Original Research

Addressing the Lack of Widespread Metabolomic Profiling of Clinically Diseased Conditions Through the Use of Surgical Pathology Specimens

Frank Chen, MD, PhD;[1]* Bei Yang, MD, PhD;[1] Lin Liu, MD, PhD;[1] Bo Xu, MD, PhD;[2] Dean Troyer, MD;[3] Weiguo Liu, MD, PhD;[4] Chris D'Angelis, MD, PhD;[5] Rafal Kozielski, MD;[1] Troy Wood, PhD;[6] Alexandra Izydorczak, PhD;[6] Wilfrido Mojica, MD[1,7]

[1] *Department of Pathology and Anatomical Sciences, University at Buffalo, Buffalo, NY*
[2] *Department of Pathology, Roswell Park Comprehensive Cancer Center, Buffalo, NY*
[3] *Department of Pathology, Eastern Virginia Medical School, Norfolk, VA*
[4] *Department of Pathology, New York University Langone Medical Center, NY*
[5] *Department of Pathology, Veteran's Administration Western New York Healthcare System, Buffalo, NY*
[6] *Department of Chemistry, University at Buffalo, Buffalo, NY*
[7] *Department of Pathology, Niagara Falls Memorial Medical Center, Niagara Falls, NY*

Our goals are to primarily determine if metabolites present in tissue can be extracted from surgical pathology specimens without altering morphology, and if the extracted fluid can be used for evaluation by mass spectrometry technology. Our secondary goal is to determine if this pre-analytic step can be incorporated into the surgical pathology workflow of a clinical laboratory.

We immersed unfixed tissues in methanol followed by routine formalin fixation paraffin embedding. We then compared morphology of tissues immersed in methanol to matching tissue not exposed to methanol. We also examined methanol extracts for suitability as substrate for analysis by mass spectrometry. Further, we determined the amount of time this additional step requires. Our results show that only minor changes are noted in methanol fixed tissues but are insignificant and not sufficient to alter diagnostic value. Methanol extracts with analytes are present for evaluation, with different tissue types possessing different profiles. Addition of this pre-fixation step only adds under 15 minutes of dedicated work. In conclusions, tissues can be pre-fixed in methanol to create extracts suitable for mass spectrometric analysis without significant changes in morphology. The potential merit of adding supplementary bio-informative data for research and clinical studies exceeds the value of the time commitment needed to create these extracts.

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INTRODUCTION

Metabolomics is an emerging field of health science research with important translational implications. It can be defined as the comprehensive study of small molecules and the interactions between them in cells, biofluids, tissues and organisms. It already is present in everyday clinical practice, as evidenced by the measurement and monitoring of a patient's blood glucose level and other key metabolites by health care providers. Yet these well-known molecules represent only a minute fraction of the metabolome. In the most recently updated version of the Human Metabolome Database, an online record of all known metabolites in the human body, over 251,000 known entities are cited as present in over 50,300 different pathways.[1] Identifying the metabolites and pathways involved in human disease are crucial to better understand the in-depth processes such as cell differentiation, maturation, and the immune responses involved in pathologic conditions.[2,3] In conditions such as cancer, the reprogramming and dysregulation of these pathways are cited as integral for tumor development, and thus hold enormous potential as a diagnostic tool in identifying cancer.[4,5] Identification of these oncometabolites hold significant clinical value, especially if they can be detected in the peripheral blood like other currently assayed metabolites.[6,7] However, the field is hindered by the enormity of this task, and difficulties associated with acquiring sufficient relevant sample material for evaluation. Although peripheral blood samples can be easily acquired for analytic studies, cohorts equaling 600 or more subjects are cited as necessary for metabolomic studies to overcome variations due to gender, age, body-mass-index, blood pressure and smoking.[8] An alternative approach is a tissue-based investigation of the metabolome. The advantages of such an approach would include the specificity associated with the focality of the disease, and elevated concentrations relative to

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***Corresponding Author:** 115 Flint Road, Department of Pathology and Anatomic Sciences, University at Buffalo, Buffalo, NY 14221. (Email: fchen@kaleidahealth.org)

their diluted levels once secreted into biofluids.[9] A major drawback is the need for tissue homogenization, a situation where tissue sampling for metabolites is not agreeable with the field of surgical pathologic diagnostics and with problems of its own.[10] Although work has been done on tissues that have undergone the traditional processing of formalin fixation, paraffin embedded (FFPE), problems with recovery, loss of specific groups of metabolites and degradation can be encountered.[11-13] Previously, Huan et al[14] described a non-destructive, methanol-based process capable of extracting metabolites while preserving histopathology in prostate tissue specimens. In this study, we sought to expand the investigation of this technique to beyond prostate tissue and assess whether it could be integrated into the anatomic surgical pathology workflow. Our aim was to determine if any changes at the cytologic and/or architectural level are incurred in a variety of tissues that are processed in a clinical pathology laboratory when subjected to methanol extraction compared to the routine FFPE protocol, and if tissue metabolomic data can be generated.

Table 1. List of specimens collected for this study, the clinical procedure performed, the portion of the specimen from which matched methanol extracts and immediate FFPE tissue specimens were examined microscopically, and the final surgical pathologic diagnosis of the specimen(s).

Case #	Organ	Surgical procedure	Tissue evaluated microscopically	Clinical diagnosis
1	Prostate	Transurethral resection of prostate	Prostate chips	Benign prostatic tissue with glandular and stromal hyperplasia
2	Small bowel and mesentery	Segmental resection	Desmoid tumor	Desmoid tumor
3	Placenta	C-section	Chorionic villi/ placental disc	Third trimester placenta
4	Uterus	Robotic-assisted laparoscopic total hysterectomy	Leiomyoma	Leiomyoma
5	Placenta	C-section	Chorionic villi/ placental disc	Third trimester placenta
6	Endometrium	Curettings	Decidualized endometrial tissue	Exaggerated placental site nodule
7	Stomach	Laparoscopic sleeve gastrectomy	Gastric mucosa and wall	No specific pathologic findings
8	Urinary bladder	Transurethral resection of bladder tumor	Bladder tumor tissue	Low grade urothelial cell tumor
9	Uterus and fallopian tubes	Robotic-assisted laparoscopic total hysterectomy and bilateral salpingectomy	Fallopian tubes	No specific pathologic findings
10	Uterus and fallopian tubes	Robotic-assisted laparoscopic total hysterectomy and bilateral salpingectomy	Fallopian tubes	No specific pathologic findings
11	Colon	Segmental resection	Colonic mucosa and wall	Diverticulosis and diverticulitis
12	Testis	Orchiectomy	Testicular tumor	Germ cell tumor
13	Urinary bladder	Transurethral resection of bladder tumor	Bladder tumor tissue	Low grade urothelial cell tumor
14	Urinary bladder	Transurethral resection of bladder tumor	Bladder tumor tissue	Low grade urothelial cell tumor

Table 2. Questions posed to the participating pathologists in the comparative evaluation of the tissues fixed first in methanol and subjected to FFPE, and the matching tissue that immediately underwent traditional FFPE (no exposure to methanol).

Query	Yes	No
Do morphologic differences exist?	0	7
Is tissue morphology compromised?	0	7
Are detailed differences present?	0	7
Is the diagnostic value maintained?	0	7
Do you have a preference for either?	3	4

METHODS

The specimens used in this study were received in the laboratory and distanced in time and space from the originating patient. No risk was associated to the originating patient or the surgical procedure performed. The laboratory procedures performed were approved by an institutional review board. Fourteen cases received fresh in the Department of Pathology were examined and matched areas of tissue were sectioned, placed in cassettes, and either placed directly in 10% neutral buffered formalin or in methanol. These tissue sections came from large biopsy procedures or resection specimens and were inclusive of placental, prostate, bladder, gastric, intestinal, uterine, fallopian tube, and testicular origin (**Table 1**). In keeping with the protocol described by Huan et al, tissue from which methanol extracts were obtained from were placed in methanol and remained there for 2 hours, with gentle rotation and at room temperature, thereafter, followed by transferring the tissue to a container with 10% neutral buffered formalin. The methanol in the container was then spun down in microcentrifuge tubes. The supernatant was aliquoted to new microcentrifuge tubes and then immediately placed in a -20ºC freezer. These methanol extracts served as the basis of analysis by mass spectrometry.

The tissue in the cassettes, after initial fixation with or without methanol, underwent conventional processing to generate paraffin-embedded tissue sections, and sectioned at a thickness of 5 microns, collected on glass slides, and stained with hematoxylin and eosin and cover-slipped. Extra tissue sections were sectioned and distributed to the pathologists involved in this study for microscopic examination. The staining protocol involved 6 minutes in hematoxylin and 2 minutes in eosin. Each pathologist was asked to evaluate the two procedurally undisclosed slides for each case, one with methanol treatment and the other without, and to provide their

opinions with additional comments to the following queries on the blinded specimens: 1) were there any significant morphologic difference between the 2 tissues processed by either straight formalin fixation or first by incubation with methanol for 2 hours; 2) was any of the tissue compromised; 3) was there any noticeable difference in the high power evaluation of the tissues (i.e., better detail in nuclear or cytoplasmic features); 4) could a diagnostic assessment be achieved from the examined slide; and 5) was there a preference for either slide?

The methanol extracts then underwent investigational analysis by mass spectrometry. Metabolomic studies are generally ascribed as using either an "untargeted-discovery-global" or "targeted-validation-tandem" approach. The former seeks to fully interrogate the sample and allows for quantification of metabolites, pattern identification and metabolic fingerprinting.[15] This approach is typically run on a column with good resolution for purposes of obtaining a broad overview of the analytes present. A more detailed characterization of metabolites that may be of interest can be further investigated by high-resolution tandem mass spectrometry (MS/MS). The latter is hypothesis testing and useful for the validation of prior untargeted analysis. For purposes of the current proof-of-concept study, an untargeted approach was utilized.

The methanol extracts from the tissues collected over time were stored in a -20ºC freezer until analysis. For liquid chromatography MS/MS (LC-MS/MS) analysis, the extracts were diluted using 500µL of sample in 500µL of methanol from Sigma Aldrich (St. Louis, MO, USA). The samples were filtered before and after the dilution with a VWR (Radnor, PA, USA) 0.45µm polypropylene filter into a glass vial. All samples were run on an Ultimate3000 HPLC with a Waters Atlantis® dC18 column (3 µm, 100 Å, 2.1 mm x 150 mm) (column A) coupled to a Thermo Fisher LTQ XL mass spectrometer. The flow rate was 0.18 mL/min with solvent A: 0.1% formic acid in water, and solvent B: 100% methanol. The gradient was modified from Huan et al. to the following: 0.0 minutes with 20% B, then increased to 20-to-35% B from 0.0-4.0 minutes, then increased to 35-to-65% B from 4.0- 22.0 minutes, then increased to 65-to-99% B from 22.0-30.0 minutes, and then held at 99% B from 30.0-34.0 minutes with a 1µL sample injection. The resulting data was processed with Freestyle 1.8 SP2 (Thermo Scientific, Bellefonte, PA, USA).

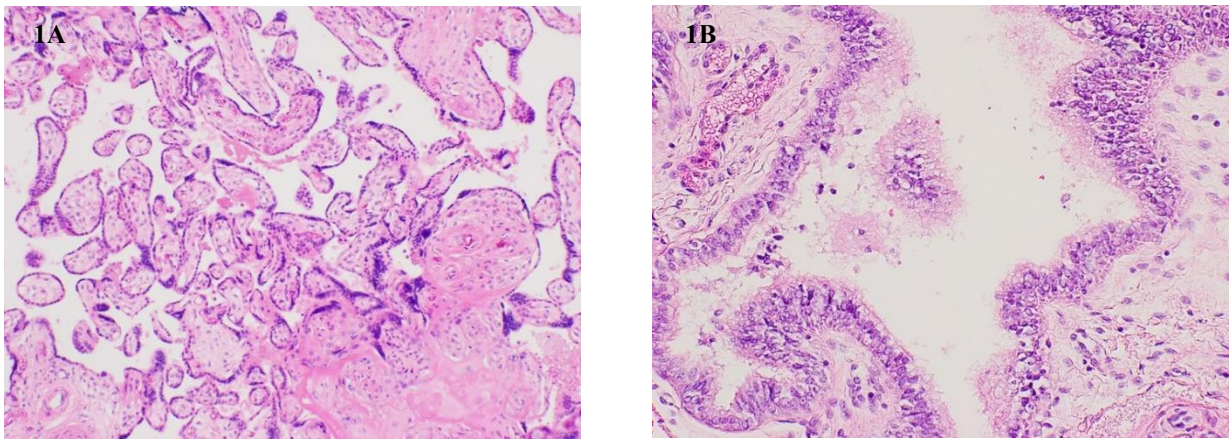

Figure 1. Section of tissue taken from a placenta and incubated first in methanol, followed by formalin fixation and conventional FFPE processing (**1A**, hematoxyklin and eosin stain, 10X) and of fallopian tube tissue (**1B**, hematoxylin and eosin stain, 20X) for demonstration purposes of preservation of both tissue architecture and cytomorphology

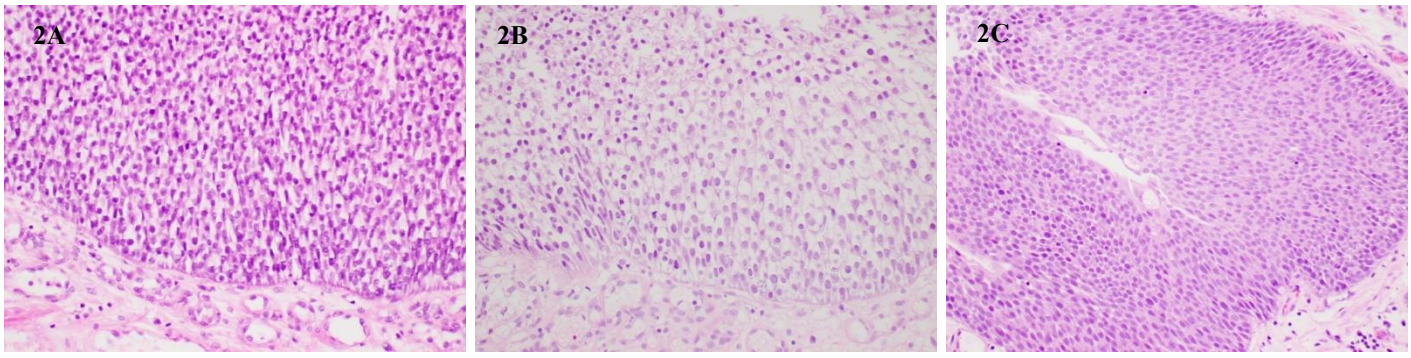

Figure 2. Matched tissue sections of urothelial cell carcinoma from a TURBT specimen fixed with formalin alone (**2A**) or first with methanol for 2 hours followed by formalin and traditional FFPE processing (**2B**). Some qualitative issues regarding unevenness and intensity of staining were noted from some of the pathologists who reviewed the slides. Tissue from the same block as 2B but stained for an extended period of time in both hematoxylin and eosin (**2C**). All stains hematoxylin and eosin and at 10X.

RESULTS

The qualitative microscopic evaluation performed by the participating pathologists was uniform and in agreement with regards to the preservation of the specimens and the maintenance of the diagnostic value between the conventionally processed FFPE specimens and the tissue with the initial, additional step of methanol extraction incorporated (**Table 2**). Overall, comparable cytologic and architectural features were appreciated in the matched tissue sections (**Figure 1**). Differences in the details, specifically some vacuolization of the cytoplasm in some tissues and an unevenness in staining intensity of the nuclei and cytoplasm were noted in some tissues fixed first with methanol, but not all. All pathologists who evaluated the tissue sections however, agreed that these differences in the details did not compromise their ability to evaluate and make a diagnosis based on the tissue section. However, in order to determine if the staining could be improved, sections from the methanol fixed blocks were re-cut and re-stained, but for longer periods of time for both hematoxylin and eosin. The additional staining times increased to 10 minutes for hematoxylin and 5 minutes for eosin, and an additional section for 15 minutes in hematoxylin and 7 minutes in eosin. Re-examination of these re-stained slides, in parallel to the originally stained protocol (6 minutes in hematoxylin and 2 minutes in eosin), demonstrated improved staining intensity in the re-stained slides (**Figure 2C**).

Table 3. Mass spectrometry data on the mass-to-charge (*m/z*) ratios of selected analyte peaks between 3 different types of originating tissue for comparative purposes. The larger nominal level indicates higher levels relative in that specific tissue relative to the other tissues in each row.

m/z	Prostate	Placenta	Uterus/FT
227.21±0.5 Da	1.16E4	3.56E3	2.76E3
203.20 ±0.5 Da	4.90E3	6.28E2	2.30E3
256.08 ±0.5 Da	4.04E3	4.51E1	3.63E1
177.23 ±0.5 Da	1.75E3	2.43E3	2.22E3
173.20 ±0.5 Da	6.99E2	2.30E3	1.92E3
219.13 ±0.5 Da	2.17E3	5.89E2	4.05E3
169.69 ±0.5 Da	8.14E2	7.59E2	1.22E3

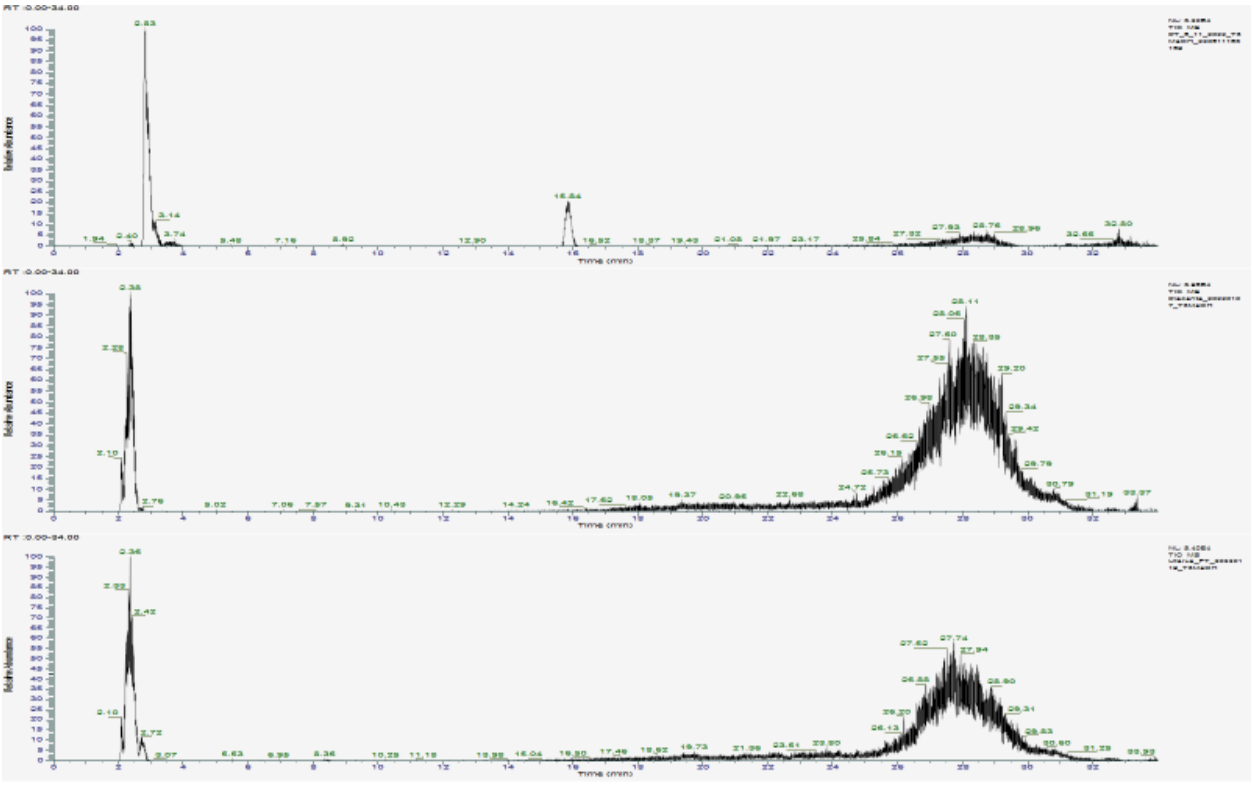

Figure 3. Total ion chromatogram (TIC) of prostate tissue (top), placenta (middle), and fallopian tube (bottom) to demonstrate the presence of unique metabolomic profiles between the different types of tissue.

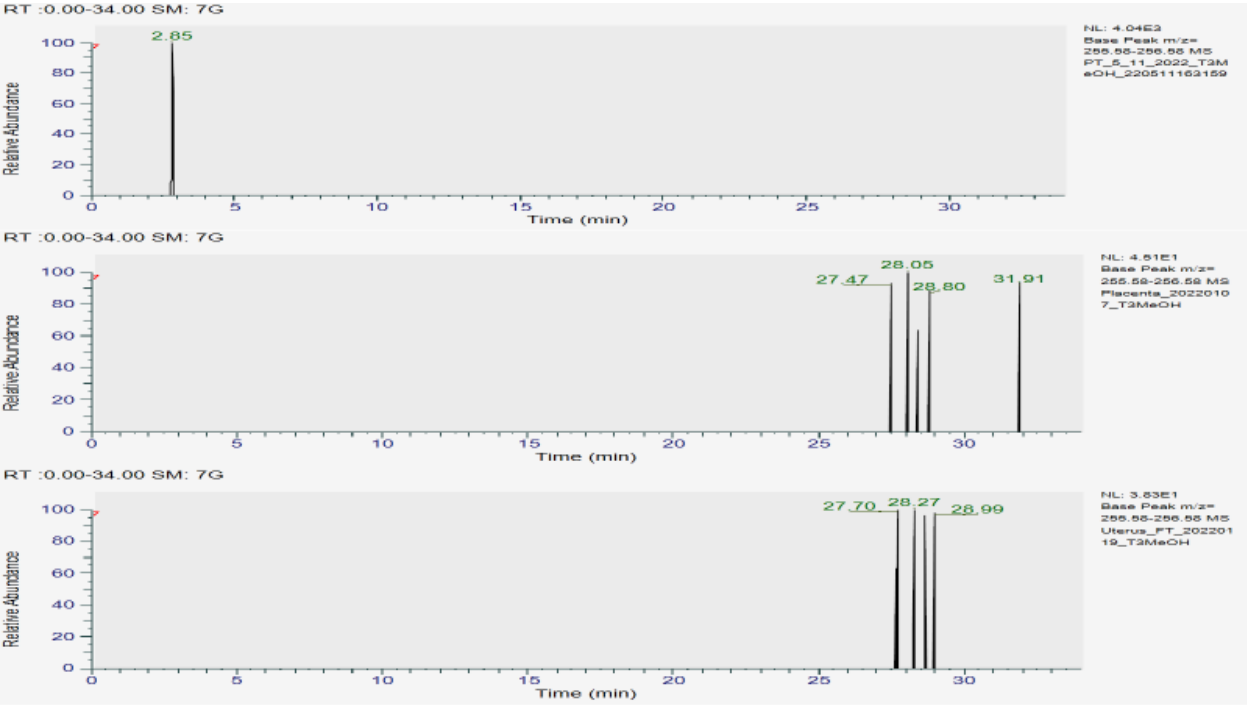

Figure 4. Smoothed, extracted ion chromatograms (EIC) for *m/z* 256.08±0.5 Da of prostate (top), placenta 20220107 (middle), and fallopian tube (bottom).

An untargeted approach was used to create an optimal separation method for the analytes in the methanol extracts. Analyte profiles were then collected to generate a total ion chromatogram (TIC). Examination of the TIC's derived from specific tissues produced profiles with differential attributes. From prostatic tissue taken for urinary obstruction, peaks were observed between 2.0 -4.0 minutes, 9.0 minutes, 15.0-16.0 minutes, 25.0-30.0 minutes and 31.0-33.0 minutes. In contrast, the methanol extracts from the third trimester placental tissue possessed peaks between 2.0-3.0 minutes, 15.0-32.0 minutes, 33.0 minutes and 34.0 minutes, and those from non-neoplastic fallopian tube tissue had peaks at 2.0-3.0 minutes, 8.0 minutes, 15.0-32.0 minutes and 33.0 minutes (**Figure 3**). This raw profile data, however, does not lend itself to the identification of specific analytes. The latter would require runs on a higher mass resolution mass spectrometer (HR-MS) and tandem mass spectrometry for the in-depth separation and retention of analytes for proper identification.

From the TIC, a list of 52 mass-to-charge (m/z) possible analytes were selected for comparative evaluation to characterize their nominal, or relative abundance in the sample. **Table 3** is illustrative of the findings from 7 m/z analytes with different nominal levels from the 3 tissues examined. Using one of these m/z analytes for illustrative purposes, specifically 256.08, it can be seen that the MeOH extracts from the placental and fallopian tube possessed levels of this particular analyte with a m/z ratio of 256.08 in far higher abundance than that from the prostate. The corresponding extracted ion chromatogram (EIC) for each tissue at 256.08 highlights the overall intensity of each m/z peak smoothed at the base peak with a Gaussian curve over 7 points (**Figure 4**). These findings point to differences in the analytes present in the methanol extracts between the different tissue sources. This work, however, is incomplete due to the small sample size studied. To further identify differences in specific metabolites found to be present and/or absent, a more in-depth study using higher resolution instrumentation will be required on a larger number of specimens. Additionally, any differences identified by these mass spectrometry studies need to be correlated with any differences noted to be present in the morphology from the corresponding parent tissue specimen. This needs to correlate with the tissue specimen forms the fundamental need to ensure that the approach in generating methanol extracts from tissues does not generate artifactual changes.

The amount of time required to perform this additional wash step to generate methanol extracts was under 15 minutes. The time needed could be broken down into what was required to cut or appropriate tissue or tissue fragments into a cassette, place that cassette into a container with methanol, place that container on a rocker for 2 hours, re-visit the specimen at the 2-hour end point to transfer the supernatant to a new tube, and place that new tube into a freezer.

DISCUSSION

Metabolomics is an emerging, highly promising field that may one day be able to provide an abundance of clinically relevant data to patient care. Additionally, it may ultimately come under the purview of the clinical pathology laboratory and therefore pathologists should be cognizant of its existence and potential to contribute to medicine. There are a number of issues hindering the research into its application and integration into the clinical realm, one being establishing profiles specific to normal and diseased tissues. Surgical pathology specimens appear to be an ideal source to facilitate this approach, but from the perspective of pathologists, must be done in such a way as not to compromise the diagnostic value of the tissue specimen. This rules-out homogenization of the tissue specimen. Even if homogenization were to be employed, the benefit of recovering larger metabolites may be outweighed by the introduction of additional problems, specifically, the loss of some analytes as well as the inability to detect them due to the presence of matrix effects. If one were to compare homogenized tissue chromatograms versus non-homogenized approaches, such as the methanol extraction employed in this manuscript, differences may become evident, such as the presence of smaller, more intact and more polar analytes in the latter with a bias towards surface analytes compared to the former and its ability to access analytes deep within the tissue. This may be appropriate and applicable to neoplastic tissues, which have been reported to differentially express cell surface glycolipids.[16] While all these issues remain the subject of more formal and in-depth investigations and are beyond the scope of this work, our goal herein was to demonstrate that surgical biopsy and resection specimens received in the pathology laboratory could prove useful in contributing to the development and expansion of our understanding of tissue-based metabolomics. Identification of specific analytes and the performance of targeted metabolomics protocols require further in-depth analysis, but can be undertaken only if adequate numbers of samples are available for examination. Understanding the pathways involved within tissues, and the derangements taken in pathologic tissue could prove extremely useful in the burgeoning era of Precision Medicine, particularly if it could be correlated to the morphology of disease as we currently interpret it. In this study we expanded prior work on metabolomic profiling from surgical pathology specimens done by Huan et al to determine if this approach could be incorporated into the pathology laboratory workflow without compromising morphology to tissue specimens other than that coming from the prostate. For the surgical specimens subjected to this modified processing step and as evaluated by a small cohort of pathologists, the minor differences noted in the cytomorphology were acceptable with no obvious alteration in architecture or the ability to render a diagnosis. Manipulating the fresh specimen by adding a short incubation step involving methanol is a small inconvenience compared to the ability to lend further insight into our scientific understanding of the underpinnings involved in normal and diseased tissue. The minor issues in the appearance of the stained tissue sections could be easily compensated for. The nearly identical morphological features present in the matched tissue specimens open the possibility to extending the utility of tissue specimens beyond surgical pathology to now include metabolomics profiling.

CONCLUSION

In summary, we believe that since there is often a significant amount of excess tissue present in surgical pathology specimens, particularly resection specimens and even in some large biopsy specimens, pathologist should be fully engaged and prescient to the future, and give some consideration to processing some of this tissue as previously described, so as to generate bankable material for the investigation and establishment of metabolomics-associated normal and disease profiles and the identification and characterization of potential biomarkers.

CONFLICT OF INTEREST DISCLOSURES

The authors have no conflict of interest to disclose.

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