

# The impact of hydration changes in fresh bio-tissue on THz spectroscopic measurements

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## Abstract

We present a study of how residual hydration in fresh rat tissue samples can vastly alter their extracted terahertz (THz) optical properties and influence their health assessment. Fresh (as opposed to preserved) tissue most closely mimics *in vivo* conditions, but high water content creates many challenges for tissue handling and THz measurement. Our THz measurements of fresh tissue over time highlight the effect of tissue hydration on tissue texture and dimension, the latter directly influencing the accuracy of calculated optical properties. We then introduce lyophilization (freeze drying) as a viable solution for overcoming hydration and freshness problems. Lyophilization removes large amounts of water while retaining sample freshness. In addition, lyophilized tissue samples are easy to handle and their textures and dimensions do not vary over time, allowing for consistent and stable THz measurements. A comparison of lyophilized and fresh tissue shows for the first time that freeze drying may be one way of overcoming tissue hydration issues while preserving tissue cellular structure. Finally, we compare THz measurements from fresh tissue against necrotic tissue to verify freshness over time. Indeed, THz measurements from fresh and necrotic tissues show marked differences.

## 1. Introduction

The use of terahertz (THz or T-rays) spectroscopy in diagnosing the health of fresh bio-tissue is one potential application where terahertz science may complement existing medical diagnostic modalities. Terahertz spectroscopy of bio-tissue is still a burgeoning research area with no formalized standard for handling and preserving samples. Different preservation methods have been reported in the existing terahertz literature: from standard pathological formalin fixing to frozen samples. Every preservation method alters a sample in one way or another, thus it

is important to choose an appropriate method for a specific study. For example, cross-linking agents such as formalin aim to preserve tissue by destroying the natural protein enzyme action and digestion process that occurs in decomposition. Formalin cross-links proteins to halt this process and water is removed from the tissue to further encourage protein denaturation. Formalin fixed samples are therefore not suitable for THz protein sensing studies. However, formalin preserves the histomorphology of tissue, hence formalin fixed samples can still be probed successfully with THz, e.g., for distinguishing between healthy and malignant tissues (Knobloch *et al* 2002).

Similarly, tissue samples frozen in domestic freezers (slow freezing) no longer retain their protein structures because the process of slow freezing forms ice crystals between cells, resulting in an irreversible destruction of subcellular structure. Slow frozen tissue however may be suited for THz analysis of tissue density and distinguishing bone–tissue boundaries via THz imaging (Ferguson *et al* 2002).

At present, THz cannot be used for *in vivo* diagnostics of tissue other than skin, thus diagnosis of minute excised fresh tissue samples is the best way to employ THz for pathological diagnostic purposes. Fresh tissue most closely resembles *in vivo* conditions as there is minimal structural destruction so, in theory, collective structural responses can be captured in the THz frequency domain to determine the health of the tissue. Studies of excised human and animal tissue as mentioned previously have revealed that THz spectroscopy may be used to identify differences between healthy and malignant tissues (Woodward *et al* 2002, 2003a, 2003b), and distinguish between organ types (Fitzgerald *et al* 2003, Berry *et al* 2003, He *et al* 2006). There is still, however, insufficient information on sample handling and storage techniques, with one particularly important aspect of fresh tissue lacking discussion: tissue hydration. Considering the human body contains around 60% water (Mullins 2001) and THz is strongly absorbed by water (Thrane *et al* 1995), tissue hydration can vastly alter THz spectroscopy measurements. This paper will present a study on fresh rat tissue with details of our experimental protocol and focus on how tissue dehydration over an extended time interval affects extracted optical properties. We will also discuss how tissues can be kept fresh from the moment of excision to measurement, and will propose a viable solution for overcoming hydration and freshness problems.

## 2. Sample preparation techniques

### 2.1. Fresh tissue excision, slicing and storage

Fresh rat tissue samples used in this study were obtained from routine autopsies unrelated to this study. Since necrosis sets in fairly quickly after excision, it was important to consider how tissue is stored from the onset of our study. Immediately after excision, tissue samples used in this study are submerged in Hank's buffer—a glucose-enriched buffer that can sustain tissue for a limited period. The buffer is kept cold in a trough seated on a bed of ice.

Types of tissue obtained for this study are diaphragm, kidney (cortex), liver, colon and stomach. Ideally, all tissues should be cut into slices with uniform thickness. However, this is not realistically possible due to handling issues. Diaphragm, colon and stomach samples are used 'as is' because they cannot be sliced any thinner in their fresh, supple state. Kidney and liver samples tend to be thicker, so several thinner slices are possible.

Several earlier trials to slice fresh tissue with uniform thickness included a vibratome (FHC Inc.), a microtome (IEC), a commercially bought rodent brain slicer (Braintree Scientific Inc.) and custom-made slicers. One millimetre thick slices are preferred as samples can be handled most easily at this thickness. The vibratome works best, but the process of cutting

one tissue sample takes several hours and is deemed unsuitable. The custom-made slicer comprising of two razor blades spaced by a 1 mm washer works surprisingly well and with some practice, slices with relatively uniform thickness are achievable. The tissue slices are then resubmerged in cold Hank's buffer and refrigerated with the buffer until the time of THz measurement.

## 2.2. Mounting fresh tissue for measurement

A standard THz-time domain system (TDS) in transmission mode is used in this study. System specification is provided in section 3. The direction of the THz beam is parallel to the optical table, thus samples have to be mounted vertically. It is necessary to design a method of keeping the tissue slices upright. Commercially purchased petri dishes were used in earlier investigations whereby fresh tissue slices were mounted by applying minimal amounts of commercial superglue at the edges. Petri dishes are found to be unsuitable because the fresh tissue pulls away from the surface of the dish during measurement, creating air pockets between the tissue and dish. These pockets result in etalon artefacts in the measured THz signal.

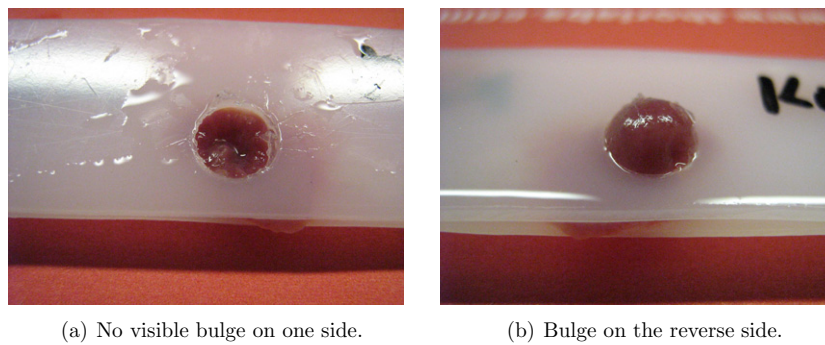
Since the tissue is only secured at the edges to the dish, the problem of the tissue pulling away from the dish cannot be overcome. This is particularly acute in the middle region of the tissue. One alternative is to sandwich the tissue between two plates, but without squeezing the tissue out of shape. It was discovered that air pockets still formed between the tissue and the plates, so this technique was not used.

The ideal scenario would be to measure tissue in air, with only air as the reference. A novel way of achieving this is to make a small hole on each mounting plate, and then sandwich the tissue between the two plates. The middle of the tissue is aligned with the two holes so that the middle of the tissue is effectively suspended in air. Assuming the holes are wider than the THz beam, it can pass through the tissue directly without interference from the plates.

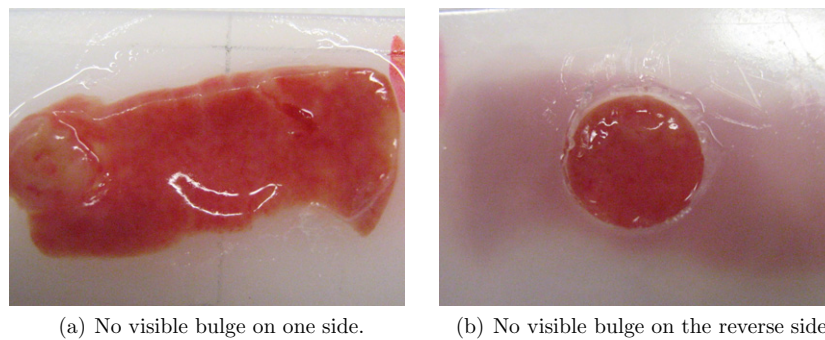
Plates are made from 1 mm thick polyethylene sheets (Goodfellow, high density sheet polyethylene,  $0.95 \text{ g cm}^{-3}$ ). Each plate is 60 mm by 20 mm in size with a 5 mm diameter hole punched in the middle of each plate. This diameter is larger than the THz beam waist of 3 mm. Polyethylene is chosen because it is stiff enough to keep the tissue in shape, yet pliable enough to be cut easily with scissors and holes can be punched out with a regular office hole puncher. Polystyrene was also trialled but being stiffer than polyethylene, it was very brittle and cracked during cutting and drilling.

The sandwiching technique is initially employed in this study. Elastic bands are used at both ends of the plates to secure the tissue between the two plates. It is found that the process of sandwiching causes the tissue to bulge at one of the holes. This is seen in figure 1. The uneven thickness of the bulge potentially affects calculations of tissue optical properties, hence this sandwiching method is unsuitable.

An improved technique is to mount the tissue using minimal drops of commercial superglue at the edges so that only one plate is required. This is the technique employed in this study. Prior to THz measurement, fresh tissue slices are placed gently on the plates without any visible stretching of the tissue, and positioned so that the hole is completely covered by the tissue as shown in figure 2. Minimal drops of superglue are then applied at the edges. It is found that none of the tissue slices droop into the hole during the course of the experiment, hence all tissue surfaces are assumed in this study to be reasonably perpendicular to the incoming THz beam.



**Figure 1.** The double plate sample holder. Kidney sandwiched between two 1 mm polyethylene plates with (a) no bulging on one side and (b) severe bulging on the reverse side.



**Figure 2.** The single plate sample holder. (a) Liver tissue covering the transmission hole completely, affixed using superglue. (b) The reverse side, demonstrating the sample remains flat without bulging.

### 3. Experimental method

The transmission-mode THz-time domain spectroscopy (TDS) system used in this study is based on the generation of a THz pulse using an ultrafast (100 fs) 800 nm Ti:Sapphire laser (Coherent Mira 900). Coherent detection was obtained via optical rectification in a 2 mm thick zinc telluride (ZnTe) crystal emitter and a 1 mm thick ZnTe crystal detector. Details of a THz-TDS system are given in Siegel (2002).

Dry nitrogen gas is used to keep the humidity in the THz test chamber below  $\sim 3\% \pm 3\%$  and temperature close to ambient (22–23 °C). The reference THz signal  $E_{\text{ref}}(t)$  is that of nitrogen without any sample; the signal bandwidth<sup>3</sup> in this case is 2 THz.

Fresh tissue samples are packed in a styrofoam box together with ice packs and transported for 20 min to the THz laboratory. Upon arrival, the samples remain in the styrofoam box until the commencement of THz measurement. Ice packs are replaced as necessary. Each fresh sample is removed from the buffer just prior to measurement and excess buffer is allowed to drip off the tissue by tilting the polyethylene plate. The tissue is then mounted vertically at the THz focal point with the THz beam directed through the holes in the polyethylene

<sup>3</sup> In this paper, bandwidth is defined as the range of frequencies over which the THz signal is above the noise floor.

plates. Each THz sample measurement  $E_{\text{sample+ref}}(t)$  is averaged over five scans. At least two measurements of each sample are taken consecutively. To capture any changes in the sample hydration, measurements are repeated for each sample at intervals of 15–20 min.

The THz sample frequency response  $E_{\text{sample}}(\omega)$  is extracted by dividing the Fourier transform of  $E_{\text{sample+ref}}(t)$  by the Fourier transform of  $E_{\text{ref}}(t)$ :

$$E_{\text{sample}}(\omega) = \frac{\mathcal{F}\{E_{\text{sample+ref}}(t)\}}{\mathcal{F}\{E_{\text{ref}}(t)\}} = \frac{E_{\text{sample+ref}}(\omega)}{E_{\text{ref}}(\omega)}. \quad (1)$$

With the knowledge of sample thickness  $d$ , the absorption coefficient  $\alpha$  can be calculated based on the Beer–Lambert law:

$$\begin{aligned} E_{\text{sample+ref}}^2(\omega) &= E_{\text{ref}}^2(\omega) e^{-\alpha d} \\ \left[ \frac{E_{\text{sample+ref}}(\omega)}{E_{\text{ref}}(\omega)} \right]^2 &= E_{\text{sample}}^2(\omega) = e^{-\alpha d} \\ \alpha &= \frac{-\ln[E_{\text{sample}}^2(\omega)]}{d}. \end{aligned} \quad (2)$$

The refractive index  $n$  is found from

$$n = \frac{(\phi_{\text{sample+ref}}(\omega) - \phi_{\text{sample}}(\omega))c}{\omega d} + 1, \quad (3)$$

where  $c$  is the speed of light in vacuum and  $\phi_x$  is the phase of  $E_x(\omega)$ .

Section 4.1 emphasizes that the sample thickness  $d$  of fresh tissue does not stay constant over the course of this study due to sample dehydration. Sample thickness is therefore measured either immediately before or after each THz measurement. Referring to equation (2), inaccurate values of  $d$  result in plots of  $\alpha$  having incorrect gradients. This is an important consideration because the gradient of  $\alpha$  is often the only unique and distinctive spectral property of complex biological systems, such as bio-tissue, in the THz regime.

## 4. Results and discussion

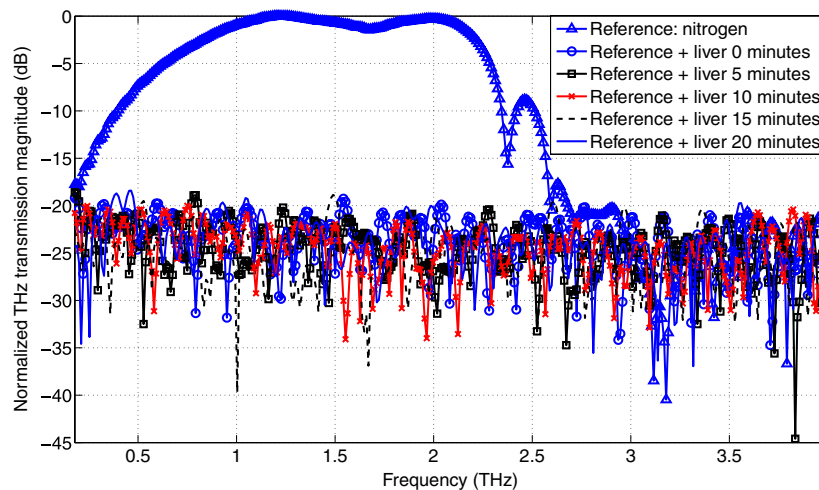
### 4.1. Changes over time

Since fresh tissue samples are stored in buffer prior to THz measurement, they contain very high quantities of water. It is therefore not surprising that the first run of measurements had extremely poor signal-to-noise ratio, resembling that of noise. The noise floor of the THz system used in this study is  $-23$  dB; as seen in figure 3, the magnitude of measured THz transmission signal for the first 20 min is buried in this noise floor. The importance of this observation becomes apparent when the absorption coefficient  $\alpha$  is calculated: figure 4 shows  $\alpha$  calculated based on 1 mm thickness<sup>4</sup>.

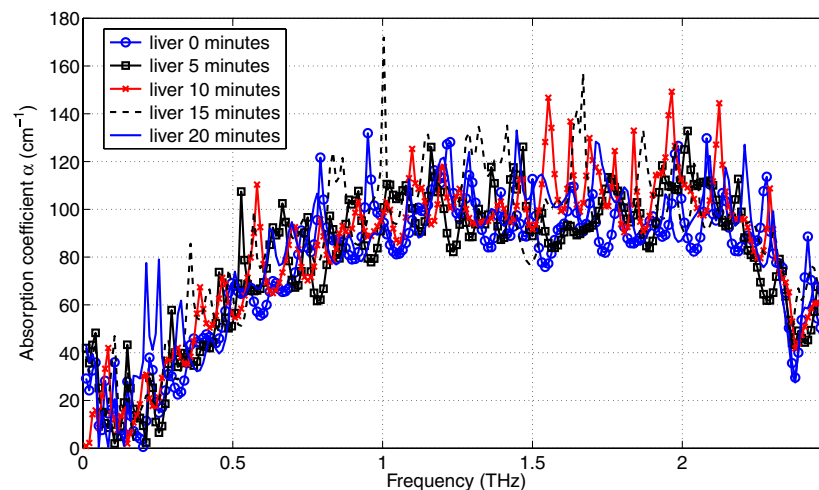
At first glance, these plots of  $\alpha$  in figure 4 do not appear abnormal considering water has similar absorption properties in the THz range (Thrane *et al* 1995). However, the measured THz transmission signal is buried in the noise floor ( $-23$  dB). This means that the dynamic range<sup>5</sup> of the measurement is inadequate, therefore  $\alpha$  has little meaning in this context (Jepsen and Fischer 2005). Optical properties of tissue should therefore not be extracted unless the transmitted THz signal is above the noise floor.

<sup>4</sup> To prevent deterioration, moist samples were handled minimally. There were no visible changes in the texture or appearance of the samples over the first 20 min hence it is assumed that sample thickness remained consistent at 1 mm.

<sup>5</sup> In this paper, dynamic range refers to the height of the THz signal above the noise floor in the frequency domain. This is consistent with that used by Jepsen and Fischer (2005).



**Figure 3.** Initial THz transmission signal of fresh liver is lost in the noise floor.

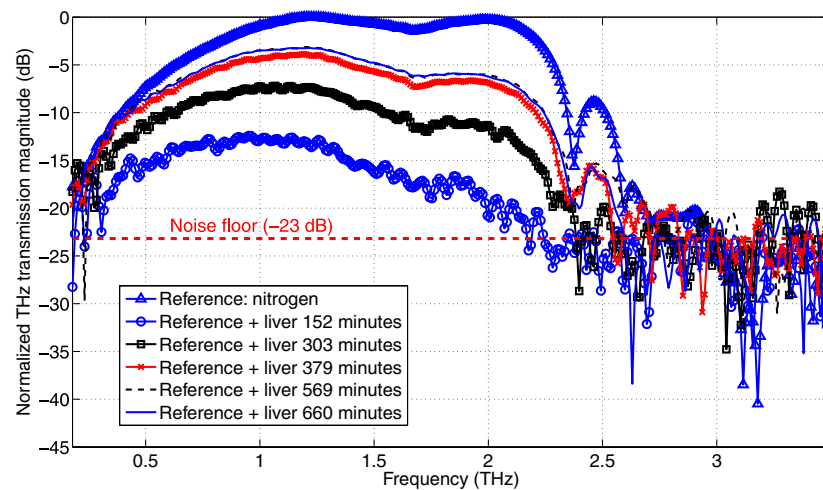


**Figure 4.** With the THz transmission signal in the noise floor, the calculated absorption coefficient is irrelevant.

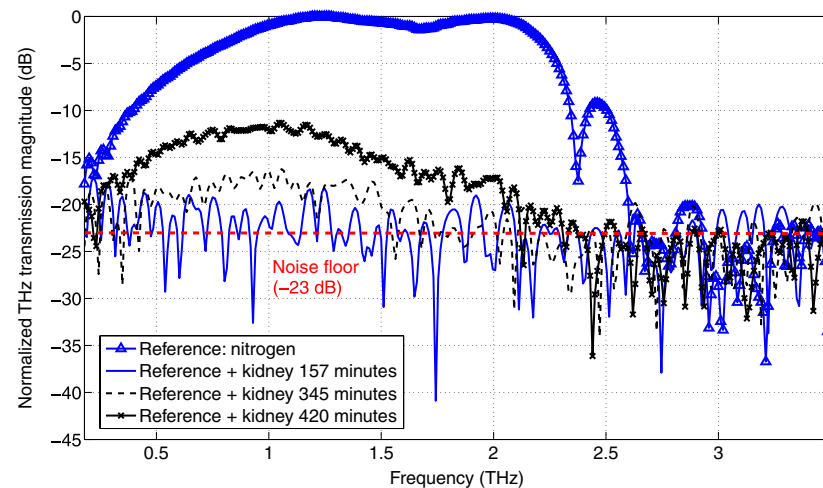
Continued exposure of tissue to the nitrogen-purged atmosphere causes the tissue to dehydrate. With less water, the strength of the transmitted THz signal increases, widening the usable THz bandwidth as a result. A comparison of figures 3 and 5 show a significant improvement in the usable bandwidth after 152 min.

In this study, all moist tissue samples were intentionally exposed to nitrogen for a prolonged period of time in order to monitor changes in the THz transmitted signal due to the level of tissue hydration. The rate of dehydration was found to vary according to tissue type, with denser tissues such as kidney, colon and diaphragm drying very slowly. This is apparent from figures 5 and 6 where the measured THz signal of kidney between 0.5 and 1 THz only rises above the noise floor after 5.5 h, whereas liver between 0.3 and 2 THz takes under





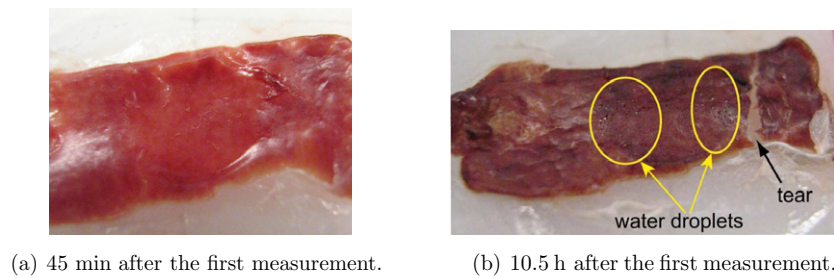
**Figure 5.** Prolonged exposure to nitrogen causes tissue to dehydrate, thus improving the usable bandwidth and system dynamic range. The measurements stabilize after 569 min (9.5 h).



**Figure 6.** Dense tissue such as kidney dehydrates slowly. When compared to liver, kidney of the same thickness (when fresh) takes over twice as long to dehydrate.

2.5 h to do so. In summary, bio-tissue samples of similar thickness (when fresh) dehydrate at different rates and their usable THz bandwidths are incomparable.

The extended exposure of tissue to nitrogen raises questions pertaining to the structural and biological integrity of the samples: do the samples disintegrate as water dehydrates? Optical images of one of the liver samples are presented in figures 7(a) and (b). The colour, shape and texture of the sample are possible indicators of biological integrity. It is apparent that the sample retains its shape after 45 min, but there is visible shrinkage. With continued shrinkage, the sample develops tears as it pulls away from the edges that were secured to the polyethylene plates with superglue. It is interesting to note that after 10.5 h there are



**Figure 7.** (a) Dehydrated liver sample after exposure in the nitrogen-purged chamber. (b) The sample becomes thin, brittle and prone to tears. Note that after 10.5 h, water droplets are still present on the surface of the sample.

still water droplets on the surface of the sample—evidence of the high quantity of water in bio-tissue. Referring to figure 5, the improvement in the dynamic range of the measured THz signal converges after 569 min (9.5 h); the rate of dehydration appears to plateau, indicating that the removal of the remaining water in the tissue becomes increasingly difficult over time. Terahertz measurements of bio-tissue exposed to prolonged air or nitrogen drying will therefore still include the influence of water. This is another impetus to explore other tissue preparation methods, such as lyophilization, that can reduce or remove water from tissue. Lyophilization will be introduced in section 5.2.

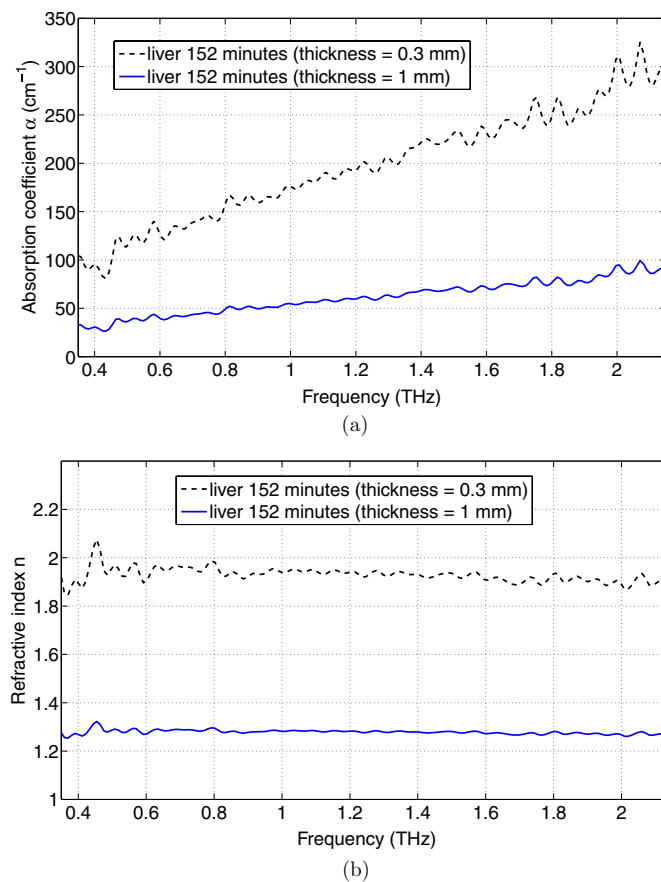
#### 4.2. Errors in extracted optical properties due to variation in thickness

In section 4.1, sample shrinkage is highlighted. Shrinkage affects the thickness of samples, which in turn influences the calculated optical properties as shown in equation (2). Sample thickness therefore needs to be monitored. In a study of fresh skin tissue, sample thickness was maintained through the use of metal spacers (Wallace *et al* 2006). This technique is tenable if the THz system is fast enough (30 scans per second as reported in their paper). It however does not generalize to longer scanning times, which is necessary for higher signal-to-noise ratio. Wallace *et al* report a signal-to-noise ratio of 4000:1. Most conventional THz-time domain spectroscopy (TDS) systems are capable of very high signal-to-noise ratios over 100 000:1 (Zhang 2002) but have scan times of a few minutes, as was the case for our experiment. As borne out from our experiments, spacers may still not resolve our sample thickness issue because spacers are no guarantee against tissue shrinkage.

Figure 8 illustrates the impact of using an incorrect value for sample thickness on a fresh liver sample that started off with 1 mm thickness. When the frequency response of the sample rises above the noise floor at 152 min, the sample's thickness is measured again and found to be 0.3 mm—a significant reduction in thickness. If 1 mm is used, the incorrect extracted optical properties are considerably lower than the actual values.

Although it is necessary to monitor sample thickness, repeated contact between the fresh sample and measuring instrument, such as callipers, may accelerate sample deterioration. The supple nature of fresh tissue also makes accurate measurements of thickness difficult, resulting in large errors in sample optical properties as seen in figure 8. Post-processing techniques dependent on tissue optical properties, such as classification, would be severely hindered by these errors. As a tissue sample dries, its texture changes to one that is more robust to handle, allowing quicker and more accurate measurements of sample dimensions. Sample handling and measurement issues are additional reasons to move towards dry bio-tissue samples.





**Figure 8.** Error in extracted optical properties of liver due to incorrect thickness. The fresh liver sample starts at a thickness of 1 mm and shrinks to 0.3 mm after 152 min. The top dashed curves are the correct versions, and the bottom solid curves show the incorrect result of assuming constant thickness of 1 mm. (a) Error limits of extracted absorption coefficient. (b) Error limits of extracted refractive index.

## 5. Lyophilization: a possible solution to hydration problems in fresh tissue

As seen in sections 2.1 and 4, fresh tissue can be difficult to handle and prepare, while the variation of hydration level results in THz measurements that are highly dependent on the duration of tissue exposure to nitrogen. In this section, alternative sample preparation techniques that are less susceptible to hydration changes will be introduced, and a possible solution will be presented and compared against results from fresh tissue samples.

### 5.1. Snap frozen tissue

Two common methods of retaining the ‘freshness’ of excised tissue are to either immediately fix the sample in formalin and then embed it in paraffin, or snap freeze (rapid freeze) it below  $-75^{\circ}\text{C}$  in a refrigerant such as liquid nitrogen, isopentane or isobutane. As mentioned in section 1, formalin fixing may destroy protein structure. Tissue structure can

also be destroyed through slow freezing in a domestic freezer because of the formation of ice crystals in extracellular spaces, resulting in damage to cell membrane and constituents (Gage and Baust 1998, Schäfer and Kaufmann 1999).

Snap freezing of tissue, such as organs, usually involves immersing the tissue in a cryomould containing a freezing medium, such as OCT (Tissue-Tek). The mould is then placed into the refrigerant. The freezing medium, which snap freezes together with the tissue, provides mechanical support for the frozen tissue and allows easier handling during slicing with a microtome. Since ice is more transparent to THz (Ashworth *et al* 2006), it is expected that THz signals from frozen samples will have much higher signal-to-noise ratio than that of fresh tissue. However, snap frozen samples thaw quickly at room temperature, so samples must be measured inside a cryostat at temperatures below  $-75^{\circ}\text{C}$  to prevent repeated thawing and refreezing, which may damage the tissue samples.

One problem encountered in earlier trials with frozen tissue is the thinness of slices. In the most standard pathology analysis, frozen tissue samples are cut into thin slices with a microtome and mounted on microscopic slides for viewing under an optical microscope. Most microtomes can therefore achieve thicknesses of up to  $50\text{ }\mu\text{m}$ —the same order of wavelength in the optical range ( $\lambda = 10^{-6}\text{ m}$ ) but three orders less than in the THz range ( $\lambda = 10^{-3}\text{ m}$ ). Terahertz measurements from our earlier trials with  $20\text{ }\mu\text{m}$  thick sliced frozen samples mounted on polystyrene slides are not successful because the measured sample signal is similar to that of the empty polystyrene slide, hence little information is present. This is expected because  $20\text{ }\mu\text{m} \ll 1/\alpha$  (where  $1/\alpha$  is the penetration depth of the sample), thus there is insufficient bulk for interaction with the THz signal (Born and Wolf 1999). Microtomes capable of cutting thicker slices exist but were not available for this study.

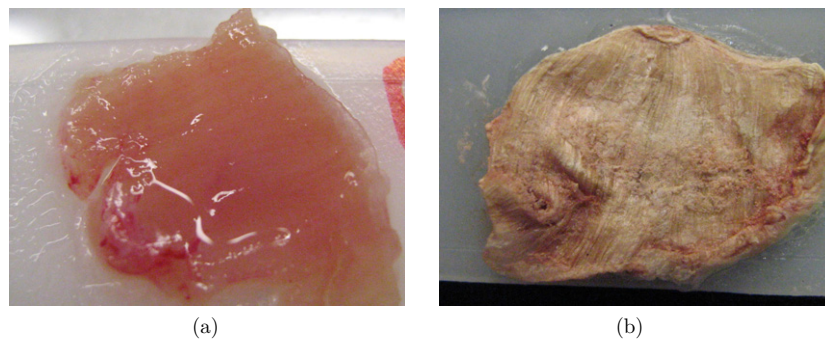
### 5.2. Lyophilizing tissue

Fresh samples preserve biological structure but contain high quantities of water whereas frozen samples thaw quickly, making THz measurements cumbersome (e.g., cryostat). One sample preparation technique that can encompass all the benefits of fresh and frozen methods is freeze drying (lyophilization). Lyophilization involves snap freezing a sample as described in section 5.1 and then placing the sample in a low-pressure (or high-vacuum, 10–100 Pa), low-temperature ( $-74^{\circ}\text{C}$ ) environment that causes water to sublime. This process removes a high percentage of water from the sample (reports vary between 90% and >99% water removal depending on sample type) while retaining its structural and molecular integrity.

Lyophilization is a well-established reproducible drying technique for commercial food and pharmaceutical products, thus controlled techniques are well developed and understood. An advantage of the use of lyophilization is that standards in its use are defined and assist in maintaining reproducibility over an extended period of time (Thorpe *et al* 2002, Hymas *et al* 2005). If tissue thickness and lyophilization time are kept constant, then lyophilized samples will be reproducible. In terms of tissue integrity after lyophilization, human blood platelets have been reported to successfully survive freeze drying (Wolkers *et al* 2001, Tang *et al* 2006). Tissue preserved through lyophilization appears to be ideally suited for THz spectroscopy.

### 5.3. Mounting lyophilized tissue for measurement

To prevent the tissue slices from curling up during snap freezing, it is still necessary to sandwich the tissue slices that are to be lyophilized, but polyethylene plates without holes are used instead. Once sandwiched, the samples are dropped into a bath of isobutane that is seated in dry ice to keep the isobutane at  $-60^{\circ}\text{C}$ . After snap freezing, the samples are



**Figure 9.** Lyophilized tissue becomes stiff hence is easier to handle than fresh tissue. (a) Fresh diaphragm. (b) Lyophilized diaphragm.

transferred into a lyophilizer (Virtis Freezemobile 25EL) and lyophilized via manifold drying for 48 h at  $-73.5^{\circ}\text{C}$ , 212 mTorr ( $\sim 28$  Pa). Since lyophilized materials become hygroscopic and exposure to moisture may cause destabilization, lyophilized tissue samples are stored in plastic screw cap test tubes that are sealed at the mouth with sealing film (Parafilm), and capped tightly. The test tubes are then refrigerated in a domestic refrigerator at  $\sim 4^{\circ}\text{C}$  until the moment of THz measurement.

As illustrated in figure 9, lyophilized tissue becomes stiff and can be mounted with ease in the THz path. To ensure that similar sample handling conditions are employed for all tissue types in this study, lyophilized tissue samples are also mounted on polyethylene plates with holes using superglue at the edges.

#### 5.4. Comparison between lyophilized and fresh tissue

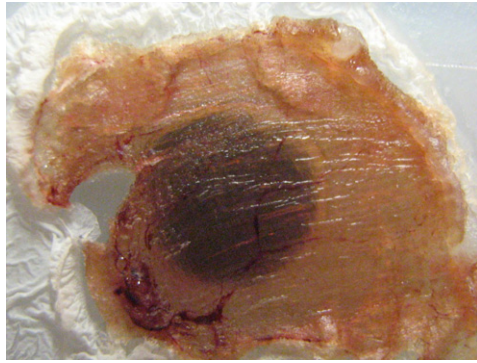
In sections 4.1 and 4.2, the impact of hydration changes in fresh tissue was presented. Dry tissue was recommended as an alternative to fresh, and THz spectroscopic measurements of nitrogen-dried tissue were shown to have better bandwidths and dynamic ranges. The duration of nitrogen drying varied according to the density of the tissue, affecting the time taken for the improvement in bandwidth and dynamic range to plateau.

For dense samples, such as stomach, diaphragm and kidney, the duration of drying required before no improvement in the THz signal is observed can be in the order of several days. Recalling section 5.2, lyophilization is a quicker and more effective method of drying samples. Lyophilized samples are also easier to handle and measure (section 5.3). Comparing figures 9 and 10, the appearance of lyophilized tissue more closely resembles that of fresh tissue, whereas nitrogen-dried tissue appears translucent and paper-like.

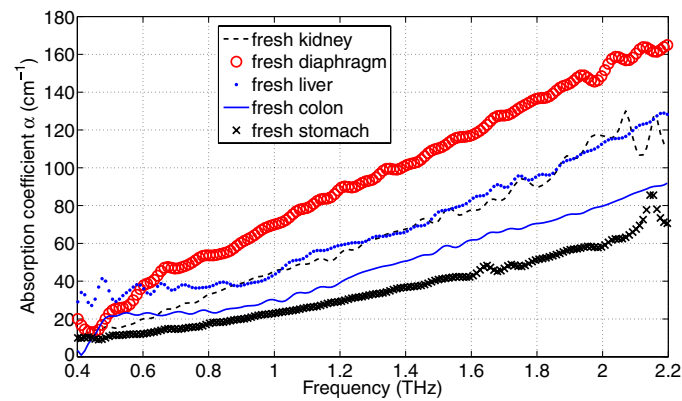
Figure 11 presents the THz absorption coefficient of various tissue samples in their nitrogen-dried and lyophilized forms. The nitrogen-dried tissue samples are dried until there is no observable change in hydration. The lyophilized tissue samples are measured in their natural form immediately after removal from the test tubes (section 5.3).

With the exception of diaphragm tissue, there is a lack of differentiability in the THz absorption coefficients of fresh nitrogen-dried tissue. For example, plots of fresh nitrogen-dried kidney and liver overlap. Errors in the slope of these plots are unlikely, because the thickness of nitrogen-dried tissue can be measured accurately. Thickness of each sample was averaged over five measurements taken at different points on the sample.

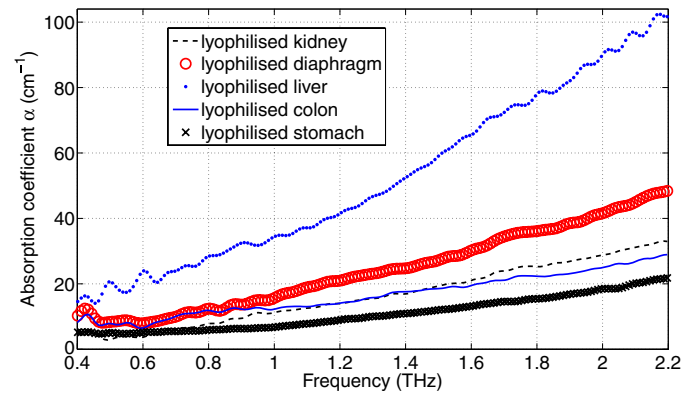
As mentioned in section 4.1, the rate of water removal via nitrogen drying slows down over time. For nitrogen-dried diaphragm tissue, its high THz absorption is indicative of high



**Figure 10.** Nitrogen-dried fresh diaphragm: in addition to thinning and shrinkage, nitrogen-dried tissue samples appear translucent. In this example, the hole in the polyethylene plate is visible through the sample.



(a) Absorption coefficient of fresh nitrogen-dried tissue.



(b) Absorption coefficient of lyophilised tissue.

**Figure 11.** (a) The THz bandwidth of nitrogen-dried samples is narrower with noise ripples visible from 1.7 THz onwards. (b) Wider THz bandwidth of lyophilized samples and distinct separation of the plots at the higher THz range. Note that the ordinate scale of part (b) differs from that of part (a).

residual water content; this is expected as fresh diaphragm tissue, being muscle tissue, is extremely dense thus is expected to dehydrate in nitrogen at a slower rate than the other tissue types.

Water removal via lyophilization appears to be effective as demonstrated by the fact that lyophilized diaphragm tissue has lower THz absorption than nitrogen-dried diaphragm tissue. This is also the case for the kidney, colon and stomach tissue samples. However, for liver, the absorption is only slightly reduced such that lyophilized liver is left with the strongest absorption of all the lyophilized samples. Since strong water absorption can be ruled out for lyophilized tissue, one hypothesis for the strong absorption of lyophilized liver is that liver tissue is made of thousands of small units called lobules (Hebel and Stromberg 1986, Standring *et al* 2005), which may scatter incident THz. A similar hypothesis has been proposed by Wallace *et al* (2006). Reconciling THz spectra with tissue histology will be the focus of a future study.

The gradients from the lyophilized samples differ although not distinctly at the lower THz range. The bandwidth of all lyophilized samples extend beyond 2 THz, whereas nitrogen-dried diaphragm, kidney and stomach dip below the noise floor at around 1.7 THz with increasing noise ripples as shown in figure 11(a).

### 5.5. Necrotic samples as a gauge for tissue freshness

In this study, changes in the hydration of fresh tissue are observed over many hours, hence it is necessary to examine a separate batch of control samples to gauge tissue freshness over time. A third batch of tissue slices is prepared according to the steps described in section 2.1, but instead of Hank's buffer, this batch is placed in non-glucose-based phosphate buffer (phosphate buffered saline or PBS) to intentionally promote necrosis. The tissue slices are packed, transported and mounted as described for fresh tissue in section 3 but are refrigerated with the PBS buffer in a domestic refrigerator until the moment of THz measurement.

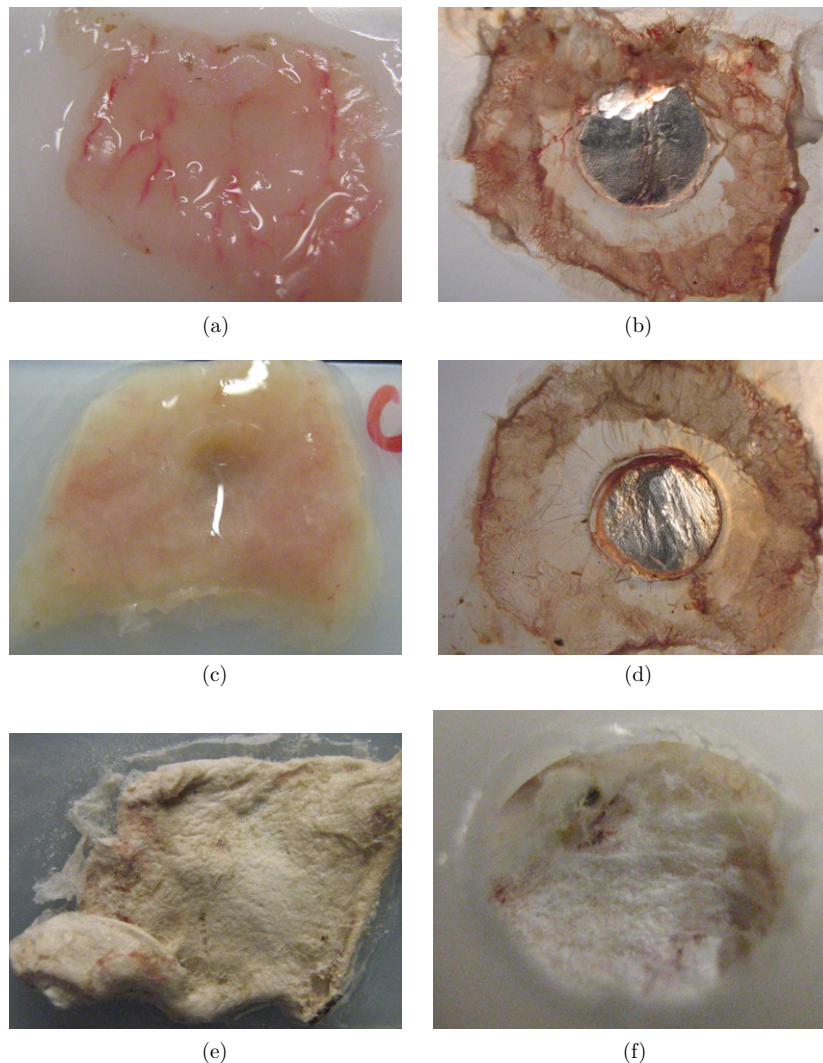
### 5.6. Comparison between necrotic, lyophilized and fresh tissue

Optical images of fresh and necrotic colon before and after nitrogen drying are shown in figures 12(a)–(d). There are visible changes in the colour and texture of necrotic colon. Bad odour is another evidence of necrosis. All necrotic samples in this study have visual and olfactory cues of necrosis.

The THz absorption coefficients of nitrogen-dried, lyophilized and necrotic samples are presented in figure 13. As highlighted in section 5.5, necrotic samples are submerged in PBS buffer until the time of THz measurements. Like fresh tissue, necrotic samples contain large quantities of water therefore nitrogen drying is also required. Necrotic tissue is similar to fresh tissue insofar as the nitrogen-drying process is concerned. As seen from figures 12(b) and (d), nitrogen drying causes shrinkage and thinning in both fresh and necrotic tissue, whereas lyophilized tissue shown in figures 12(e) and (f) retains much of the original shape and texture of fresh tissue.

With the exception of stomach, there are clear differences between the THz absorption of nitrogen-dried fresh and nitrogen-dried necrotic tissue in figure 13. This is encouraging as this indicates that (i) Hank's buffer has successfully sustained the fresh tissue used in this study and (ii) fresh tissue does not become necrotic during the nitrogen-drying process. Nitrogen-dried fresh tissue is therefore still suitable for THz spectroscopy after the prolonged drying time.

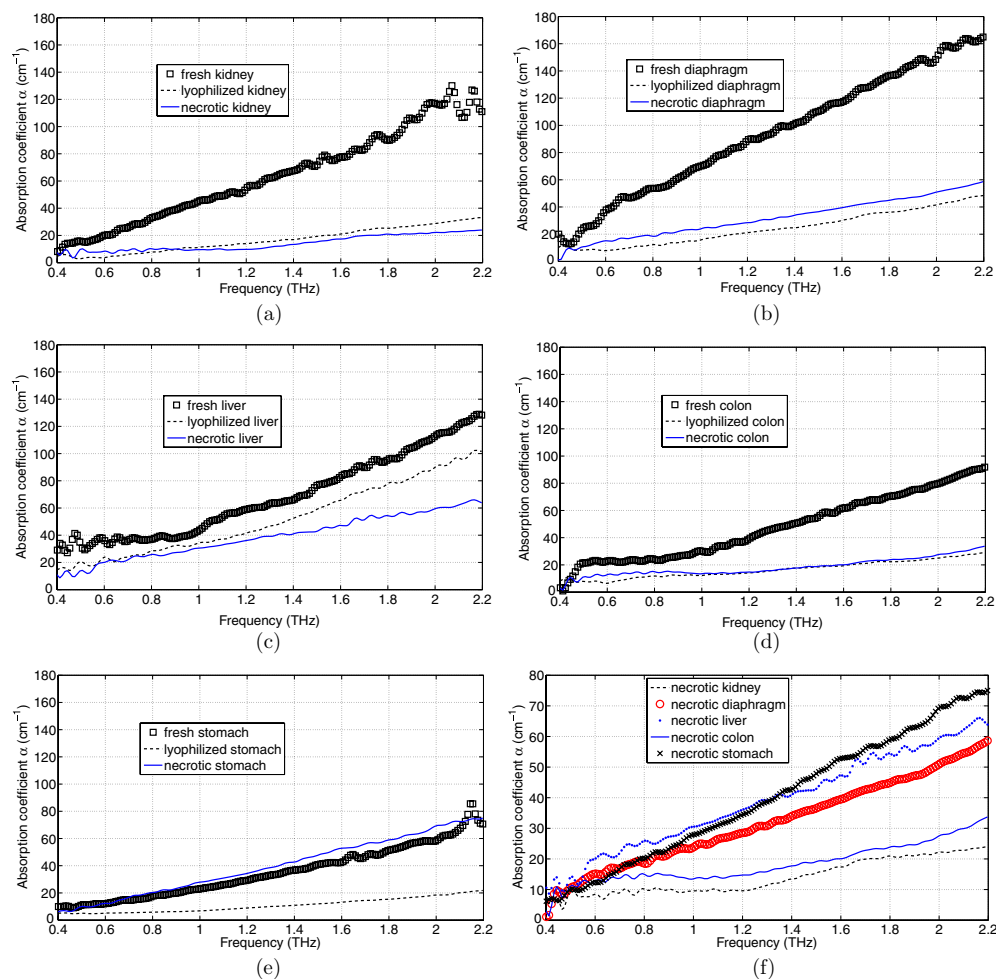




**Figure 12.** (a)–(d) Shrinkage and thinning in both fresh and necrotic tissue due to nitrogen drying. (e)–(f) Tissue structure is remarkably preserved in lyophilized tissue. (a) Freshly excised colon: note the hole in the polyethylene plate behind the sample cannot be seen. (b) Nitrogen-dried fresh colon: the hole in the polyethylene plate behind the sample can now be seen because of the sample's translucency. (c) Necrotic colon showing turbid discolouration and texture; the hole in the polyethylene plate behind the sample cannot be seen. (d) Nitrogen-dried necrotic colon: the hole in the polyethylene plate behind the sample can now be seen because of the sample's translucency. (e) Lyophilized colon: the sample does not become translucent and retains much of the shape and thickness of fresh colon. Some shrinkage is visible but of less severity than for the nitrogen-dried samples. (f) Lyophilized colon viewed from underside: tissue structure is retained as seen by the fibres in the sample.

Terahertz absorption of lyophilized tissue appears to be similar to that of nitrogen-dried necrotic tissue, which could either indicate that lyophilized tissue has become necrotic or that necrotic tissue becomes drier than fresh tissue when exposed to nitrogen. Necrosis is unlikely to have developed in lyophilized tissue given its minimal water content plus the





**Figure 13.** THz absorption coefficients of nitrogen-dried fresh, lyophilized and nitrogen-dried necrotic samples. All samples, with the exception of stomach, show clear differences in THz absorption between nitrogen-dried fresh and nitrogen-dried necrotic tissue. (a) Nitrogen-dried fresh, lyophilized and nitrogen-dried necrotic kidney. (b) Nitrogen-dried fresh, lyophilized and nitrogen-dried necrotic diaphragm. (c) Nitrogen-dried fresh, lyophilized and nitrogen-dried necrotic liver. (d) Nitrogen-dried fresh, lyophilized and nitrogen-dried necrotic colon. (e) Nitrogen-dried fresh, lyophilized and nitrogen-dried necrotic stomach. (f) All nitrogen-dried necrotic samples.

storage conditions used in this study. The low THz absorption of necrotic tissue could be due to protein denaturation, which alters water binding capacity, resulting in dryer samples. However, discussion of the histology of necrotic tissue is outside the scope of this paper.

## 6. Conclusion

This study has presented lyophilization as an alternative to fresh tissue for THz spectroscopic measurements. Problems with handling fresh tissue are highlighted and shown to contribute

to large errors in calculation involving tissue thickness. The high water content of fresh tissue also creates time variability in the THz bandwidth and dynamic range. Complete water removal via nitrogen drying gets progressively difficult and residual water is inevitably included in THz spectroscopic measurements of nitrogen-dried fresh tissue. Ease of handling lyophilized tissue, fast and effective removal of water, and structural preservation are benefits of lyophilization. The only problem encountered in this study involving lyophilized tissue is that the liver sample developed cracks. This is likely due to incorrect lyophilization duration. All samples were lyophilized for 48 h—this could have been too long for certain samples such as liver. Further investigation is needed to explore optimal lyophilization durations for specific tissue types and if possible, quantify tissue hydration. Changes in weight before and after measurements may be good indicators of the extent of dehydration. With improved protocols for sample handling and lyophilization, and together with complementary information from fresh tissue and histology, lyophilized tissue may help move us several steps closer to fully understanding THz interaction with tissue.

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