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A High-Throughput Method for Targeted Metabolomics Analysis of Different Tissue Samples using the Absolute*IDQ*™ Kit

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Introduction

In recent years technology improvements have greatly enhanced the field of metabolomics and nowadays hundreds of endogenous metabolites can be quantified in body fluids like plasma and serum in a high-throughput manner using targeted metabolomics approaches [1-3]. Accurate and reproducible determination of metabolite concentrations in tissue samples are of high importance to characterize animal models and to identify metabolic changes that occur in different tissue types in specific diseases. However, the extraction of metabolites from tissue is often one of the most labor intensive steps for metabolomics studies.
Here we report the development of a high-throughput method allowing the rapid extraction of metabolites from multiple tissue samples. The method involves a bead-based homogenizer in combination with a simple extraction protocol and the use of the Absolute*IDQ*™ Kit. The Absolute*IDQ* kit was originally validated for plasma and can simultaneously identify and quantify a large number of endogenous metabolites [4]. The metabolite panel of the kit includes acylcarnitines, amino acids, hexose, glycerophospholipids, and sphingolipids - metabolites with significant different lipophilic and hydrophilic properties. Therefore, different extraction solvents were tested, and both reproducibility as well as suppression effects were evaluated for a range of different animal tissue types including liver, kidney, muscle, brain, and fat tissue.

Materials and Methods

The best starting material for tissue metabolomics are small pieces of tissue snap-frozen in liquid nitrogen to quench the metabolism. Most commonly, the break up of tissue is achieved by grinding frozen tissue to powder using a liquid-nitrogen cooled pestle and mortar or other devices and the subsequent extraction from this powder. We used a Precellys 24 bead-based homogenizer (PEQLAB Biotechnology GmbH, Germany) which has recently been described [5]. It can homogenize and extract up to 24 samples in parallel and has already been applied to metabolomics applications [6]. Each tissue sample is placed into individual sealed tubes containing ceramic beads. The extraction solvent is added and the homogenization is achieved in a short time (30-60 s) by a fast multidirectional motion. Cooling during homogenization is accomplished by a cooling adaptor which blows cold air (-50 °C) around the tubes.

Sample preparation

Male mice (C57Bl/6J), aged 41 weeks, were narcotized and all available blood was taken (retro orbital). The mice were sacrificed and the abdominal skin was moistened with 80 % ethanol, opened and pulled aside. Then, the abdomen was opened and the abdominal aorta and the inferior vena cava were cut through in a way that blood contact with organ surfaces was minimal. Organs were dissected as quickly as possible, carefully patted with lint free tissue paper, cut into appropriate pieces, and placed into pre-labelled tubes. Tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C until extraction. The frozen bovine tissues (F2 Charolais x German Holstein) were obtained from the Research Institute for the Biology of Farm Animals (Dummerstorf, Germany).

Table 1: Overview of analyzed animal tissue types

Species	Tissue	µL solvent/ mg tissue
Mouse	Liver	6
Mouse	Kidney (without capsule)	6
Mouse	Muscle (*M. biceps femoris*)	3
Mouse	Fat (visceral)	3
Mouse	Brain (*cerebrum*)	3
Bovine	Liver	6
Bovine	Muscle (*semitendinosus*)	3
Bovine	Fat (subcutaneous)	3

The frozen tissue samples (20-200 mg) were placed into pre-cooled (dry ice) 2 mL homogenization tubes containing ceramic beads (1.4 mm diameter). The different pre-cooled extraction solvents were added (3-9 µL/mg tissue), and the tissue was homogenized using a Precellys 24 homogenizer equipped with an integrated cooling unit. The settings applied to all tissue types were: three times 20 s, 5500 rpm, with 30 s pauses between the homogenization steps. The tubes were subsequently centrifuged for 5 min at 10.000 x g and 10 µL of the supernatants were loaded onto the 96-well kit plate.

The Absolute*IDQ* kit was prepared as described in detail in the User Manual. The extracts were analyzed using a API 4000™ triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex). The standard flow injection method of the Absolute*IDQ* kit comprising two 20 µL injections (one for positive and one for negative detection mode) was applied for all measurements. Quantification was achieved by multiple reaction monitoring (MRM) detection in combination with the use of isotope-labeled and other internal standards.

Results and Discussion

To develop a broadly applicable method, a selection of commonly used tissue types was tested. Tissue types and animal species are listed in Table 1. Several parameters were evaluated in different experiments. For better comparison, analyzed tissue samples were mostly obtained from the same animal.

Reproducibility of homogenization method

The reproducibility of homogenization using the Precellys 24 homogenizer was tested by performing three independent homogenizations using tissue samples of the same animal. Each homogenate was analyzed three times resulting in nine technical replicates

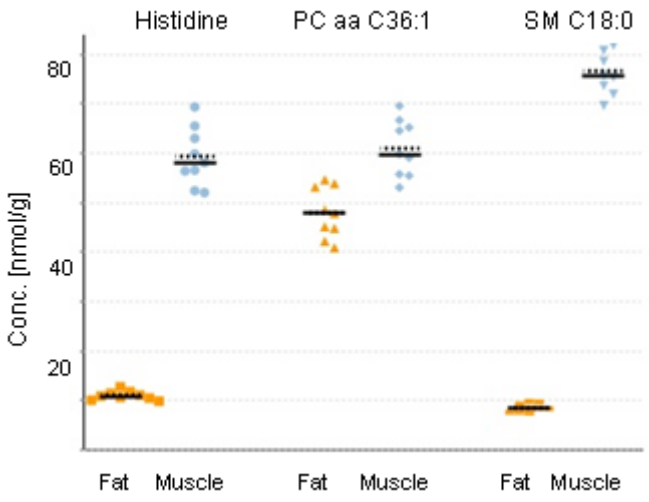

***Figure 1**: Scatter Plot of technical replicates. Three independent homogenizations using tissue samples of the same animal are shown (each extract loaded on three wells). Example results are shown for the amino acid histidine, one phosphatidylcholine (PC aa C36:1) and one sphingomyelin (SM C18:0). Methanol was used for tissue extraction. The variances for bovine liver tissue were similar (not shown).*

In addition, the experiment was performed using three different tissue types (bovine fat, bovine muscle, bovine liver) to assess potential differences due to the different textures of the tissues. Figure 1 shows the values of the nine replicates for some metabolites from different compound classes. It can be seen that results obtained for independent homogenization experiments are very similar to each other and fall in the range of the method's general variance. Hence, a very reproducible homogenization can be achieved by using this method.

Extraction Solvents

The Absolute*IDQ* kit has been validated for plasma. During preparation, 10 µL of plasma is loaded on a filter paper of the kit plate and dried in a stream of nitrogen. Extraction of the metabolites is then achieved using methanol containing 5 mM ammonium acetate. Therefore, methanol was also the first choice for our tissue experiments, but other solvents were also tested: ethanol, 10 mM phosphate buffer (pH 7.5), different mixtures of ethanol and methanol with 10 mM phosphate buffer, and a mixture of ethanol/dichloromethane. The often used methanol/chloroform/water extraction [6] method was not tested, since this method results in a biphasic separation where each fraction needs to be dried separately. Our aim was a method suitable for high-throughput applications thus avoiding any phase separation and drying steps. The different extraction/homogenization solvents were tested by analyzing replicates (n = 6) for all tissues and by comparing yield and CVs for all metabolite classes.
The results revealed that there is no solvent that works best for all kinds of tissues and all metabolite classes. Not all tissues behaved similarly with regard to the different extraction solvents however, for the majority of the metabolites to be analyzed with the kit (phosphatidylcholines, lyso-phosphatidylcholines, sphingomyelins and acylcarnitines) methanol gave the best overall results. For the amino acids, carnitine and for hexose, the use of 10 mM phosphate buffer resulted in clearly higher yields. In Table 2, yields of the extraction with methanol in comparison to phosphate buffer are shown for amino acids, hexose and carnitine. Although some general trends are seen, it is also evident that the yield depends on tissue type. The other tested solvents, like mixtures of ethanol or methanol and phosphate buffer (85/15 or 70/30 v/v), did not result in higher yields for the amino acids compared to the methanol extraction, but gave lower yields for many lipids.
For most tissue types, 3 µL solvent per mg tissue was used, but for liver and kidney 6 µL per mg tissue gave better results. By using only 3 µL solvent for liver and kidney stronger ion suppression effects were observed. For better comparison, the values in the figures of this application note have been normalized to nmol/g tissue - the simplified assumption was made that 1 mg frozen tissue is equivalent to a volume of 1 µL.

Table 2: Extraction yield [%] using methanol in comparison to phosphate buffer *(The medians of the concentration values obtained in 10 mM phosphate buffer were set to 100 %).*

Metabolite	Liver	Kidney	Muscle	Brain	Fat
Carnitine	32	62	126	57	93
Arg	n. d.*	10	48	25	11
Gln	58	27	96	105	49
Gly	60	39	89	59	43
His	49	23	79	63	31
Met	26	23	77	57	30
Orn	6	17	45	75	21
Phe	32	18	68	47	25
Pro	58	11	83	48	37
Ser	18	11	76	83	26
Thr	19	17	77	65	45
Trp	82	54	95	94	80
Tyr	32	24	76	57	40
Val	48	24	75	53	30
xLeu	38	20	75	36	24
Hexose	52	75	39	87	103

* *n.d. – not determined*

Internal standards / Ion suppression effects

The internal standards (IS) of the kit are essential for quantification. Therefore, the signal intensities of the MRM pairs of the IS in tissue samples were compared to the values obtained for human plasma and to the values of the zero samples. The zero samples are used to calculate the limit of detection (LOD). LOD is defined by the median value of the zero samples multiplied by three. The preferred extraction solvents methanol and 10 mM phosphate buffer were applied as zero samples. The comparison provides information about ion suppression effects in tissue samples. The observed suppression was dependent on the analyte class. All IS for acylcarnitines, glycerophospholipids and sphingolipids in the tissue samples gave higher intensities compared to plasma samples, but lower intensities than the zero samples. A significant increase of intensities of a specific MRM pair compared to the zero samples would indicate potential interferences of unknown compounds in the tissue extract, but such an effect was not observed for any IS in the tissue samples.
For amino acids, the intensities of the IS were mainly in a similar range as in plasma. However, for some of them intensities were considerably lower both in phosphate buffer and in methanol. Especially the IS for the amino acids arginine (Arg) and ornithine (Orn) showed significantly lower values. Therefore, the obtained results for these two amino acids in tissue samples should be evaluated with caution. In case of liver tissue the MRM pair of the internal standard for Arg exhibited unusually high ion suppression and thus the results for this metabolite cannot be used.

Coefficient of variation (CV)

For the analyzed tissue types, the intra-day CVs of all metabolites above LOD were compared. For the acylcarnitines, amino acids and hexose, all CVs were below 15 %. The CVs of several phosphatidylcholines and lysophosphatidylcholines were above 20 %. This was not consistent with all tissue types. Therefore, when starting with tissue experiments it is recommended to perform some preliminary tests using identical tissue samples (n = 6) to evaluate the CVs for the given tissue and preparation.

Comparison of different tissue types

Here some general remarks are given regarding the metabolomics analysis of different tissues. In Figure 2 and 3, concentration values of some representative metabolites in the five different mouse tissues are shown. It can be seen that the concentration ranges of the chosen metabolites vary considerably in different tissues.

Amino acids

For most of the amino acids, highest concentrations were found in liver and kidney tissue, and lowest concentrations were found in fat tissue, as shown below for phenylalanine. One exception was glutamine, for which the concentration was highest in brain tissue (Figure 2).

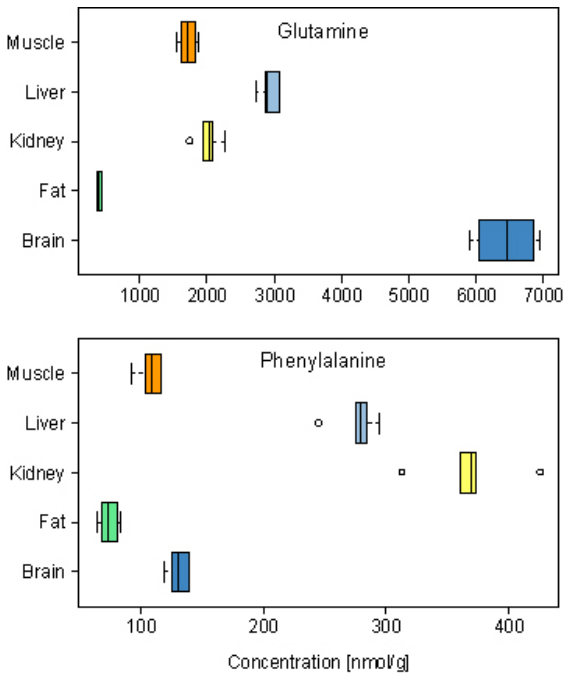

Figure 2: *Box plots showing concentration values of the amino acids glutamine and phenylalanine in different mouse tissues. The median value is represented as a solid line (n=6).10 mM phosphate buffer was used as extraction solvent.*

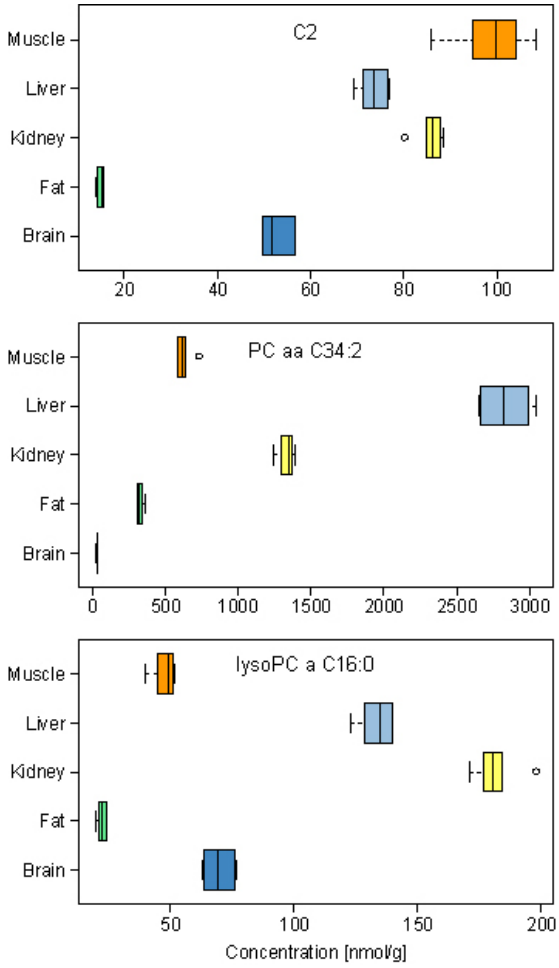

Figure 3: *Box plots showing concentrations of selected metabolites in different mouse tissues. The median value is represented by a solid line (n=6). Methanol was used as extraction solvent.*

Acylcarnitines

In general, a high number of acylcarnitines were above LOD in the investigated tissue samples with the exception of fat tissue, in which only few of them were above LOD. For carnitine and acetylcarnitine (C2), the highest concentrations were found in muscle tissue (Figure 3). In bovine muscle, the concentrations were much higher compared to mouse muscle and lay in the µmol/g range. If concentrations were outside of the evaluated quantification ranges (defined in the Analytical Specifications for plasma), such values would be considered as semi-quantitative. Alternatively, dilution experiments can be performed to test if the assay is linear up to very high concentration values. This has been tested for bovine muscle tissue and revealed linear behavior for both carnitine and acetylcarnitine up to µmol/g level.

Lipids

Generally, the concentrations of phosphatidylcholines, lyso-phosphatidylcholines and sphingomyelins were highest in kidney and liver tissue as shown for two representative examples in Figure 3. However, there were several exemptions, in which certain lipids were higher in brain, e.g. SM 18:1, lyso PC C18:1, or muscle tissue, e.g. PC aa C32:0, PC aa C32:2, PC ae 38:0. The concentrations of many lipids were lowest in fat tissue since most of the lipids in fat are stored in the form of triglycerides, a class of metabolites that is not analyzed by this kit.

Conclusions

The data presented in this application note demonstrate that targeted metabolomics analysis of tissue samples can be performed with the Absolute*IDQ* kit in a reproducible and high-throughput manner. The metabolite panel in the kit, originally validated for plasma, is also well applicable to tissue samples. In the investigated tissue types, the majority of the metabolites were found in concentrations clearly above LOD and such data should give an excellent overview of the metabolic status in different tissues. We recommend using methanol as extraction solvent for tissues due to the best results for most kit metabolites. In cases where the yield of amino acids/hexose is of high interest a second separate tissue extraction using 10 mM phosphate buffer should be applied.

References

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