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TITLE:

On-Site Sampling and Extraction of Brain Tumors for Metabolomics and Lipidomics Analysis

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brain tumor, metabolomics, lipidomics, biomarker search, solid phase microextraction, LC-MS

SUMMARY:

The manuscript presents on-site sampling of human brain tumors with solid phase microextraction followed by their biochemical profiling towards the discovery of biomarkers.

ABSTRACT:

Despite the variety of tools available for cancer diagnosis and classification, methods that enable fast and simple characterization of tumors are still in need. In recent years, mass spectrometry has become a method of choice for untargeted profiling of discriminatory compound as potential biomarkers of a disease. Biofluids are generally considered as preferable matrices given their accessibility and easier sample processing while direct tissue profiling provides more selective information about a given cancer. Preparation of tissues for the analysis via traditional methods is much more complex and time-consuming, and, therefore, not suitable for fast on-site analysis.

The current work presents a protocol combining sample preparation and extraction of small molecules on-site, immediately after tumor resection. The sampling device, which is of the size of an acupuncture needle, can be inserted directly into the tissue and then transported to the nearby laboratory for instrumental analysis. The results of metabolomics and lipidomics analyses demonstrate the capability of the approach for the establishment of phenotypes of tumors related to the histological origin of the tumor, malignancy, and genetic mutations, as well as for the selection of discriminating compounds or potential biomarkers. The non-destructive nature of the technique permits subsequent performance of routinely used tests e.g., histological tests, on the same samples used for SPME analysis, thus enabling attainment of more comprehensive information to support personalized diagnostics.

INTRODUCTION:

Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) are the main methods used for the real time analysis of brain lesions. Brain tumor differentiation is generally based on histopathology with additional staining and advanced immunohistochemical techniques. According to the updated guidance on central nervous brain tumors issued by the World Health Organization (WHO) in 2016, genetic tests are crucial for the differentiation and classification of these tumors[1]. Differentiation and classification of tumors allow physicians to choose the most effective treatment for a given type of tumor thereby expanding the life expectancy of the patient. Unfortunately, despite the availability of such advanced methods to assist physicians in selecting an optimum therapy for their patients, the life expectancy of patients diagnosed with glioblastoma (IV grade glioma) is only about 15-16 months[2]. Even with the sophistication and increased accuracy of the said imaging and histological methods as diagnostic tools, there is still a great need for new techniques capable of offering complementary information to aid physicians in decisions regarding the course of treatment. Over the past years, several new approaches based on mass spectrometry have been proposed for intraoperative analysis of cancer[3,4]. The potential of solid phase microextraction (SPME), the method presented herein, as a rapid on-site analysis tool, has already been demonstrated in a variety of studies[5]. The current manuscript shows one of the clinical applications of the method, untargeted metabolomics and lipidomics of human brain tumors. Untargeted investigations present an important starting point in the discovery of potential biomarkers. Once established, such biomarkers can then be used as diagnostic references to differentiate among tumors using the same technology coupled to on-site instrumentation.

SPME is an equilibrium-based sample preparation technique that extracts small molecules from sample matrices with the use of small amounts of extraction phase. In SPME’s most traditional configuration of the device (probe), a fiber is coated with an appropriate extraction phase and immobilized on a solid support i.e., a metal wire[5,6]. Biocompatible coatings and devices (probes) enable extraction directly from complex biological matrices without sample pretreatment e.g., homogenization and filtration. Through the extraction process, analytes are partitioned between the extraction phase and sample matrix in proportion to their initial concentrations. If extraction is carried out long enough, then equilibrium is achieved. While extraction at equilibrium provides the highest possible sensitivity and reproducibility, pre-equilibrium extraction is also possible and even preferable in some cases i.e., in vivo sampling, where time-restrictions associated with the on-site sampling (e.g., operating or emergency rooms) necessitate fast extractions. The extraction time profile of a given analyte is generally influenced by the physicochemical properties of the analyte, the matrix being sampled, the type of sorbent used, and several other extraction conditions. The plethora of factors governing their extraction kinetics makes it practically impossible to ensure equilibrium extraction of all compounds when untargeted analyses such as metabolomics or lipidomics are performed. For the above-mentioned reasons, the extraction time of the current protocol was set arbitrarily to ensure satisfactory sensitivity and coverage of metabolites on one hand, and practicality for on-site use on the other.

It should be emphasized that the very small size of probes used for the extraction of sample from tissues only causes minimum tissue damage while the sampling procedure itself does not consume any tissue but very small amounts of small molecules from the sampled area; therefore, the same sample can be further used for routine tests i.e., histological or genetic, enabling the attainment of essential and complementary information from the same sample. Such complementary, comprehensive data would enable a better understanding of tumor biology, hopefully facilitating the discovery of new treatment targets. Exploiting this method further increases the possibility of on-site intraoperative diagnostics when determining target biomarkers.

Below we present protocols for sampling of brain tumors on-site for metabolomics and lipidomics analyses and data processing.

PROTOCOL:

The study presented herein was approved by Bioethics Committee of Collegium Medicum in Bydgoszcz at Nicolaus Copernicus University in Toruń (KB 628/2015). Remember to always wear a lab coat and any other required personal safety equipment, such as (but not limited to) safety gloves and glasses. Do not touch the extraction phase of the solid phase microextraction (SPME) probes.

1. Preparation of the SPME devices

1.1. Use probes (fibers) with mixed-mode and C18 coatings for metabolomics and lipidomics, respectively. Collect two sets of samples, one for metabolomics and one for lipidomics.

1.2. Adjust the coating of the probe to an optimum length by trimming the SPME probe. In the current study, the selected coating length was 7 mm. Select the length of the fiber according to the size of the tumor under study, ensuring that the entire sorbent can be immersed in the tumor (**Figure 1**).

1.3. Condition the coatings of the probes by soaking them in methanol: water 50:50 v/v mixture for a minimum period of 1 h before the extraction procedure. Transport fibers to the site of sampling (e.g., hospital) in a vial containing the conditioning solution.

2. Sample collection procedure

2.1. Do not wash or pretreat the tumor in any way prior to SPME extraction.

2.2. Start the sampling as soon as possible after the tumor removal (2 min in the presented study).

NOTE: Adjust the time depending on the on-site set-up for a given facility (distance of researcher's working site from the operating table) and keep it constant for the entire study. Minimizing the elapsed time between tumor removal and start of the extraction is crucial for the capture of unstable metabolites that degrade after blood circulation is cut off from the studied tissue.

2.3. Perform sampling at room temperature. Alternatively, place the sample on ice when the extraction is carried out. In either case, maintain the same conditions for the whole set of samples.

2.4. Take the probe out from the vial.

2.5. Wash fibers with liquid chromatography-mass spectrometry (LC/MS) grade water for 5 s by immersing them in LC/MS grade water. Do not let the sorbent dry prior to the fiber insertion to ensure good reproducibility of data.

2.6. Insert the fibers into the brain tumor tissue as far apart as possible, ensuring that the entire extraction phase is located inside the tumor.

NOTE: It is recommended that extractions be carried out in replicate in order to determine the heterogeneous nature of the tumor (**Figure 2**). Three replicates per sample are recommended.

2.7. Leave the probe for 30 min in the tissue (measured with a timer).

2.8. Use blank controls to eliminate sources of error related to the presence of artefacts stemming from sources other than the sampled tumor. To obtain blank controls, subject fibers to the same analytical workflow, as described above, but without the sampling step (insertion in the tissue or any other sample/matrix). In the data processing step, compile the analytes extracted from these fibers into an "exclusion list" to exclude signals derived from contaminants stemming from solvents or fiber manufacturing. It is recommended that at least 3 replicates of blank are used.

NOTE: To check for the risk of contamination, it is necessary to perform sampling from gloves, tables, apparatus or any other surfaces that may pose a contamination risk. In such cases, fiber preparation, time of extraction, and desorption protocols are the same as that for the samples.

2.9. While extraction is being carried out, label the vials to be used for storage of the probes after extraction.

2.10. After 30 min, remove fiber(s) from the brain tumor.

2.11. Wash fibers with water for 3 s by immersing them in LC/MS grade water to remove residues of blood or cell debris from the probe so the final extract contains only small molecules. A longer washing step is not recommended as it may lead to the loss of polar compounds.

2.12. Immobilize the fibers in the pre-slit septa of the high-performance liquid chromatography (HPLC) vial cap by piercing the septa from the bottom with the non-coated end of the fiber.

2.13. Put the fibers immobilized in the cap in separate HPLC vials and place them in the selected transportation container.

2.14. Perform steps 2.11-2.13 for fibers dedicated to blank controls.

3. Transport and storage

NOTE: Several options are available for transporting samples to the laboratory. It is recommended that a liquid nitrogen Dewar or polystyrene box filled with dry ice be used for transportation; alternatively, ice packs can be used for immediate and quick transportation.

3.1. Place the vials with fibers in the transportation container.

3.2. Upon laboratory arrival, immediately place vials with SPME fibers in a -80 ˚C or -30 ˚C freezer. Do not store fibers longer than 3 years at -30 ˚C or 5 years at -80 ˚C.

4. Sample preparation for metabolomics analysis

NOTE: This step should only be performed once all samples for an experiment have been collected.

4.1. Before instrumental analysis, prepare desorption solvent mixture: acetonitrile:water 80:20 v/v.

4.2. Take out the vials containing the mixed-mode fibers from the freezer. Use these for metabolomics analysis.

4.3. Label vials to be used for desorption.

4.4. Pipette 300 µL of the desorption solution (prepared in step 4.1) into glass inserts placed in 2 mL vials.

4.5. Perform desorption from each fiber placed in a separate insert by fully immersing the coating in the desorption solvent, then agitating it for 120 min at 1,200 rpm using vortex.

4.6. After 120 min (once desorption is completed) remove caps with probes.

4.7. Prepare QC sample by mixing 10 μL aliquots of each sample from the sample set. The sample set size depends on the experimental design. It is important to analyze all samples as one batch.

4.8. Close the vials with new caps.

4.9. Place the vials in the autosampler (4 ˚C) of the liquid chromatography high resolution mass spectrometer (LC-HRMS) and move to step 5.

NOTE: Randomize injections order of the samples including control blanks. Inject QC sample after every 8-10 samples to monitor the stability of the instrument.

5. Metabolomics analysis using reversed phase liquid chromatography and high-resolution mass spectrometer (RPLC-HRMS analysis)

5.1. Set up the parameters of the LC-HRMS analysis and positive ionization mode.

NOTE: The parameters used in the current study in positive mode were as follows: scan range: m/z 80-1000; resolution 70 000; acquisition performed using AGC (1,000,000 ions); inject time to C-trap: auto; spray voltage: 1.5 kV; S-lens RF level: 55%; S-lens voltage: 25 V; skimmer voltage: 15 V; capillary temperature: 300 °C; sheath gas: 40 a.u.; aux gas: 15 a.u.; aux gas heater temperature: 300 °C. This chromatographic method was adapted from Vuckovic et al.[7]. Injection volume: 10 μL.

5.2. Set up the parameters of the LC-HRMS analysis and negative ionization mode.

NOTE: The parameters used in the current study in negative mode: scan range: m/z 80-1000; resolution 70 000; acquisition performed using AGC (1,000,000 ions); inject time to C-trap: auto; spray voltage: 2.5 kV; S-lens RF level: 55%; S-lens voltage: -25 V; skimmer voltage: -15 V; capillary temperature: 256 °C sheath gas: 48 a.u; aux gas: 11 a.u.; aux gas heater temperature: 413 °C. This chromatographic method was adopted from Vuckovic et al.[7]. Injection volume: 10 μL.

5.3. Calibrate the instrument as recommended by the manufacturer.

NOTE: In the current study, the instrument was calibrated using external calibration every 48 h, resulting in a mass accuracy <2 ppm.

5.4. Start the analysis by clicking the **Start** button in the software operating the instrument.

5.5. When the analysis is complete, replace the RPLC column with the HILIC column, change the mobile phases and go to step 6.

6. Metabolomics analysis using hydrophilic interaction liquid chromatography and high-resolution mass spectrometer (HILIC-HRMS analysis)

6.1. Set up the parameters of the LC-HRMS analysis and positive ionization mode.

NOTE: The parameters used in the current study in positive mode were as follows: scan range: m/z 80-1000; resolution 70 000; acquisition performed using AGC (1,000,000 ions); inject time to C-trap: auto; spray voltage: 1.5 kV; S-lens RF level: 55%; S-lens voltage: 25 V; skimmer voltage: 15 V; sheath gas: 60 a.u.; aux gas: 40 a.u.; aux gas heater temperature: 425 ˚C; capillary temperature: 325 ˚C. Chromatographic method was adapted from Vuckovic et al.[7]. Injection volume: 10 µL.

6.2. Set up the parameters of the LC-HRMS analysis and negative ionization mode.

NOTE: The parameters used in the current study in negative mode were as follows: scan range: m/z 80-1000; resolution 70 000; acquisition performed using AGC (1,000,000 ions); inject time to C-trap: auto; spray voltage: 1.3 kV; S-lens RF level: 55%.; S-lens voltage: -25 V; skimmer voltage: -15 V; capillary temperature: 263 ˚C; sheath gas: 60 a.u.; aux gas: 30 a.u.; aux gas heater temperature: 425 ˚C. Chromatographic method was adapted from Vuckovic et al.[7]. Injection volume: 10 µL.

6.3. Calibrate the instrument as recommended by the manufacturer.

NOTE: In the current study, the instrument was calibrated using external calibration every 48 h, resulting in a mass accuracy <2 ppm.

6.4. Start the analysis by clicking the **Start** button in the software operating the instrument.

7. Sample preparation for lipidomics analysis

NOTE: This step should only be performed once all samples for the experiment have been collected.

7.1. Before starting the analysis, prepare desorption solvent mixture: isopropanol:methanol 50:50 v/v.

7.2. Take out the vials containing the C18 fibers dedicated for lipidomics analysis from the freezer.

7.3. Label vials to be used for desorption.

7.4. Pipette 200 µL of the desorption solution (prepared in step 7.1) to silanized glass inserts placed in 2 mL vials.

NOTE: Non-silanized inserts can be also used, but their use may result in poor reproducibility of compounds with high logP, as such compounds can non-specifically attach to glass walls.

7.5. Perform desorption from each fiber in a separate insert by fully immersing the coating in the desorption solvent, then agitating it for 60 min at 1,200 rpm using vortex.

7.6. After 60 min when desorption is completed remove caps with probes.

7.7. Prepare QC sample by mixing 10 µL aliquots of each sample from the sample set. The sample set size depends on the experimental design. It is important to analyze all samples as one batch.

7.8. Close the vials with new caps.

7.9. Place the vials in the autosampler (4 ˚C) of the liquid chromatography high resolution mass spectrometer (LC-HRMS) and move to step 8.

8. Lipidomics analysis using reversed phase liquid chromatography and high-resolution mass spectrometry (RPLC-HRMS analysis)

8.1. Set up the parameters of the LC-HRMS analysis and positive ionization mode.

NOTE: The parameters used in the current study in positive ion mode were as follows: scan range: m/z 100-1000; acquisition performed using AGC (1,000,000 ions); inject time to C-trap: auto; spray voltage: 3.5 kV, S-lens RF level: 55%; S-lens voltage: 25 V; skimmer voltage: 15 V; capillary temperature 275 ˚C; sheath gas: 30 a.u.; aux gas: 10 a.u.; spare gas: 2 a.u.; probe heater temperature 300 ˚C. LC parameters used were: phase A: methanol:water, 40:60 with 10 mM ammonium acetate and 1 mM acetic acid ; phase B: isopropanol:methanol, 90:10 with 10mM ammonium acetate and 1 mM acetic acid.; the gradient: 0 min – 20% B; 1.0 min – 20% B; 1.5 min – 50% B; 7.5 min – 70% B; 13.0 min – 95% B; 17.0 min – 95% B; 17.1 min – 95.5 % B; 23.0 min – STOP; C18 Column, 3.5 µm, 2.1 mm x 75 mm; flow: 0.2 mL/min; oven temperature: 55 °C; injection volume: 10 µL.

8.2. Set up the parameters of the LC-HRMS analysis and negative ionization mode.

NOTE: The parameters used in the current study were as follows: HRMS parameters for negative ion mode: scan range: m/z 100-1000; acquisition performed using AGC (1,000,000 ions); inject time to C-trap: auto; spray voltage: 3.5 kV, S-lens RF level: 55%; S-lens voltage: -25 V; skimmer voltage: -15 V; capillary temperature 275 ˚C; sheath gas: 30 a.u; aux gas: 10 a.u.; spare gas: 2 a.u; probe heater temperature 300 ˚C. Chromatographic method: same as in 8.1.

8.3. Calibrate the instrument as recommended by the manufacturer.

NOTE: In the current study, the instrument was calibrated using external calibration every 48 h, resulting in a mass accuracy <2 ppm.

8.4. Start the analysis by clicking the **Start** button in the software operating your instrument.

8.5. When the analysis is completed, replace the RPLC column with the HILIC column, change the mobile phases and go to step 9.

9. Lipidomics analysis using hydrophilic interaction liquid chromatography and high-resolution mass spectrometer (HILIC-HRMS analysis)

9.1. Set up the parameters of the LC-HRMS analysis and positive ionization mode.

NOTE: The parameters used in the current study in positive ion mode were as follows: scan range: m/z 100-1000; acquisition performed using AGC (1,000,000 ions); spray voltage: 1.5 kV; S-lens RF level: 55%; S-lens voltage: 25 V; skimmer voltage: 15 V; .capillary temperature 325 °C; sheath gas: 60 a.u.; aux gas: 30 a.u.; spare gas: 2 a.u.; probe heater temperature 320 °C. LC parameters used were: phase A: 5 mM ammonium acetate in water; phase B: acetonitrile; the gradient: 0 – 2min – 4% B; 15.0 – 20% B; 15.1 – 4% B, 21.0 min – STOP; 3 µm 100 mm x 2.1 mm column; flow: 0.4 mL/min; oven temperature: 40 °C; injection volume: 10 µL.

9.2. Set up the parameters of the LC-HRMS analysis and negative ionization mode.

NOTE: The parameters used in the current study in negative ion mode were as follows: scan range: m/z 80-1000; acquisition performed using AGC (1,000,000 ions); spray voltage: 1.5 kV, S-lens RF level: 55%; S-lens voltage: -25 V; skimmer voltage: -15 V; capillary temperature 320 °C; sheath gas: 50 a.u.; aux gas: 21 a.u.; spare gas: 3 a.u.; probe heater temperature 320 °C. The chromatographic method was the same as described in 9.1.

9.3. Calibrate the instrument as recommended by the manufacturer.

NOTE: In the current study, the instrument was calibrated using external calibration every 48 h, resulting in a mass accuracy <2 ppm.

9.4. Start the analysis by clicking the **Start** button in the software operating the instrument.

10. Data processing and statistical analysis

10.1. Process data using software compatible with the format of the raw data files.

10.2. Perform statistical analysis using the processed data.

NOTE: The type of test depends on the scientific hypothesis and design of the study. In the current study, Principal Component Analysis, Partial-Least Square-Discriminant Analysis, and one-way ANOVA were used.

REPRESENTATIVE RESULTS:

Exploiting solid phase microextraction as a sample preparation method in combination with liquid chromatography coupled to high-resolution mass spectrometry and an advanced data processing software allowed us to successfully characterize the metabolome and lipidome of human brain tumors. The probe, the size of which was equivalent to an acupuncture needle, caused minimum damage to the studied tissue and no tissue consumption, therefore, enabling the further use of samples for histological or genetic studies. Satisfactory separation of the selected groups was obtained for both reversed phase and HILIC columns, and for both ionization modes in metabolomics and lipidomics analyses. The use of both separation methods not only in metabolomics, but also in lipidomics analysis provided valuable complementary data. The reversed phase column separate lipids with respect to their carbon chains length and the presence of unsaturated bonds, whereas the HILIC column is useful for profiling lipid groups, especially phospholipids[8].

The reproducibility of the instrumental analysis was found to be very good based on the tight clustering of QC samples on the principal component analysis plot (**Figure 3A**, the three QC samples injected every eight patients' samples along the sequence overlap). Moreover, extraction blanks used for negative control analysis were found to separate well from real samples. The wide range of analytes extracted by the probes facilitated the discovery of representative species, thus successfully allowing for differentiation between human brain tumors based on their histological origin, malignancy, and other factors (e.g., genetic). **Figure 3B** shows lipidomics data for samples collected from patients with gliomas and meningiomas. Enabling differentiation between these tumors, which were characterized by different histological origin and malignancy, was an important goal of the study, as meningiomas are generally considered as benign tumors, while gliomas are one of the most malignant. Additionally, in **Figure 4** presenting metabolomics data, gliomas were divided based on their degree of malignancy into high and low. These sub-groups were compared with the molecular phenotype of meningiomas. In both cases, prominent separation of clusters was observed. Nowadays, diagnostics of glioma primarily relies on determining specific genetic mutations in tumor samples. Therefore, results obtained were compared to genotyping data. **Figure 5A** presents separation of the samples with detected co-deletion 1p19q and samples where the mutation was not observed.

Statistical analysis also permits selection of compounds in the studied groups. These compounds might be considered as potential biomarkers in cases where an appropriately large cohort is sampled. However, more in-depth analysis, including conclusive confirmation of detected compounds by fragmentation and comparison of detected species with analytical standards, is required to draw definitive conclusions of a biological nature. Examples of such discriminant metabolites are presented in **Figure 3C** and **Figure 5B**. The identities of these compounds, i.e., sphingomielyn: SM d36:1 and proline were confirmed by comparing fragmentation patterns of the metabolites from the sample and authentic standards.

FIGURE AND TABLE LEGENDS:

Figure 1: SPME fibers prepared for the extraction process.

Figure 2: Extraction of meningioma using SPME probes.

Figure 3: PCA and box plots for glioma and meningioma. Principal component analysis plot containing (**A**) all analyzed samples including blanks, extraction QC, blanks, meningiomas, gliomas; (**B**) containing only studied groups (after exclusion of blanks and QCs); (**C**) box whisker plot for sphingomielyn: SM d36:1 differentiating patient with glioma and meningioma. Lipidomics data.

Figure 4: PCA for HGG, LGG and meningioma. Principal component analysis plot showing differentiation between (**A**) high grade gliomas (HGG) and meningiomas (MEN) [9] and (**B**) low grade gliomas (LGG) and meningiomas. Metabolomics data reprinted from Via Medica ref[9] with permission.

Figure 5: PCA and box plots for glioma with and without deletion. (**A**) Principal component analysis plot showed differences in patients with and without co-deletion 1p19q; (**B**) box whisker plots for proline differentiated patients with and without co-deletion 1p19q; n-without deletion, y-with deletion. Metabolomics data

DISCUSSION:

Untargeted metabolomics and lipidomics are commonly used in studies focused on identifying tumor biomarkers. However, in most cases, researchers look for compounds that can be used for screening of the disease. Consequently, the preferred biological samples are blood or urine due to their relatively easy access. Analysis of tumor tissue is mainly performed to understand the mechanisms behind the disease, characterize different tumor types, etc. On-site analysis of tumor biomarkers is rarely performed, as such applications require extensive sample preparation. Alternatively, strategies based on real-time analysis of tissue profiles without pre-selection of specific biomarkers are earning the attention of the medical community[3,4]. The solution presented herein provides another perspective on tissue processing on-site by unveiling the type of information that can be obtained via such methods.

The combination of sampling, sample preparation, and extraction renders SPME as a very useful tool for on-site analysis. Moreover, lack of tissue consumption during sampling enables further use of the same samples for biomarker analysis and routine tests (genotyping, histological analysis), therefore, adding new information to the results of standard testing. The sampling device has a very simple design, its operation is very easy, and no special training is required to perform the extraction itself. However, achieving reliable results requires much more than just proper handling of devices. To properly perform the experiment, one needs to understand the extraction process, the nature of the sample, and be aware of potential mistakes that can influence the data.

It is important to consider the heterogeneity of cancerous tissue[10]; sampled tumors may contain parts undergoing necrosis, calcification, and hypoxia, and each of these processes will be reflected in the metabolome and lipidome attained, thus influencing results. Therefore, it is recommended that spatial resolution sampling be carried out by insertion of several fibers in different parts of the cancerous tissue, or alternatively, that a longer coating be used to penetrate the whole of the tumor so as to obtain averaged information on the tumor. If the spatial resolution sampling method is carried out, fibers can be all desorbed into one desorption solvent; this would not only allow for the attainment of overall information on the tumor, but also increase the sensitivity of the analysis. Alternatively, desorption of individual fibers into separate vials would enable investigations to figure out the internal diversity of the brain tumor, which consists of the core built of cancerous cells, and the outer zone, which is the border of healthy tissue. Deeper parts of the tumor are usually more damaged by the processes related to cancer[11]. However, investigators must keep in mind that this option compromises method sensitivity and the overall number of detectable compounds. In the current work, a 7 mm coating was used; this length was considered optimum for various sizes of tumors included in the study. The coatings penetrated the tumors, and thus provided non-special resolution, but averaged data across the sample. Regardless of the protocol selected, it is important that the same protocol be followed during the entire study, including the number of fibers used for individual sampling, the length of the coating, extraction time, and all other factors delineated in this work.

It is important to control the quality of the analysis. The pooled QC (see steps 4.7 and 7.7 in the protocol) should be prepared and used for monitoring instrument stability during the run of the entire sample batch. The blank controls (see step 2.8) can be later used to prepare an “exclusion list” to eliminate signals of contaminants originating from solvents or fiber manufacturing. On special occasions, such as a risk of contamination, it is necessary to perform sampling from gloves, tables, apparatus or any other surfaces that may pose a contamination risk. In such cases, fiber preparation, time of extraction and desorption protocols are the same as for the samples.

Metabolomic and lipidomic analyses are focused entirely on small molecules (less than 1,500 Da) appearing in an organism or specific components of the organism, such as specific organs, tissue, fluids, cells, etc. Metabolomic and lipidomics offer a snapshot of biochemical changes occurring in the body, and in the case of cancer, they integrate information related to the genome, histology, and malignancy of the tumor. These omics sciences create a connection between physiology and phenotype as metabolites are higher up in the biochemical ladder than proteins or genes[12]. By understanding the metabolome and lipidome of cancerous tumors, we come closer to discovering the phenotype among all -omics sciences as these branches of study offer more in-depth knowledge of dynamic changes of molecules as a response of living organisms to various stimuli. As presented in this work, the data obtained in one sampling corresponds to the histology of the cancer, its degree of malignancy, and it reflects changes occurring at the genome level. In gliomas, as the type of cancer of interest in this study, the information hidden in the genome is particularly important, as a personalized treatment is developed based on the results of genetic tests. Particular mutations are prognostic markers of the outcomes of the chemo- or radiotherapy. As demonstrated here, the selection of biomarkers reflecting a given mutation is possible with the proposed strategy. Mutation markers, as well as additional descriptors types such as those that indicate the degree of malignancy of the tumor can also be used to support routine diagnostic methods.

Ex vivo chemical biopsy with the use of solid phase microextraction fibers is the first step in the application of the method to intraoperative diagnostics. The method can be easily adopted for in vivo sampling pending permission from appropriate Ethical Boards. In such cases, sterilization of SPME devices must be performed according to the accepted sterilization procedures of the hospital where sampling is to be carried, i.e., autoclaving or ethylene oxide sterilization. The pre-conditioned and sterilized fibers must be kept in sealed packages labeled with a sterilization expiry date. It is important to note that fibers should not be cleaned with the use of surfactants. Such a procedure can cause unspecific changes in sorbent composition, thus impacting the extraction of analytes. In the studies described herein, a 30 min extraction period was used, but other reports validate that shorter times can yield satisfactory results in in vivo studies[13]. Huq et al. showed that analyte equilibrium time is achieved faster in tissue, as a complex matrix, than in simple matrices[14]. However, the reproducibility of the obtained results can be compromised under shorter extraction periods as more analytes will be extracted under pre-equilibrium conditions; therefore, precise time control must be implemented.

Both omics sciences exploited as part of this work have excellent potential as biomarker discovery tools. Once biomarkers are selected or a chemometric model is established, medical diagnostics based on the determination of target metabolites via methods applicable for on-site investigations, such as the SPME approach described in this work, can be developed and implemented as part of routine diagnostics.

The protocol proposed in the present manuscript describes how to perform untargeted metabolomic and lipidomic analyses of cancer tissue using solid phase microextraction for screening of potential biomarkers. It is designed to enable extraction of representative compounds, differentiation of tumors, and identification of discriminatory compounds characterizing a given cancer i.e., potential biomarkers. The untargeted analyses with SPME described in this article represent a starting point in the development of quick intraoperative diagnostics, where a selected panel of compounds can be determined without the necessity for screening of all compounds present in the sample. In the interest of fast diagnostic results, SPME probes used for on-site extraction could be directly coupled to analytical instrumentation located in the hospital facility. Simple extractions performed with minimum sample preparation followed by chromatography-free analysis would significantly shorten overall time from hours to a few minutes, as already described for drug monitoring[15].

ACKNOWLEDGMENTS:

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Authors' contributions: JB: optimization of sample preparation and LC-MS parameters,

performance of SPME-LC-MS experiments, data analysis, statistical analysis and data interpretation and manuscript preparation related to lipidomics part; PZG: coordination and performance of majority of samplings in hospital, optimization of sampling and sample preparation parameters, performance of SPME-LC-MS experiments, data analysis, statistical analysis and data interpretation, manuscript preparation related to metabolomics part; MG – assistance in optimization of sample preparation, LC-MS method and data analysis related to lipidomics part; KG: co-performance of SPME samplings and optimization of sampling and sample preparation, SPME-LC-MS analysis related to metabolomics part; KC: performance of several SPME samplings in hospital, assistance in optimization of sampling, sample preparation and data analysis related to metabolomics part; KJ: performance of several SPME samplings in hospital, assistance in lipidomics analysis; DP: performance of surgical procedures, recruitment of the patients; JF: performance of surgical procedures, recruitment of the patients; MH: performance of surgical procedures, coordination of clinical part of the research; BB: concept, coordination supervision of the project and manuscript preparation, performance of several samplings

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

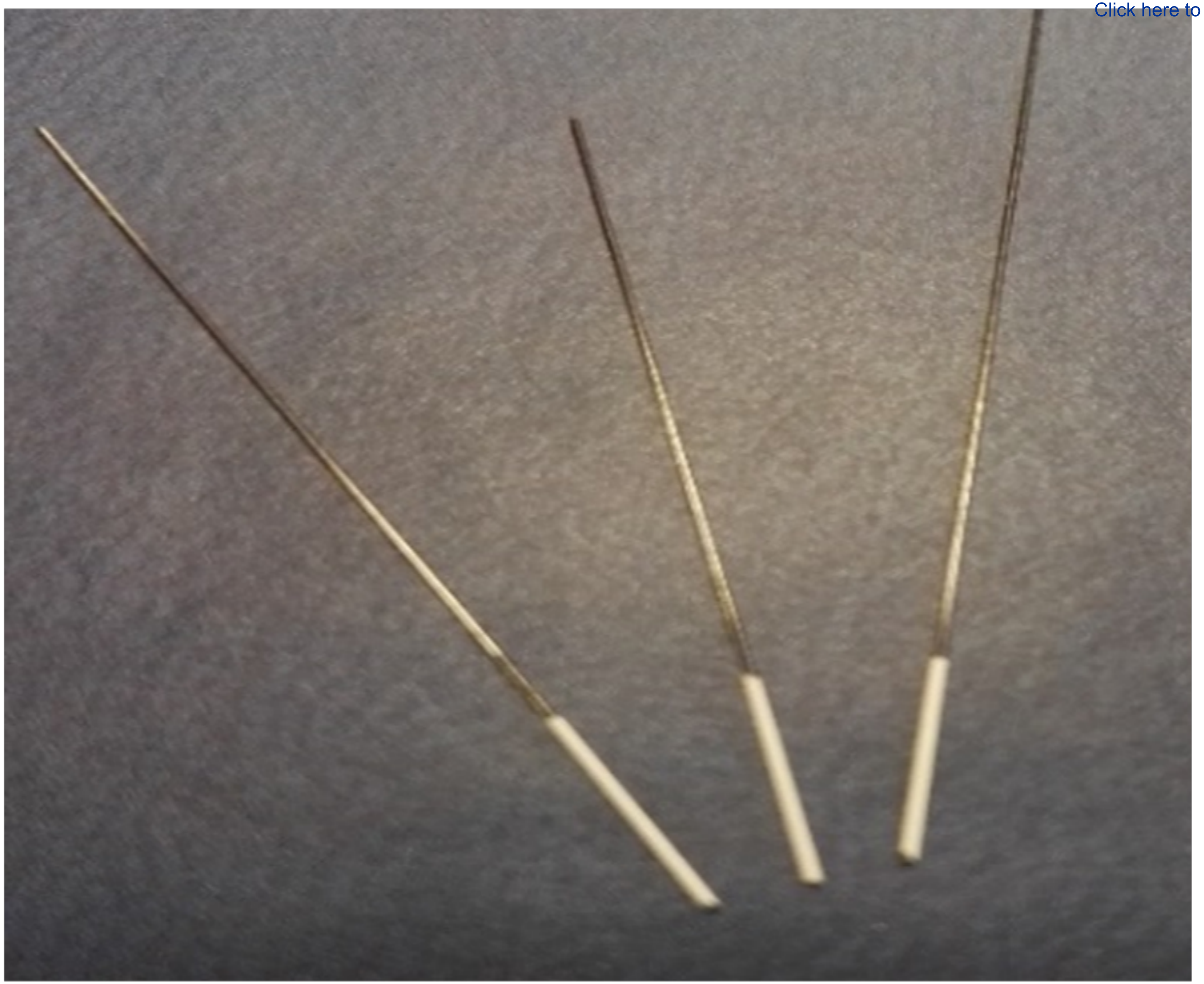


Figure 2

Figure 3

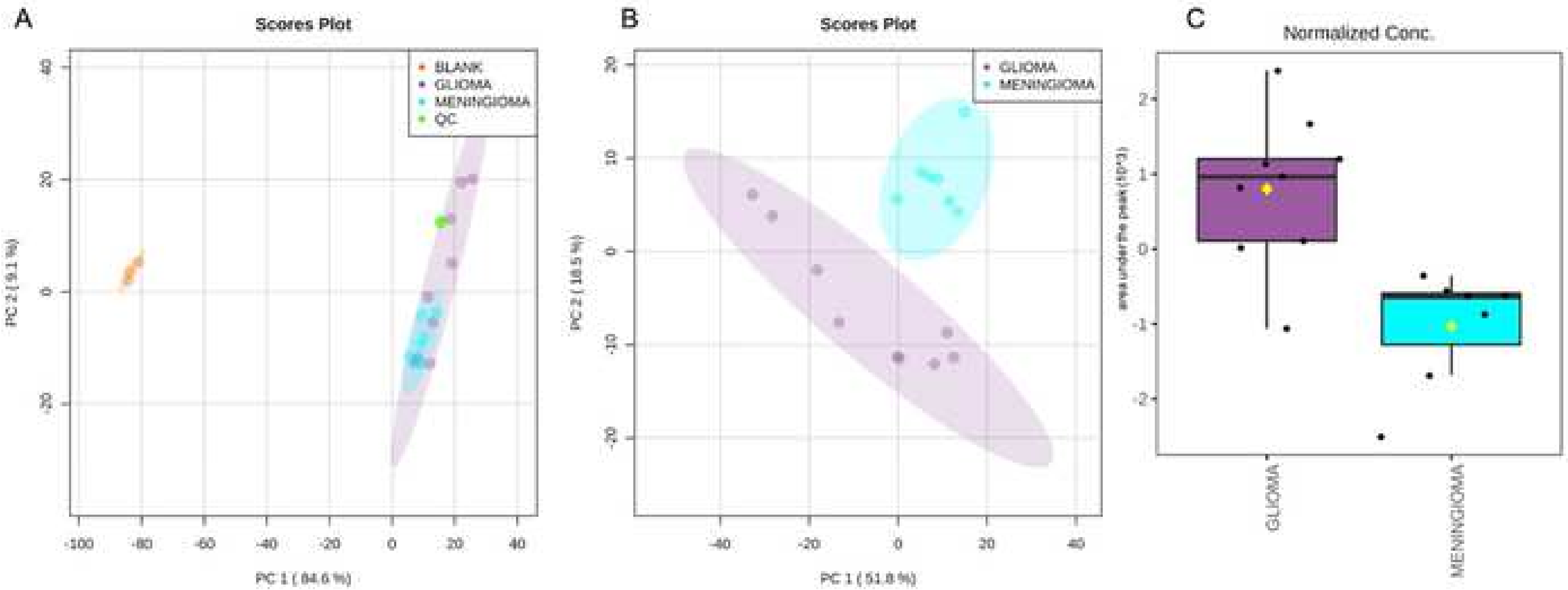
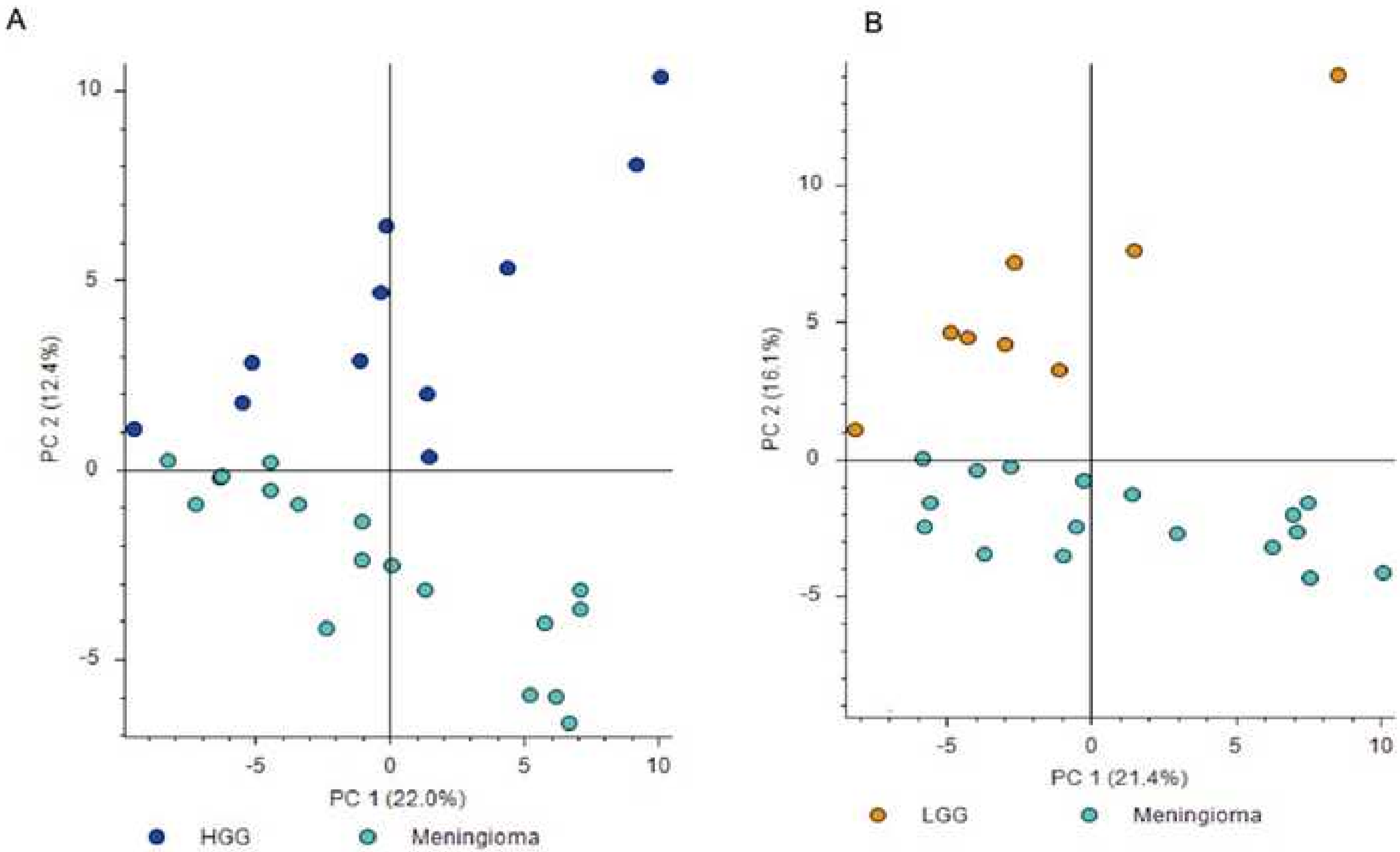

A
Scores Plot
BLANK
GLIOMA
MENINGIOMA
QC
40
20
0
-20
PC 2 (9.1 %)
-100
-80
-60
-40
-20
0
20
40
PC 1 (84.6 %)
B
Scores Plot
GLIOMA
MENINGIOMA
20
10
0
-10
-20
PC 2 (18.5 %)
-40
-20
0
20
40
PC 1 (51.8 %)
C
Normalized Conc.
2
1
0
-1
-2
area under the peak (10^3)
GLIOMA
MENINGIOMA

Figure 4


A
10
5
0
-5
PC 2 (12.4%)
-5
0
5
10
PC 1 (22.0%)
● HGG
● Meningioma
B
10
5
0
-5
PC 2 (16.1%)
-5
0
5
10
PC 1 (21.4%)
● LGG
● Meningioma

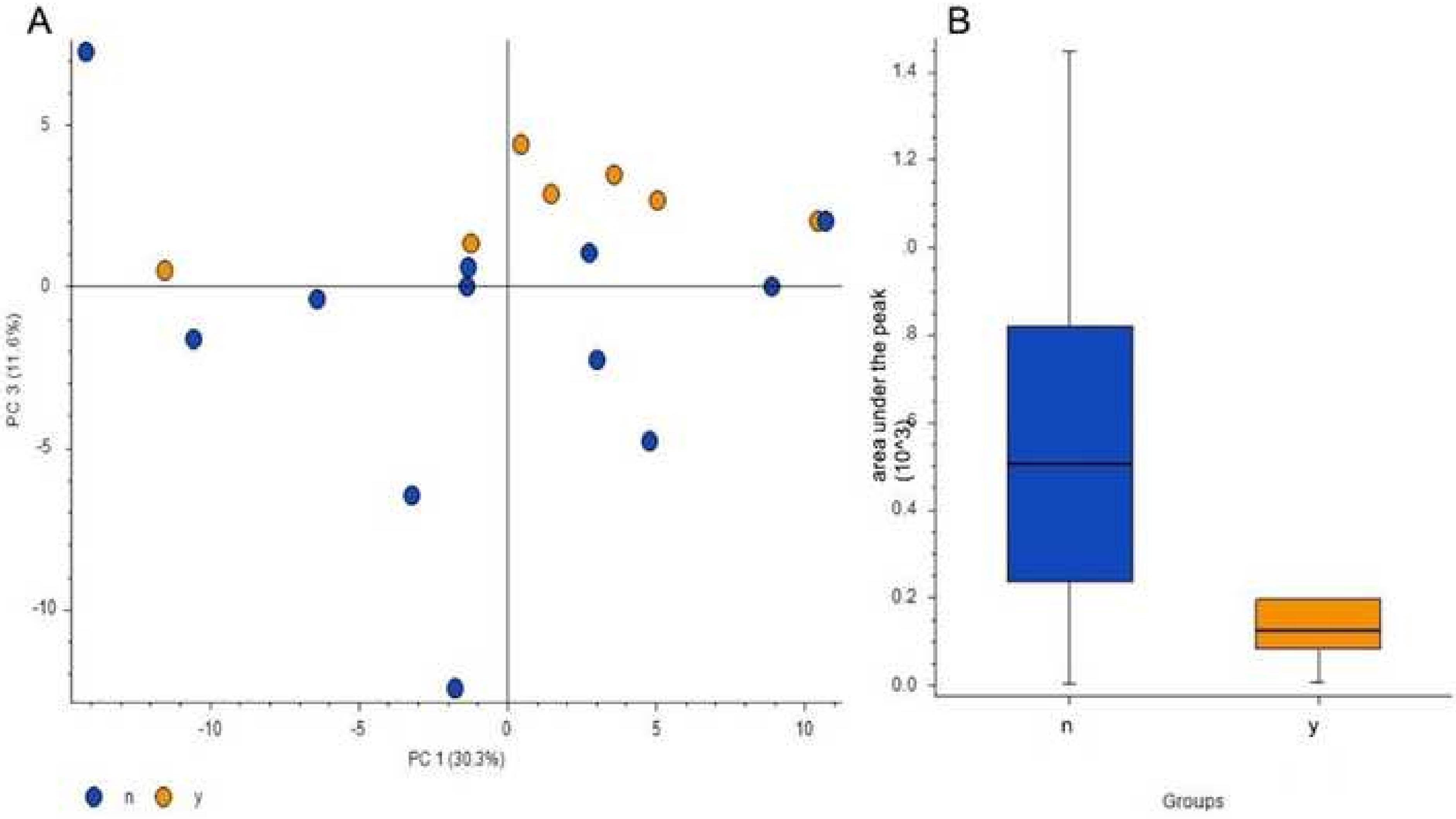

A
PC 3 (11.6%)
5
0
-5
-10
-10
-5
0
5
10
PC 1 (30.3%)
● n ● y
B
area under the peak (10^3)
1.4
1.2
.0
.8
.6
0.4
0.2
0.0
n
y
Groups

Table of Materials

Name of Material/Equipment	Company	Catalog Number
acetic acid	Honeywell	49199
acetonitrile	Honeywell	34967
ammonium acetate	Honeywell	14267
BenchMixer MultiTube Vortexer	Benchmark Scientific	BV1010*
caps	Agilent	5183-2076
Compound Discoverer 2.1	Thermo Scientific	
Discovery HS F5 Supelguar Cartridge 2 cm × 2.1 mm, 3 µm	Supelco	567571-U
Discovery HS F5, 2.1 mm x 100 mm, 3 µm	Merck	567502-U
formic acid	Honeywell	56302
glass vial inserts 250ul , deactivated	Agilent	5181-8872
glass vial inserts 350ul	Agilent	5188-5321
glass vials 1.5ml	Agilent	5183-2030
glass vials, 2 mL (amber, deactivated)	Agilent	5183-2072
glass vials, 2mL	Agilent	5182-0715
HILIC Luna 3 µm, 200A, 100 x 2.0 mm	Shim-Pol	PHX-00D-4449-B0
isopropanol	Honeywell	34965
LipidSearch 4.1	Thermo Scientific	
Metaboanalyst	Xia Lab @ McGill	
methanol	Honeywell	34966
Pierce LTQ Velos ESI Positive Ion Calibration Solution	Thermo Scientific	88323
Pierce Negative Ion Calibration Solution	Thermo Scientific	88324
Q Exactive Focus hybrid quadrupole-Orbitrap MS	Thermo Scientific	726049
SecurityGuard cartridge for HILIC, 4 mm × 2.0 mm	Phenomenex	KJ0-4282
SeQuant ZIC-cHILIC 3µm,100Å 100 x 2.1 mm	Merck	1506570001
SeQuant ZIC-HILIC Guard Kit 20 x 2.1 mm	Supelco	1504360001
SPME LC fiber probes, C18	Supelco	custom order
SPME LC fiber probes, mixed Mode	Supelco	custom order
UltiMate 3000 HPLC systems	Thermo Scientific	5200.0345

water	Merck	1153331000
XSelect CSH C18 3.5µm 2.1x75mm	Waters	186005644
XSelect CSH C18 VanGuard Cartridge, 3.5 µm, 3.9x5 mm	Waters	186007813

Comments/Description
Mobile phase additive, LCMS grade
HPLC solvent, LCMS grade
Mobile phase additive, LCMS grade
Vortex mixer
Blue scrw tp,pre-slit PTFE/Si septa
software for data processing
Guard Column
HPLC Column
Mobile phase additive, LCMS grade
HPLC Column
HPLC solvent, LCMS grade
software for data processing
online software for statistical analysis (Chong, J., Wishart, D.S. and Xia, J. (2019) Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. Current Protocols in Bioinformatics 68, e86)
HPLC solvent, LCMS grade
Mass Spectrometer
Guard Column
HPLC Column
Guard column
comercial probes: 57281-U; probes used in the experiment were not needle assembled and were precut to the length described in the pr
HPLC system

HPLC solvent, LCMS grade
HPLC Column
Column cartidge

otocol

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Dear Dr. Bajaj

The authors would like to thank for your input and valuable comments which helped to improve our manuscript. All comments and suggestions have been addressed.

1. The editor has formatted the manuscript to match the journal's style. Please retain as use the attached file for revision.
We apologize for missing the formatting. Thank you for the correction.

2. Please address all the specific comments marked in the manuscript.
All comments have been addressed.
Answers to some specific questions/comments:

- The "fiber" is synonym of the "probe" or "device" and coating is a part of the probe consist of extraction phase immobilized on the fiber with biocompatible glue. We made a correction in the text to make it more clear for the reader.
- We have add more detailed description to sample prep part of the protocol. Regarding the instrumental analysis, it has been shortened based on the Reviewers' comments. They suggested to focus on sampling/sample prep part as this is the novelty and it can be followed by instrumental analysis already established in a given lab. Therefore, to find a compromise between yours and Reviewers' comments we have added main steps to the parts following steps related to sample preparation.
- In the previous submission to JoVE I have asked Benjamin Werth, who has invited me to submit our work to JoVE, about contribution. This is his reply: "There is not an explicit rule on where to include those in our template, but it is common to include author contributions in the 'Acknowledgements' section at the end of the manuscript. Things like co-first authors, equal contributions, etc. can be indicated with asterixis in the 'Authors and Affiliations" section." Therefore, I appreciate if you re-consider placing information about author's contribution to Acknowledgment. It is important to my PhD students, who include the article in their thesis.

3. Once done please ensure that the highlight is no more than 2.75 pages (no less than 1 page) including headings and spacings.
The steps in the protocols to be included in the video have been highlighted.

4. Please proofread the manuscript well before submission.
The manuscript has been proofread by the authors and professional English editor

5. Please cite the original work as mentioned by the reviewers wherever applicable.
The references have been added to the revised version as per Reviewers' suggestions.

6. Please sort the materials table in alphabetical order.
The Table has been modified as requested.

Sincerely,
Barbara Bojko, PhD, D.Sc.

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The permission for re-use of Figure 6 from your article: A new strategy for brain tumour metabolomic analysis published in Medical Research Journal 2018; 3 (1): 1–8 in Journal of Visualized Experiments (JoVE) has been granted.

Best regards

Marta Majerczyk