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ORIGINAL ARTICLE

Paulina Zofia Goryńska[1], Kamila Chmara[1], Krzysztof Goryński[1], Dariusz Paczkowski[2], Marek Harat[3,2], Barbara Bojko[1]

[1]Department of Pharmacodynamics and Molecular Pharmacology, Faculty of Pharmacy, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland.
[2]Department of Neurosurgery, 10th Military Research Hospital, Bydgoszcz, Poland
[3]Department of Public Health, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland

A new strategy for brain tumour metabolomic analysis

Corresponding author:

Barbara Bojko
Zakład Farmakodynamiki
i Farmakologii Molekularnej,
Collegium Medicum UMK,
ul. dr. A. Jurasza 2
85-089 Bydgoszcz, Poland
e-mail: bbojko@cm.umk.pl

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ABSTRACT

Introduction: Nowadays, diagnosis of brain tumours is mainly carried out via neuroimaging techniques. The most widespread methods for routine analysis include computer tomography and magnetic resonance imaging. While such methods are useful to localise tumours, they are unable to offer a conclusive diagnosis of the tumour type. A final diagnosis can only be made via a histological examination of tissue after tumour resection, or, in cases where the location of the tumour is not amenable to resection, after a biopsy of the tumour is carried out. Untargeted metabolite analysis is a relatively new approach to diagnostics, capable of establishing wide characterisation of endogenous metabolites of a given system, a method that can be applied to improve identification of tumour types via biomarker discovery. In this regard, sample collection and preparation can be said to be the most important step in metabolomic studies.

Material and methods: In the current study, a solid phase microextraction (SPME) protocol for metabolomics, which has been successfully applied towards metabolite analysis in various biological materials in the last few years, was optimised for brain tumour tissue metabolomic analysis. In the current study, the described approach was applied to human brain tumours. Aiming to incur minimal tissue damage, the probes used for sampling were of diameter ca. 0.2 mm. Aiming to optimise the method towards enhanced recovery of the extracted metabolites, various desorption solvents were tested in an optimisation study. The final protocol was used for analysis of a pilot cohort of patients with glioma and meningioma tumours.

Results: The results showed that a protocol where chemical biopsy was performed directly from resected tumour with 7-mm-long coating SPME probe and desorption was done using 0.3 mL of a mixture of acetonitrile and water 80:20 v/v was superior to other tested protocols. The optimised method allowed for successful differentiation between the two types of brain tumours studied: meningioma and glioma. Despite the relatively small cohort group involved in the study, several compounds were tentatively identified as statistically significant metabolites responsible for this differentiation.

Conclusions: The presented preliminary data demonstrate a potential of the proposed method as a low invasive diagnostic tool for on-site analysis.

Key words: brain tumour, solid phase microextraction, metabolomics, mass spectrometry

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Introduction

Despite the considerable scientific progress made in the neuro-oncology field within recent decades, brain tumours continue to carry a high rate of mortality and morbidity, with many types of brain tumour still considered to be incurable and terminal. For instance, despite undergoing radiation therapy, the average life expectancy of patients diagnosed with glioblastoma multiforme, the most aggressive type of brain cancer, is only 12.1 months. [1] While the first classification of brain tumours was established in 1979, remaining unchanged for nearly 10 years, revised classifications have since been introduced owing to the significant amount of research carried out on the central nervous system and its diseases, including tumours [2]. While initial classifications were based on the histological origin of a given type of cancer, histology-based diagnoses have since been shown to not always match the clinical profile of a given cancer [3]. Because the

correct classification and diagnosis of tumour type and subtype play a very important role in the prediction of response to a given treatment, significant scientific interest has been paid to the identification of molecular biomarkers of brain tumours, which can be used to complement histological testing, and thus aid in more accurate diagnoses. To that extent, the genotyping and identification of certain mutations have already been proven to be a promising prognostic tool in the treatment of gliomas [3], one of the most widespread types of central nervous system tumours, constituting approximately 60% of all primary brain tumours [4,5]. However, the genotype of an individual is not the only biological determinant of a tumour; for instance, factors such as hyperglycaemia or elevated BMI may also predispose patients to the development of glioma [4, 5]. To this end, metabolite profile determinations aid in the identification of the phenotype of a given tumour, which reflects all factors that influence tissue biochemistry, including environmental, demographic, physiological, and pathological factors, among others [6]. Owing to the development of new analytical methods such as nuclear magnetic resonance (NMR), spectroscopy, and mass spectrometry (MS), today scientists are able to gain better insight into the biochemical secrets of the human body that trigger diseases, including the ones associated with the growth and progression of brain tumours [5]. Because gliomas constitute some of the most common and malignant brain cancers, elucidations of the metabolomic profiles of gliomas may broaden the therapeutic options for these types of tumours. The relatively low response of brain tumours to treatment, including that of gliomas, poses an immense challenge for clinicians and researchers. Today, implementation of radiotherapy coupled with temozolomide is the most prescribed course of treatment for many brain tumours; however, considering the still high morbidity and mortality of brain tumours, further broadening of scientific knowledge regarding different types of brain tumours, as well as their biochemical foundation, can contribute to the development of more effective treatments targeted at specific tumour types, which will in turn contribute to increased chances of survival for patients afflicted by such conditions. [7]. In addition, the timeframe of analysis also plays an important role in improved diagnosis of brain tumours; in many cases, surgeons depend on intraoperative results for diagnosis, which in some cases, like histological testing, might become obsolete. In view of the lack of sufficiently fast and precise diagnostic tools available to date, further development of real time or on-site rapid diagnostic tools is needed to guide medical personnel towards optimal diagnosis and course of treatment [8]. Certainly, fast analysis protocols would enable doctors to make immediate decisions regarding the course of treatment, which would in turn contribute to an increased chances of therapeutic success.

Solid phase microextraction (SPME) is an analytical platform with demonstrated capability, having shown great potential towards tissue analysis applications, including both *ex vivo* and *in vivo* analyses. The integrated sampling and extraction process is characterised by minimum invasiveness and lack of sample consumption because SPME can be applied to extract molecules directly from intact tissue in a negligible manner [Fig. 1]. Owing to these features, following SPME sampling, the biopsy or resected tissue can be used for further analysis, i.e. histological testing or genotyping [9]. The most common geometry of the SPME device is the fibre, which can be described as a metal alloy wire with a dimension of ca. 0.2 mm, which consists of a tip coated with an extraction phase. The device is made of biocompatible materials that prevent adhesion of biological materials to the fibre, while also averting contamination of the sample, which is particularly crucial in living tissue applications. Detailed information regarding the technology and its utilisation in bioanalysis can be found elsewhere [10–13]. In the present study, the development of a method allowing for *in situ* and *in vivo* metabolic profiling of brain tumours is presented. Here, an evaluation of the feasibility of the method to distinguish the tumour type based on the metabolic phenotype was carried out via analysis of sets of menin-

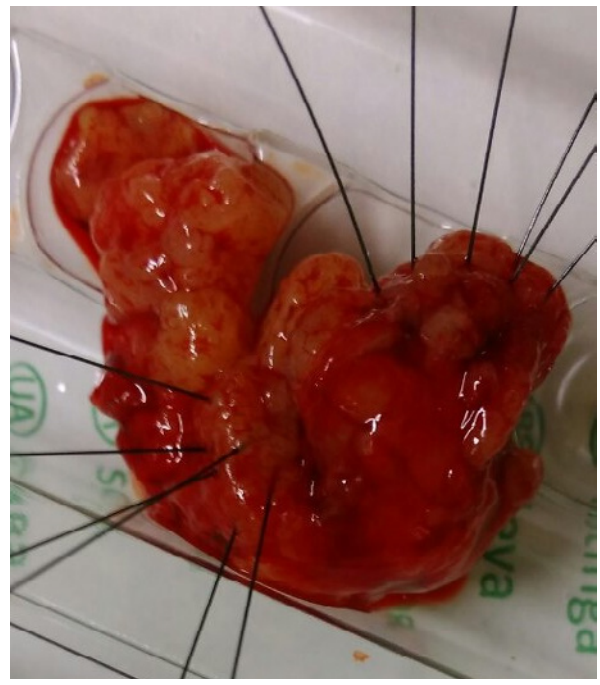

Figure 1. SPME extraction from a tumour, performed immediately after resection

gioma and glioma tumours, with results demonstrating the capability of the method to differentiate tumours based on their phenotype.

Materials and methods

The study was approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz at Nicolaus Copernicus University in Torun (KB 628/2015). Sample preparation was performed via SPME in fibre format. The coating of fibres used in this experiment consisted of a mix of C18 and strong cation exchange (SCX) particles. Coatings were preconditioned in 1.5 ml methanol/water solution 50/50, v/v for one hour before extraction. Next, the fibres were statically washed in 1.5 ml water for five seconds, and subsequently introduced to resected brain tumour tissue (Fig. 1).

The static extraction process was executed for 30 minutes, followed by a five-second static wash step with water. To select the optimum conditions, the desorption step was carried out with the use of seven desorption solvent mixtures, as follows: acetonitrile/methanol/water, 25/25/50, v/v/v (solution #1); acetonitrile/methanol/water, 50/25/25, v/v/v (solution #2); acetonitrile/methanol/water, 25/50/25, v/v/v (solution #3); acetonitrile/water, 80/20, v/v (solution #4); acetonitrile/water, 80/20, v/v further diluted with acetonitrile 50:50 v/v (solution #4.1; a reference solution, used in the previous metabolomics analysis with SPME [12]); acetonitrile/water, 20/80, v/v (solution #5); acetonitrile/water, 50/50, v/v (solution #6). The volume of desorption solvent was 0.3 mL in each case. The desorption process was continued for 120 min with agitation set at 1200 rpm, using a BenchMixer™ MultiTube Vortexer (Benchmark Scientific, Edison, USA). The LC–MS grade chromatographic solvents methanol, acetonitrile, water, and formic acid, as well as ammonium acetate used for buffer preparation were purchased from Sigma-Aldrich (Poznań, Poland).

LC-MS/MS analysis

Experiments were carried out on a Q Exactive Focus Orbitrap (Thermo Fisher Scientific) coupled to a Dionex UHPLC system. Chromatographic separation was carried out with the use of a hydrophilic stationary phase HILIC column (Luna HILIC 100 mm x 2.0 mm, 3 μm, Phenomenex) as well as in reversed-phase using a pentafluorophenyl (PFP) column (Discovery HS F5 100 x 2.1 mm, 3 μm, an in-kind gift from Supelco, Bellefonte, PA, USA). The flow rates were 400 μl min^{-1}and 300 μl min^{-1} for HILIC and PFP columns, respectively. The chosen mobile phases for the reversed phase column were water with formic acid (99.9:0.1; v:v) (A) and acetonitrile with formic acid (99.9:0.1) (B), while mobile phases for the HILIC column consisted of acetonitrile with 20 mM ammonium acetate buffer (90:10, v:v) (A) and acetonitrile with 20 mM ammonium acetate buffer (50:50; v:v) (B). The injection volume was 10 μl. The gradients of both methods used in this study were adopted from Vuckovic et al. [14]. Analyses were performed in positive and negative electrospray ionisation modes, with total run times per sample of 20 and 40 minutes for the HILIC and PFP methods, respectively. Processing and data acquisition were performed on a Compound Discoverer 2.1, an in-kind contribution from Thermo Fisher Scientific.

Statistical analysis was performed using Principal Component Analysis (PC) and ANOVA; the P-value adjustment was calculated using the Benjamini-Hochberg procedure to establish the false-discovery rate.

Results

In SPME applications, the selection of an appropriate extraction phase and desorption solvent plays a large role in the attainment of adequate extraction efficiency. In this study, a mix-mode extraction phase was selected based on its previously reported superior performance for metabolomic analysis [15]. The current work also included a desorption solution optimisation study aimed at a performance comparison of seven solvents, utilising factors such as signal intensity and number of detected features on each LC column, in positive and negative ionisation modes, as performance parameters. As can be seen in Fig. 2, analyte coverage varied at different desorption conditions. The lowest number of features appeared on the HILIC column in both positive and negative modes when desorption solutions 1 and 5 were used (56 and 38; 49 and 46, respectively, for positive and negative modes). The same trend was observed for desorption solutions 1 and 5 for the PFP column, in both ionisation modes (132 and 95; 145 and 88, respectively).

Among the compared solutions, the largest number of features detected by both columns and in both ionisation modes was observed for ACN/H_2O, 80/20 as the desorption solvent. The number of features detected by the HILIC column in positive and negative ionisation modes were 137 and 119, respectively, and analogously 394 and 149 for the PFP column in positive and negative ionisation modes, respectively. Furthermore, this desorption solution significantly increased peak areas and, consequently, the sensitivity of the method as compared to the other tested solvents. Particularly, for the reversed phase method with the PFP column, employment of this desorption solution yielded almost

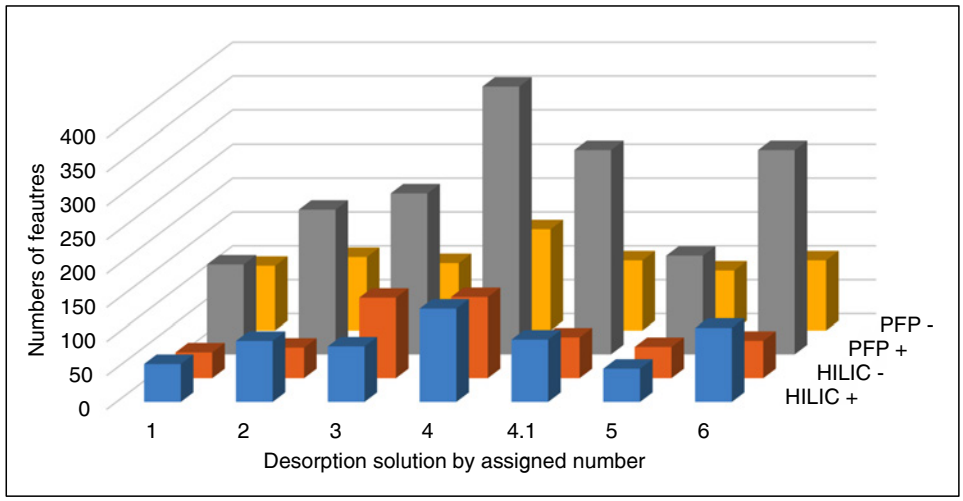

Figure 2. Comparison of number of features detected by HILIC and PFP column in positive and negative ionisation modes for six desorption solution mixtures (#1 ACN/MeOH/H_2O, 25/25/50, v/v/v; #2 ACN/MeOH/H_2O, 50/25/25, v/v/v; #3 ACN/MeOH/H_2O, 25/50/25, v/v/v; #4 ACN/H_2O, 80/20, v/v; #4.1 ACN/H_2O, 50/50, v/v further diluted with ACN 50:50 v/v; #5 ACN/H_2O, 20/80, v/v; #6 ACN/H_2O, 50/50, v/v)

twice as many compounds as compared to that attained via the other tested solutions [Fig. 2]. The S-plot shows the multiplicity of features, differentiating the tested desorption solvents as well as denoting which compounds detected by the selected desorption solvent go otherwise undetected when employing any of the other five desorption solutions. Figure 3 includes a Box-Whisker chart of one such discriminating compound marked in the S-plot [Fig. 2], as well as the chromatograms for this compound obtained for different desorption solvents.

Analyte coverage and intensity of extracted endogenous compounds for HILIC and PFP methods are presented in Figures 4 and 5, respectively.

Following protocol optimisation, pilot studies were performed on resections of tumours of patients with glioma (HGG) and meningioma tumours (n = 11 and 17, respectively). The principal component analysis plot shown in Figure 6 demonstrates clear separation of data for the two patient groups.

Discussion

Solid phase microextraction, which can be described as an equilibrium-based, non-exhaustive sample preparation method, has been successfully demonstrated for untargeted metabolomics and *in vivo* studies [16–17]. However, as the diversity of the tissues, their heterogeneity, as well as the biochemistry of the given tissue can influence method performance, additional tuning of some parameters in the general protocol might allow for an increase in detection limits, as well as aid in the attainment of superior results for chromatographic measurements. For this purpose, additional efforts were expended to optimise the SPME protocol proposed by Risticevic et al. towards the currently discussed application [18]. In the present study, a straight-forward strategy was applied to prepare a final protocol that enables the detection of more endogenous metabolites in comparison to that afforded by the originally proposed method, which in turn should enhance differentiation of various types of brain tumours. The goal of the optimisation study carried out in this work included the selection of a desorption solution that could be directly subjected to both types of chromatographic modes: HILIC and reversed phase. Furthermore, the optimisation parameters included selection of a suitable solution that dispensed with the need for extract dilution with acetonitrile, a procedure often carried out to make the extractant more compatible with the HILIC column, which requires a higher content of organic solvent to obtain acceptable peak shapes. Such a dilution significantly affects overall sensitivity and, consequently, the quality of the results. Conversely, increasing the organic content and compromising water content could potentially affect the recovery of polar compounds. As such, method optimisation necessitated a careful and comprehensive comparison of various desorption solutions.

As expected, diluted extracts yielded a much lower number of detected molecular features, as can be seen in Fig. 2. In HILIC separation, more polar compounds elute later and reverse, and fewer polar compounds elute at the beginning of the chromatographic run. As

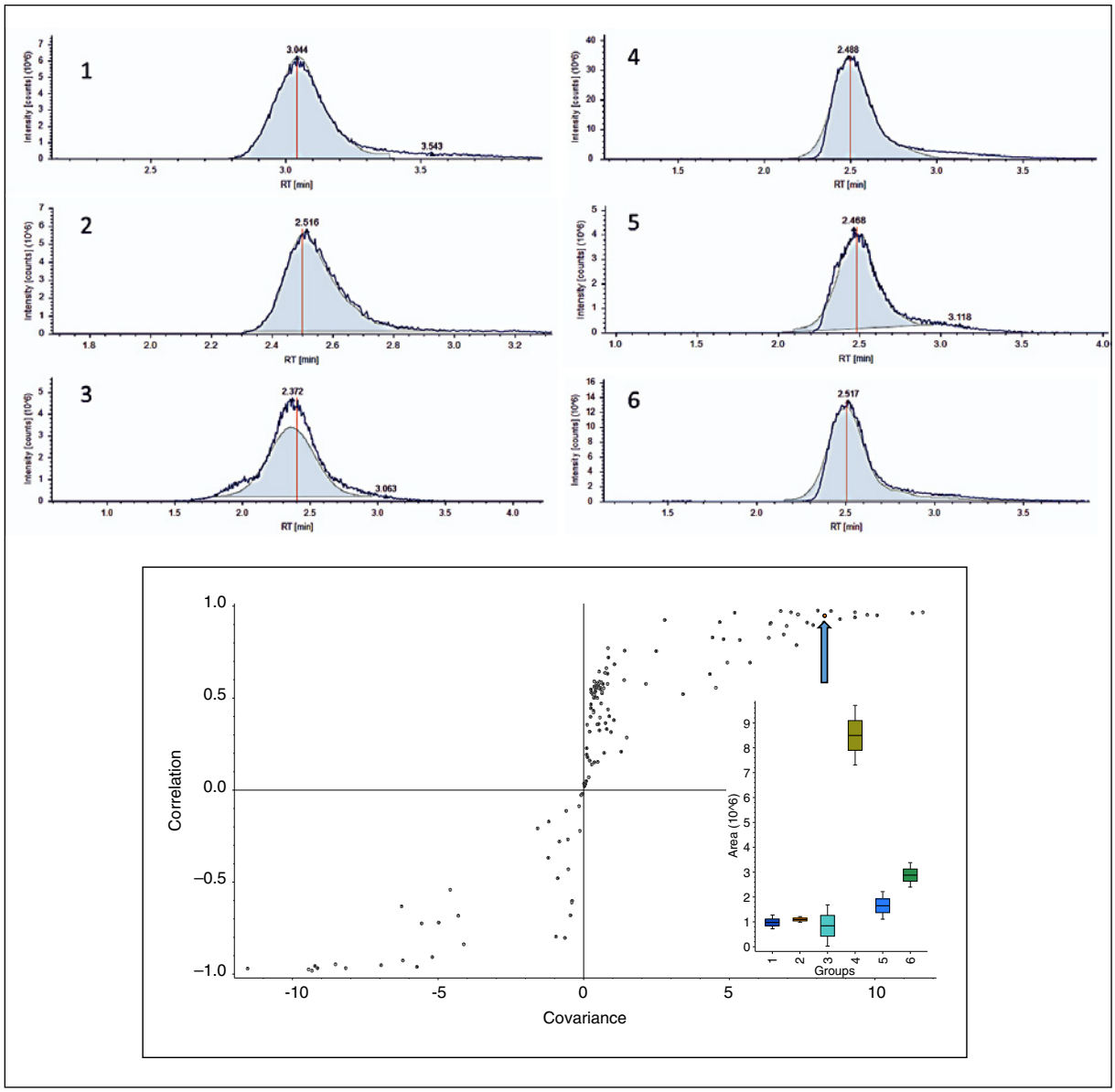

Figure 3. Endogenous compound m/z 268.0808. Top panel: Chromatograms of signal intensities for the selected compound for the six different mixtures of desorption solutions on the HILIC column in negative ionisation mode (#1 ACN/MeOH/H_2O, 25/25/50, v/v/v; #2 ACN/MeOH/H_2O, 50/25/25, v/v/v; #3 ACN/MeOH/H_2O, 25/50/25, v/v/v; #4 ACN/H_2O, 80/20, v/v; #5 ACN/H_2O, 20/80, v/v; #6 ACN/H_2O, 50/50, v/v). Bottom panel: S-plot and Box-Whisker chart for selected compound on HILIC column in negative ionisation mode

demonstrated in Fig. 4, higher content of water in the mobile phase resulted in lower sensitivities, even in cases where the extract injected on the column was not diluted. Addition of acetonitrile, on the other hand, increased not only the detectability of fewer polar compounds, but also of compounds that eluted at the end of the chromatographic gradient, i.e. most polar species. While results from the PFP column did not yield such pronounced differences between subsequent desorption solvents as those attained for the HILIC column, they nonetheless corroborated the confirmed superior performance of the ACN/H_2O 80:20 (v/v) solution as desorption solvent. As such, the ACN/H_2O 80:20 (v/v) solution was selected as desorption solvent for the final protocol.

Validation of the clinical applicability of the method was carried out using a relatively small patient cohort; nonetheless, effective separation between groups was achieved, based on the two first principal components (PC1 22% and PC2 12.4%). Although the small size of the studied cohort impedes the establishment of conclusive findings of a biological or clinical nature,

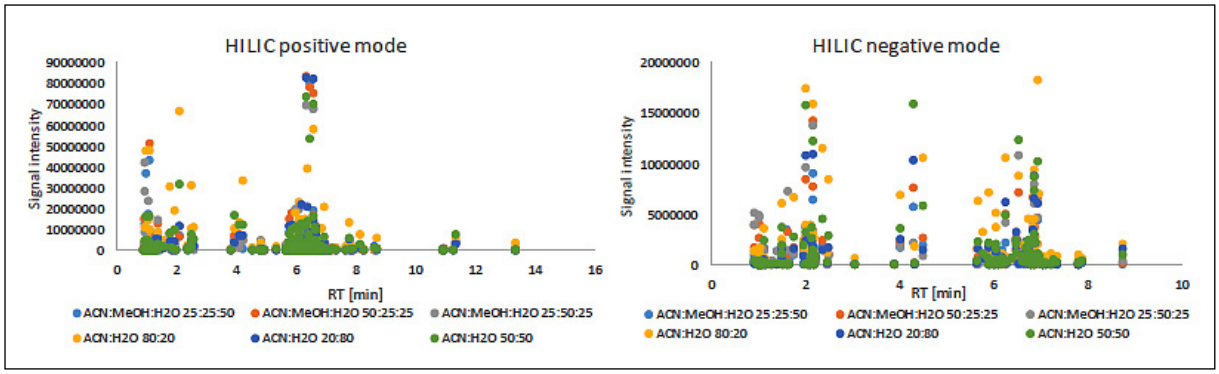

Figure 4. Comparison of analyte coverage for the six desorption solvents used, with separation carried out on HILIC column.

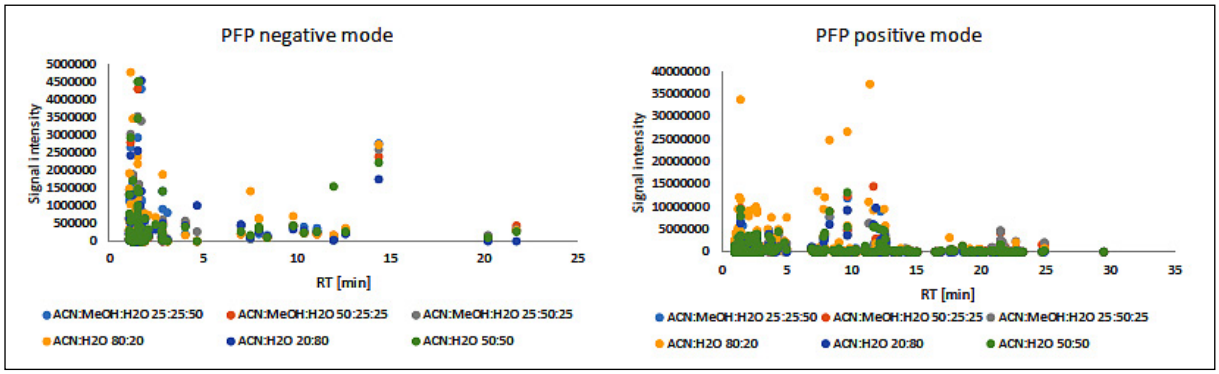

Figure 5. Comparison of analyte coverage for the six desorption solutions, with separation carried out on the PFP column.

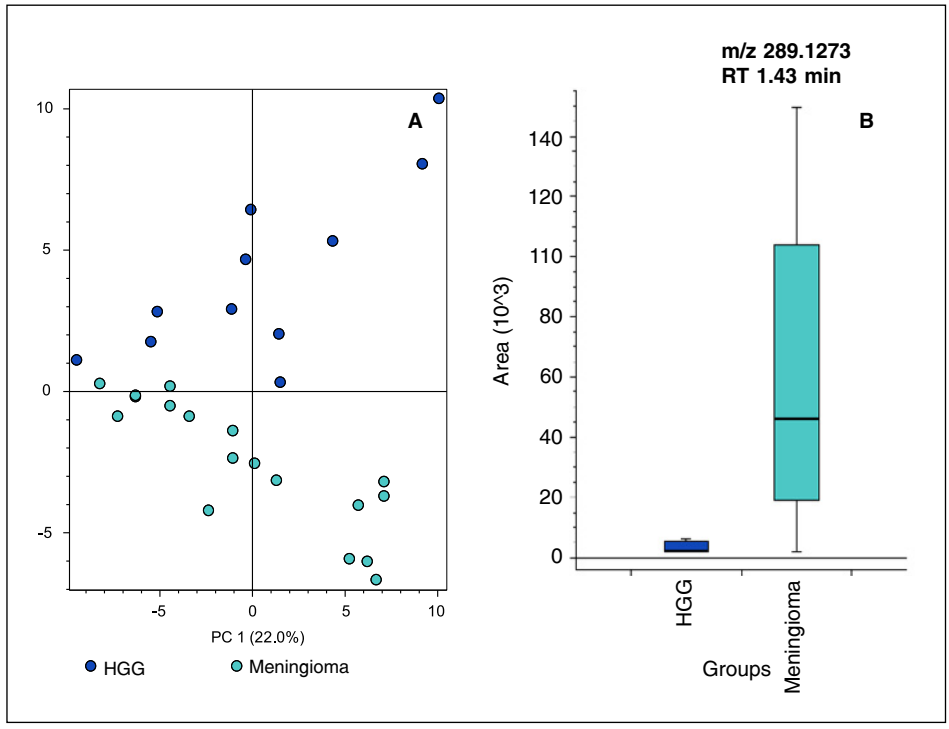

Figure 6 A. Principal component analysis showing differentiation of meningiomas (light blue) and gliomas (dark blue); **B**. example of a discriminant compound for glioma and meningioma differentiation, putatively identified as ophthalmic acid

the obtained results nonetheless revealed several compounds as possible biomarkers of these tumours. To demonstrate the applicability of the proposed approach for further biomarker studies, an example of one such discriminative metabolite is presented in Fig. 6. Identification of this compound was performed based on its accurate mass via a comparison of experimental data with databases included in the Compound Discoverer 2.1 software (which includes the Human Metabolome Database and ChemSpider, among others). This compound, tentatively identified as ophthalmic acid, has been previously reported in the literature as a biomarker of oxidative stress [19]. In the current study, this compound was shown to be present in distinctively higher levels in meningioma tissue. The proposed analytical protocol is currently being used in further studies based on a larger cohort of patients with the aims of finding potential biomarkers of high- and low-grade gliomas, as well as establishing different phenotypes of gliomas.

Conclusions

The aims of the current study were to improve the metabolomics sample preparation protocol in terms of increasing desorption efficiency, and to validate the protocol applicability for metabolomic analysis, particularly aimed at the discovery of biomarkers of different brain tumours. According to the experiments performed in the current study, the use of SPME fibres for extraction, use of the optimised desorption solution, and separation via high-resolution mass spectrometer provided an appropriate workflow for comprehensive brain tumour metabolomic analysis, using both reversed phase and HILIC chromatography in positive and negative ionisation modes. The evaluation clearly demonstrated that analyte coverage, in particular when using the HILIC method, was improved in comparison to that achieved via the previously described protocol proposed by Risticevic et al., from which the optimised protocol was adapted. The currently proposed optimised protocol increases the probability of finding unique biomarkers of tumours in future studies. To that end, the proposed strategy was applied in a validation study to find specific endogenous metabolites capable of differentiating between various types of brain tumour tissues, with clear separation attained between groups.

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Statement of competing interests: The authors declare no conflict of interests.

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