for Operator 1, the results decreased (accuracy =
802 75.6%, sensitivity = 66.7%, specificity = 84.6%) (Fig. 7b), which again necessitates the use of
803 a standardization procedure.

804 **(ii) Standardization.** DS and PDS were employed as standardization methods. The
805 number of transfer samples for DS was optimized according to the misclassification rate
806 obtained for the validation set using the secondary system (Operator 2) (Fig. 8a). An optimum
807 number of 59 transfer samples (30% of the samples in the training set of the primary system
808 [Operator 1]) was obtained, resulting in a misclassification rate of 17.8% in the validation set
809 of the secondary system. This improved the accuracy (82.2%), sensitivity (69.6%) and
810 specificity (95.5%) compared to the results without DS. Fig. 8b shows the DF plot for the PCA-
811 LDA model using the training of the primary system and prediction with the secondary system
812 after DS.

813 The number of transfer samples was maintained as 59 for PDS; and the window size
814 was optimized by using the validation set of the secondary system. An optimum window size
815 of 23 wavenumbers was selected with a misclassification rate of 22.2% (Fig. 8c). The accuracy,
816 sensitivity and specificity using PDS were 77.8%, 100% and 54.5%, respectively. Although
817 DS obtained an average better classification performance than PDS for this dataset, it also
818 generated some outliers as mentioned before. For this reason, the results after PDS seem better
819 standardized. The PCA-LDA DF plot after PDS is depicted in Fig. 8d.

820

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828 F.L.M. is the principal investigator who conceived the idea for the manuscript;
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831 together the text and finalized the manuscript.

832 Competing financial interests

833 The authors declare no competing financial interest.

834 Data availability statement

835 The datasets generated during and/or analysed during the current study are available
836 from the corresponding authors on reasonable request.

837

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1370 **Figure legends**

1371 **Figure 1. IR spectra of healthy control (absence of disease) samples varying ATR-FTIR**
1372 **instruments and operators.** Average (a) raw and (b) pre-processed IR spectra for healthy
1373 control samples measured across three different ATR-FTIR spectrometers in the same institute
1374 (A, B and C). Average (c) raw and (d) pre-processed IR spectra for healthy control samples
1375 across two different operators (Operator 1 and 2).

1376 **Figure 2. PCA scores for healthy control (absence of disease) samples varying ATR-FTIR**
1377 **instruments before and after standardization.** (a) PCA scores for healthy control samples
1378 across three different ATR-FTIR spectrometers in the same institute (A, B and C) after pre-
1379 processing but before PDS; (b) PCA scores for healthy control samples across three different
1380 ATR-FTIR spectrometers in the same institute (A, B and C) after PDS (model built with 55
1381 transfer samples and window size of 23 wavenumbers). The dotted blue circle shows 95 %
1382 confidence ellipse (two-sided). Each measurement observation (circle) corresponds to the data
1383 acquired from a unique operator.

1384 **Figure 3. Flowchart for standardization using Direct Standardization (DS).**

1385 **Figure 4. Flowchart for a standardization protocol using different experimental**
1386 **conditions.**

1387 **Figure 5. Discriminant function (DF) plots using PCA-LDA to discriminate healthy**
1388 **control (absence of disease) samples from ovarian cancer samples varying the instrument.**
1389 (a) DF plot of the PCA-LDA model for the primary system; (b) DF plot of the PCA-LDA
1390 model for the primary system predicting the samples from the secondary systems. Sample
1391 index represents the number of samples' spectra.

1392 **Figure 6. PCA-LDA results for DS and PDS standardisation models for spectra collected**
1393 **by the three different instruments.** (a) Misclassification rate in % for the validation set of the
1394 secondary system varying the number of transfer samples in % from the primary system for

1395 DS optimization; (b) DF plot of the PCA-LDA model for the primary system predicting the
1396 validation set from the secondary system after DS; (c) Misclassification rate in % for the
1397 validation set of the secondary system varying the window size for PDS optimization; (d) DF
1398 plot of the PCA-LDA model for the primary system predicting the validation set from the
1399 secondary system after PDS. Transfer samples (%) refer to the percentage of training samples'
1400 spectra from the primary instrument that are used to transform the signal obtained using the
1401 secondary instrument.

1402 **Figure 7. Discriminant function (DF) plots using PCA-LDA to discriminate healthy**
1403 **control (absence of disease) samples from ovarian cancer samples varying the operator.**

1404 (a) DF plot of the PCA-LDA model for the primary system (Operator 1); (b) DF plot of the
1405 PCA-LDA model for the primary system predicting the samples from the secondary system
1406 (Operator 2).

1407 **Figure 8. PCA-LDA results for DS and PDS standardisation models for spectra collected**

1408 **by two different operators.** (a) Misclassification rate in % for the validation set of the
1409 secondary system (Operator 2) varying the number of transfer samples in % from the primary
1410 system (Operator 1) for DS optimization; (b) DF plot of the PCA-LDA model for the primary
1411 system predicting the validation set from the secondary system after DS; (c) Misclassification
1412 rate in % for the validation set of the secondary system varying the window size for PDS
1413 optimization; (d) DF plot of the PCA-LDA model for the primary system predicting the
1414 validation set from the secondary system after PDS.

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