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Exploring Freeze-Drying as a Method for Diagnostic Optimization of Synovial Fluid Spectroscopic Data: An ATR-FTIR Analysis on Primary Osteoarthritic Patients

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Abstract

The need for cost-efficient, simple and radiation free technologies in clinical diagnostics has orientated the scientific community in the investigation of techniques such as vibrational spectroscopy. Vibrational spectroscopy due to its simplicity both in sample preparation as well as friendly use combined with the technological evolution in data processing and storage could play a promising role in diagnostics even for multifactorial diseases such as osteoarthritis via biofluid analysis. However, despite the related works aiming in synovial fluid there is not a common line in terms of sample preparation. Hence, it is important to establish an effective sample pretreatment protocol to avoid subjectivity and provide the possibility of reliable comparisons among results. In this work, freeze drying preprocessing technique was compared with natural drying in terms of diagnostic performance in 35 knee synovial fluids aspirated from primary osteoarthritic patients with 2 and 4 Kellgren-Lawrence scores. Principal component analysis combined with mean spectra analysis was implemented for this diagnostic purpose. Results have shown that natural drying technique tends to generate more distinct enhanced interclass variations among synovial dried samples compared to freeze drying, despite the latest potential in other biological samples.

Keywords: Freeze Drying; Air Drying; Biofluid; Synovial Fluid; ATR-FTIR; Osteoarthritis

1. Introduction

One of the major prerequisites in the Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) analysis of synovial fluids (SFs), as well as other biofluids, is the desiccation preprocessing step. The necessity of this step originates from the strong absorbance of water in the infrared region. Water interacts strongly with infrared radiation, rendering qualitative component analysis almost impossible due to its strong absorption bands, present in the biofluid spectrum. In detail, absorption bands resulting from the bending vibration of proteins in the range 1600-1700 cm^{-1} (Amide I) are overlapped by the stronger water absorption band at ~1645 cm^{-1} [1]. Also, characteristic is the peak at ~3500 cm^{-1} with a range extend of 3000-4000 cm^{-1}, overlapping peaks originating from phospholipids, proteins and amino acids such as cholesterol, creatine etc. [2], [3]. Finally, peak at ~2130 cm^{-1} is also a characteristic peak of water. It emanates from the coupling of the scissoring bending vibration of H-O-H and a broad liberation band and fortunately, due to its wavenumber position, it plays a less "invasive" role in the biofluid component spectrum [4].

Of the available drying methods implemented in synovial fluid analysis, the most used is natural drying at ambient room temperature (Tab. 1). Drying at high temperatures is prohibited due to the degeneration that can occur in the biological load of the fluid.

Synovial fluid has many similarities with blood. Biochemically it is characterized as an ultrafiltration of blood plasma with additional components produced by the surrounding tissues [5]. This results in a quite similar absorbance spectrum with blood plasma. For the ATR-FTIR analysis of blood plasma, several sample pre-treatment ways have been investigated to optimize the ATR-FTIR discriminatory analysis performance including freeze drying [6], [7]. Cold drying is a method that enables the drying of the sample without destroying the structural integrity of biomolecules with promising results in blood plasma analysis [7]. Unfortunately, despite the importance of synovial fluid analysis in the possible diagnosis and understanding of (so far) unknown skeletal diseases such as osteoarthritis, corresponding studies have not been conducted.

The purpose of this work is to investigate the diagnostic potential provided by freeze-drying in relation to the conventional drying method of synovial fluids in ambient room temperature for ATR-FTIR analysis by the implementation of the principal component analysis technique. This study could lay the groundwork for the construction of a common standardized protocol of proper synovial fluid management for spectroscopic analysis.

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2. Materials and methods

2.1. Study population

For this work knee synovial fluids from primary osteoarthritic patients were investigated. The samples were provided from the biobank of the General University Hospital of Alexandroupolis following the necessary protocols for personal data protection. Approval for the collection and use of synovial fluid samples was obtained from the General University Hospital of Alexandroupolis Scientific Committee (Protocol number: 23679/30-05-2022). All patients underwent clinical and radiographic examinations and were classified based on the Kellgren-Lawrence (K-L) system into four categories, each of which is related to the degree of disease progression [8]. The fluids studied originate from 15 patients with K-L:2 score and 20 with K-L:4. The specified selected cohorts result from the distinct differences observed between the two K-L radiographic scores [9]. The detailed profile of the patients who participated in the study with respect to their personal rights is presented in Supplementary Fig. S1.

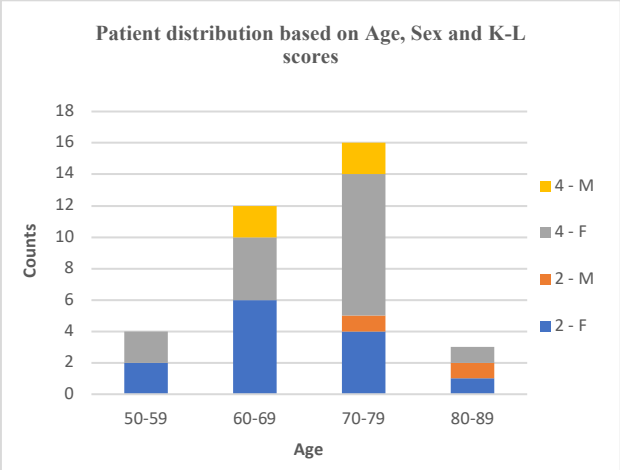

Fig. 1. The patients' profile from whom SFs were aspirated in terms of age, sex (Male-M, Female-F) and Kellgren- Lawrence classification score (2 & 4).

The collected synovial fluids were stored in 2 mL Eppendorf tubes and placed in deep freezing conditions (-80ºC) till analysis. Samples were transported between facilities for analysis into dry ice.

2.2. Drying in ambient temperature (Natural drying)

For the film formations in ambient temperature, the following procedure was performed. In short, initially, the vials were left at room temperature (22-24ºC) after removal from the freezer to thaw for 90-120 minutes. Then they were centrifuged at 3000 rpm for 10 minutes to separate blood cells and debris from the mixture. Afterward drops of SF (~100 μL) were carefully collected from the supernatant with a single-use sterilized 1 mL syringe and placed on gamma-sterilized triple-vented petri disks to allow the controlled exchange of gases with the surrounding space. The disks were left in ambient temperature for 48 hours to allow the water evaporation.

2.3. Freeze-drying

For the freeze drying of SFs, the performed process was the following. After thawing the SFs and centrifuging them similar as previously the supernatants were collected with a pipette and placed in sterile Eppendorf tubes. A hole was drilled in the caps and half of the vials were immersed in liquid nitrogen for instant cooling. The vials were then placed in the lyophilization unit (Cool Safe 4-15L; LABOGENE) overnight at -104ºC in order for SFs to dry. After drying a small fraction of each sample was placed on the Internal Reflection Element (IRE) crystal for measurement.

2.4. Spectral analysis parameters

An Attenuated Total Reflectance- Fourier Transform Infrared spectrometer (PerkinElmer Frontier 1 bounce Diamond/ZnSe) at a nominal resolution of 4 cm^{-1} was used for the measurements. Measurements were collected in the mid-infrared region, 4000-600 cm^{-1} with the final spectra resulting from the averaging of 32 scans per sample. The spectra were not smoothed but zero-corrected followed by vector normalization. Between measurements, the IRE crystal was cleaned with 2-propanol to remove organic residues. The pressure arm was used during the measurements to limit environmental disturbances and to ensure proper contact of the sample with the crystal. All measurements were performed at the same pressure value. The software used to record the data was Spectrum by PerkinElmer. The spectra were processed using in-house Python scripts.

2.5. Data analysis

The first step for the assessment of the spectral quality among the two different drying methods was to calculate the mean spectra for both cases considering the corresponding K-L sample scores. Quantitative correlation was achieved by illustrating the spectral differences among drying methods. The second step was the implementation of Principal Component Analysis (PCA) to observe the inter-class and intra-class variation among spectral data resulting from the different implemented sample processing methods. Spectra preprocessing included only normalization and zero correction of data. No differentiation or smoothing was involved to keep the initial information intact and avoid noise enhancement [10]. The region of PCA implementation was the fingerprint region (1800- 600 cm^{-1}) due to its importance in diagnostics [11] and the limited impact of water [2]. Orange software was used for the implementation and visualization of the PCA algorithm [12].

3. Results

3.1. Mean Spectra visualization

Fig. 2 and 3 demonstrate the mean spectra for the two different drying techniques considering in each case the two different K-L scores. The mean spectra resulting from the freeze-drying sample preparation tend to generate higher absorbance values. No differences are observed in the total number of peaks or arms present.

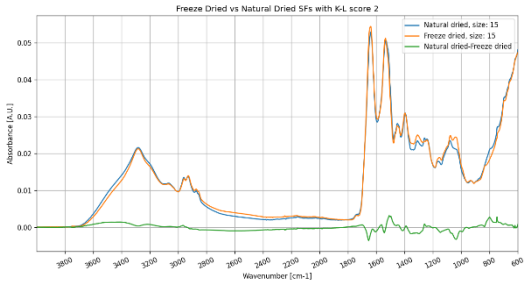

Fig. 2. The mean ATR-FTIR spectra resulting from natural dried and freeze dried synovial fluids classified with a K-L score: 2. The spectra

result from normalized and zero corrected spectra. Each spectrum results from 32 scans per sample. Resolution 4 cm^{-1}. Blue curve: Natural Air Dried mean spectrum, Orange curve: Freeze dried mean spectrum, Green curve: The difference between Natural Air Dried and Freeze dried spectra.

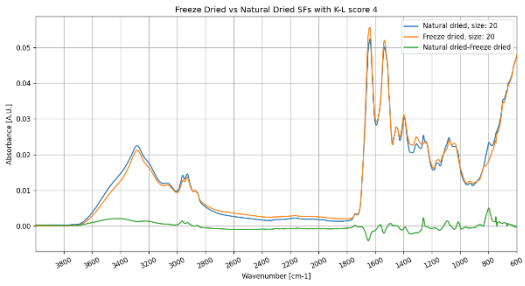

Fig. 3. The mean ATR-FTIR spectra resulting from natural air dried and freeze dried synovial fluids classified with a K-L score: 4. The spectra result from normalized and zero corrected spectra. Each spectrum results from 32 scans per sample. Resolution 4 cm^{-1}. Blue curve: Natural Air Dried mean spectrum, Orange curve: Freeze dried mean spectrum, Green curve: The difference between Natural Air Dried and Freeze dried spectra.

However, in terms of interclass variations freeze drying method tends to generate less spectral differences compared to the natural drying method between the different K-L classified groups as illustrated in Fig. 4 and 5 by the difference curves.

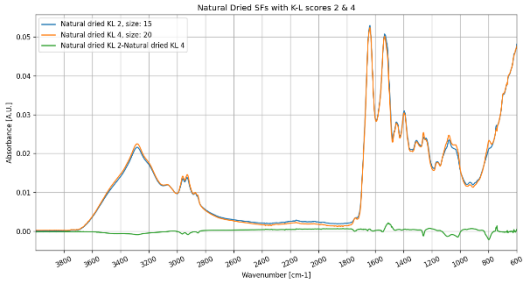

Fig. 4. The mean ATR-FTIR spectra from natural air dried synovial fluids classified with a K-L scores 2 and 4. The spectra result from normalized and zero corrected spectra. Each spectrum results from 32 scans per sample. Resolution 4 cm^{-1}. Blue curve: K-L score: 2, Orange curve: K-L score: 4, Green curve: The difference between K-L 2 and K-L: 4.

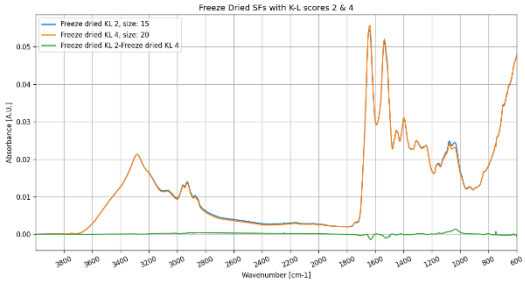

Fig. 5. The mean ATR-FTIR spectra from freeze dried synovial fluids classified with a K-L scores 2 and 4. The spectra result from normalized and zero corrected spectra. Each spectrum results from 32 scans per sample. Resolution 4 cm^{-1}. Blue curve: K-L score: 2, Orange curve: K-L score: 4, Green curve: The difference between K-L 2 and K-L: 4.

3.2. Principal component analysis

Fig. 6 and 7 illustrate the interclass variations among SFs from patients with K-L scores 2 and 4 for natural drying sample preparation and freeze drying preparation respectively for the first two principal components. For the natural drying of SFs in ambient temperature, the first principal component, PC1 accounts for 64% of the total variance and PC2 for 11%, while for the freeze drying method, PC1 accounts for 48% and PC2 for 30% of the total variance.

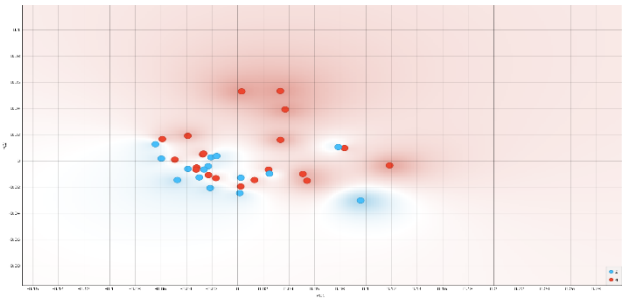

Fig. 6. The score plot of natural air dried SFs' spectra for the first two principal components, PC1, PC2 for the spectral range 1800-600 cm^{-1}. Spectra have been normalized followed by wavenumber range separation. PC1 explains 64% of the total variance, while PC2 11%. Blue dots represent spectra characterized with K-L:2 while red with K-L:4.

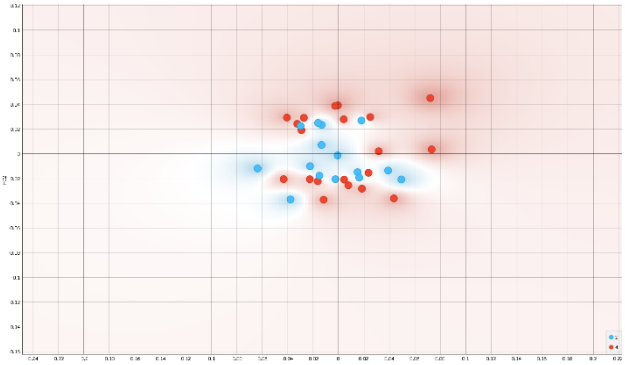

Fig. 7. The score plot of freeze dried SFs' spectra for the first two principal components, PC1, PC2 for the spectral range 1800-600 cm^{-1}. Spectra have been normalized followed by wavenumber range separation. PC1 explains 48% of the total variance, while PC2 30%. Blue dots represent spectra characterized with K-L:2 while red with K-L:4.

Intraclass variations for SFs aspirated from patients with K-L score 2 are illustrated in Fig. 7 while for K-L score 4 in Fig. 8. Tab. 2 collects the aforementioned results.

4. Discussion

ATR-FTIR has shown great performance in the diagnosis of many diseases through biofluid analysis [13], [14]. Its high signal-to-noise ratio, simple sample preparation, reproducibility, fast analysis, and small sample needs, render it a promising diagnostic tool with a clinical perspective even in multifactorial diseases such as osteoarthritis. Through synovial fluid analysis, there are a few scientific works denoting the prospective of this technique in diagnosis with variations in sample preprocessing (Tab. 1). Hence it is important to establish a well-set sample preparation protocol that can foster diagnostic performance and help future research by avoiding unnecessary effort and pointless time consumption. In this work, we evaluate the effectiveness of freeze drying technique against natural drying via PCA by comparing spectra resulting from patients with primary OA both in the early stage of disease, i.e., K-L score:2, as well as in a progressive stage, i.e., K-L:4. This technique finds great applications in pharmaceutics and food processing however,

has not been previously applied in synovial fluid analysis [15], [16].

Intraclass mean spectra synovial fluid analysis has indicated that the freeze drying technique tends to generate higher signal spectra with minimum difference in total peak number for both K-L scores. Mean spectra resulted from a total of 32x15=480 spectra for K-L:2 and 32x20=640 for KL:4 (Fig. 2 and 3). However, there is a difference in intensity mainly in the region 4000-2400 cm^{-1} due to the presence of O-H stretching and bending vibrations that enhance the signal compared to the freeze-dried spectra. This results from the trapped water during the natural drying process for the film formation [17].

Table 1. The available drying methods in the literature for synovial fluid analysis with the IR spectroscopy[a].

Drying Method	Technique	Sample Origin	References
In ambient room temperature	RAMAN	Humans	[25]
In ambient room temperature (20ºC)	TRANSMITTANCE FTIR	Horses	[26]
In ambient room temperature (22ºC)	TRANSMITTANCE FTIR	Horses	[11]
In ambient room temperature	TRANSMITTANCE FTIR	Horses	[27]
In ambient room temperature	TRANSMITTANCE FTIR	Humans	[28]
In ambient room temperature	ATR-FTIR	Humans	[29]
In ambient room temperature	RAMAN	Humans	[30]
In ambient room temperature (20º- 22ºC)	TRANSMITTANCE FTIR	Dogs	[20]
Dry air	TRANSMITTANCE FTIR	Humans	[31]
Dry air	TRANSMITTANCE FTIR/ATR-FTIR	Humans	[32]
Dry air	TRANSMITTANCE FTIR	Humans	[33]
Drying under mild vacuum	TRANSMITTANCE FTIR	Humans	[34]
Drying under mild vacuum	TRANSMITTANCE FTIR	Humans	[35]

[a]Results originate from screening of Scopus, PubMed and Google Scholar databases, with keyword combination "synovial fluid" and "spectroscopy" and ("IR" or "infrared").

The interclass analysis appears to generate the opposite denoting that the differences in the natural drying method are greater among different samples compared to freeze drying (Fig. 4 and 5). The latter notion also tends to support the principal component analysis (Tab. 2).

PCA is an unsupervised chemometric technique with wide implementation in biological studies. Interclass PCA analysis has shown that for the bio-fingerprint region spectral differences among patients with K-L:2 and K-L: 4 tend to differentiate data in the score plot in a more organized way compared to the freeze drying (Fig. 6 and 7). This difference fosters the diagnostic value of the air-drying sample preparation technique. One thing that should be stressed is the relatively low variance explained by the first two principal components. This is not something that contradicts the general notion that primary osteoarthritis is a multifactorial disease and hence many features tend to be involved in disease progression [18].

Table 2. PCA scores for the inter-class and intra-class variation of vibrational spectroscopic data originating from SFs of patients with primary Osteoarthritis classified with K-L scores 2 and 4. Samples prepared with two different drying methods, i.e., Natural Air drying and Freeze drying. All the other parameters remained constant and identical during the measurements.

Case	Variation (Inter/Intra)	PC1 (%)	PC2 (%)
Natural Air dried samples with K-L scores 2 & 4	Inter	64	11
Freeze dried samples with K-L scores 2 & 4	Inter	48	30
Natural Air dried vs Freeze dried samples with K-L score: 2	Intra	49	22
Natural Air dried vs Freeze dried samples with K-L score: 4	Intra	51	19

On the other side, intraclass variation further verifies that the two drying methods result in distinct well-separated spectral results for both cases (Fig. 8 and 9). This is to be expected since freeze drying due to phenomena such as quenching cooling, cold denaturation, freeze concentration, and phase separation tends to affect the protein folding and state differently [16] compared to the natural drying process driven by the well-described "coffee ring" effect [19].

4.1. Limitations

Like every experimental process, there are always some limitations involved. One of the main limitations is the gap between aspiration and analysis. Due to the general difficulty of obtaining fresh SFs from patients with primary osteoarthritis samples had to be stored in deep freezing conditions (-80 ºC) for a long period ~3 years before analysis. Malek et al. have tackled this parameter concluding that even though some detrimental differences are observed performance of predictive algorithms does not significantly alter [20]. However, despite the similarities in anatomy and pathophysiology of humans with dogs, primary osteoarthritis follows different molecular pathways compared to secondary [21], [22]. Hence, there is also a necessity to compare the two methods to freshly aspirated SFs, to reach safe conclusions.

One other limitation is the freeze-drying protocol implemented in this research. In this experiment, samples were left to dry under vacuum at -104 ºC after instant cooling with immersion of samples in liquid nitrogen. Changes in freeze-drying temperature may lead to different results that should also be investigated as also denoted by Sang et al. [23]. This freeze-drying protocol was highly influenced by Salmann et al. for blood sera [7] taking into consideration the well-described fact that synovial fluid is an ultrafiltrate of blood plasma [24].

5. Conclusion

In conclusion, results via both mean spectra analysis and PCA denoted that the conventional natural air drying method

tends to perform diagnostically better compared to the freeze drying method, despite the benefits associated with the latest both in structural integrity and sample size quantity.

Acknowledgments

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