

Analysis of Fenfluramine and Norfenfluramine in Mouse Brain and Cerebellum by Liquid Chromatography Tandem Mass Spectrometry using a Novel Solid-Supported Liquid Extraction

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This study outlines the application of a new synthetic supported liquid extraction (SLE) sorbent for the quantitative determination of the antiepileptic drug fenfluramine (FNN) and metabolite, norfenfluramine (NFNN), in mouse brain by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Additionally, a comparison of the synthetic SLE sorbent with diatomaceous earth (DE) was conducted, wherein the synthetic SLE sorbent eliminated a greater content of phospholipid while demonstrating higher analyte recovery with improved reproducibility. The validated method supported a two-fold dynamic range with a limit of quantitation (LOQ) of 0.05 µg/g in mouse brain, demonstrated acceptable calibration curve linearity ($r^2 > 0.99$), intra- and inter-run precision (C.V. < 10%) and accuracy (100 ± 10%), and matrix effect (100 ± 10%).

Introduction

LC-MS/MS has been widely adopted for the high throughput quantitative bioanalysis of small molecules, attributed mainly to the high selectivity, sensitivity, and sampling frequency of the approach. However, even with highly selective analyte monitoring, LC-MS/MS analysis can be deleteriously impacted by changes in ionisation efficiency due to coeluting matrix components such as salts, proteins, lipids (including phospholipids) and other various organic molecules. Such ionisation effects can influence the achievable limit of quantitation (LOQ), method reliability and reproducibility, chromatography and MS source contamination [1]. Therefore, sample preparation is required not only to extract target analytes from matrix, but also to remove unwanted components potentially impacting ionisation efficiency - the latter is often referred to as the matrix effect. The use of appropriate sample preparation techniques is dependent upon the complexity of the matrix, requirements for the detection of target analytes, and the selected instrument detection method. It is understood that sample preparation can

be both time-consuming and costly, but these are the unavoidable factors in order to gain reliable quality analytical results, and to preserve high-value instruments from damage.

Sample preparation products for bioanalysis are often based on a 96-well plate format, which allows automated / semi-automated simultaneous sample processing, thereby supporting the preparation of a large number of samples aligned with high throughput LC-MS/MS analysis. The format also accommodates the relatively small sample sizes associated with biological matrices, typically within several hundreds of microliters. Protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the techniques most commonly implemented in the preparation of biological samples for LC-MS/MS analysis, with pros and cons for each approach [2-4].

Although LLE represents a sample preparation process with advantages such as high recovery and extract cleanliness, its mainstream adoption into the modern bioanalytical lab is confounded by the disadvantages associated with automation (time consumption, labour-intensive processes, and the potential for emulsion

formation). In contrast, supported liquid extraction (SLE) as a flow-through technique has been increasingly used as an alternative approach to LLE, overcoming many of the disadvantages associated with LLE. [5] The SLE substrate provides a chemically inert but highly hydrophilic surface upon which an aqueous sample adsorbs. When the aqueous sample is loaded onto SLE substrate, a thin layer of aqueous phase is generated and coated onto the SLE sorbent surface. This thin layer of aqueous phase on the sorbent significantly increases the contact surface area during extraction. Following a brief equilibration period, analytes are extracted with a water immiscible solvent either by gravity or through the application of positive or negative pressure while the aqueous phase is retained on the sorbent. The extraction mechanism and workflow process are outlined in Figure 1. Since insignificant mixing of aqueous and organic phases occurs with the SLE workflow, emulsions are eliminated and the intimate contact between phases allows very efficient analyte partitioning, often resulting in high analyte recovery. Due to the simplicity of the SLE workflow (load, soak and elute), labour and

time demands are significantly reduced. Lastly, SLE in the 96-well plate format is especially amenable to automation, increasing overall sample throughput.

Traditionally, the sorbent used for SLE is highly purified diatomaceous earth (DE). However, as a naturally occurring material, DE consists of irregular fossilised micro-organisms. Consequently, variance in particle-size distribution can generate issues with product manufacturing and batch-to-batch quality control, in turn leading to inconsistent product performance. Figure 2 shows the scanning electron microscope (SEM) image of natural DE sorbent and synthetic SLE sorbent particles, using the same SEM settings. As shown in the Figure 2A, there is noticeable amount of debris in the DE sorbent with particle size inconsistencies. However, for synthetic SLE sorbent (Figure 2B), the particle size is much more uniform, without obvious debris. Further, the inconsistency in DE sorbent particles results in the reduced and inconsistent aqueous phase holding capacity [6,7], leading to a higher risk of sample loss and matrix breakthrough during sample loading and elution. The use of the synthetic SLE substrate allows control of the particle size distribution, in turn leading to improved consistency of method performance.

Fenfluramine (FNN) is an anti-epileptic drug whose mechanism of action is poorly understood. In order to study the distribution of FNN and the accumulation of its major metabolite norfenfluramine (NFNN) in mouse cerebellum, it was necessary to develop a sensitive assay given the

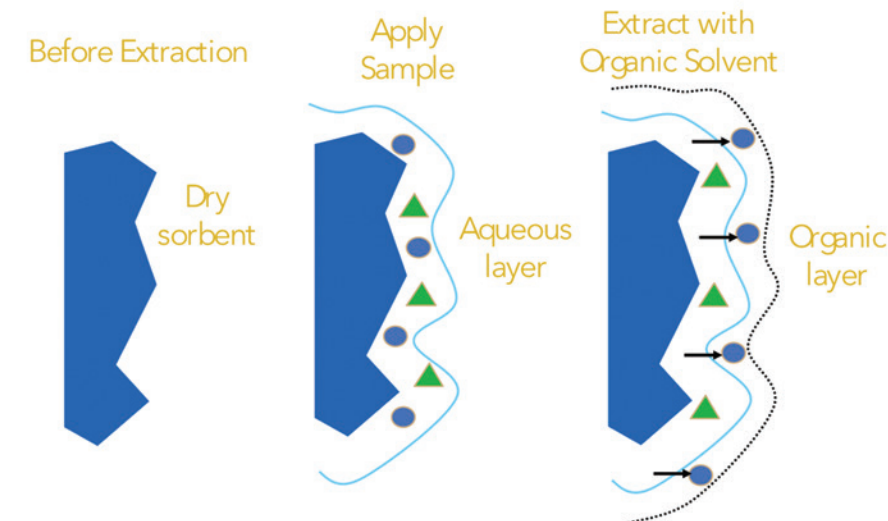


Figure 1: The process and analyte extraction mechanism of supported liquid extraction (SLE).

limitation in tissue mass (ca. 60 mg). Brain homogenate represents a matrix complexity greater than that of traditional plasma owing to significantly higher phospholipid content, and it was therefore necessary to deplete as many of these potential ion suppressors as possible in order to minimise accumulation within the LC/MS system. To this end, the novel synthetic SLE sorbent was evaluated in terms of the efficacy of phospholipid removal, recovery of FNN and NFNN, assay specificity and matrix effect, all benchmarked against traditional diatomaceous earth sorbent.

Experimental

Chemicals and Standards

High Performance Liquid Chromatography (HPLC) or Omnisolv grade solvents were sourced from Millipore Sigma, including methanol (MeOH), dichloromethane (DCM), methyl tert-butyl ether (MTBE), 1-chlorobutane, and hexane. Ethyl acetate (EtOAc) and chloroform Optima grade solvents were supplied by Fisher. Other chemicals were purchased from Sigma-Aldrich, including ammonium hydroxide (NH_4OH), concentrated hydrochloric acid (HCl), and ammonium bicarbonate (NH_4HCO_3).

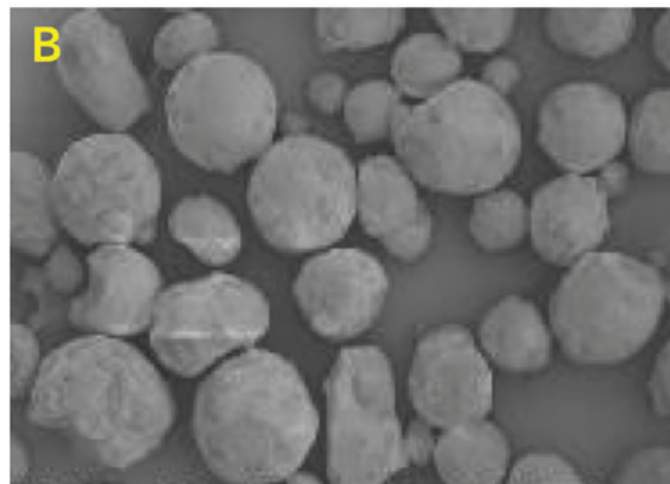


Figure 2: SEM images of DE sorbent (A) and synthetic SLE sorbent (B).

Table 1: MRM transitions for target analytes and phospholipids.

| Target analytes | Q1/Q3 |
|--------------------------------|-------------|
| FNN | 232.0/159.0 |
| FNN-D5 | 237.0/159.0 |
| NFNN | 204.0/109.0 |
| NFNN-D6 | 210.1/161.0 |
| Phospholipid | Q1/Q3 |
| Lysophosphatidylcholine (18:2) | 520.3/184.1 |
| Lysophosphatidylcholine (18:1) | 522.4/184.1 |
| Lysophosphatidylcholine (18:0) | 524.4/184.1 |
| Lysophosphatidylcholine (20:4) | 544.3/184.1 |
| Phosphatidylcholine (30:1) | 704.5/184.1 |
| Phosphatidylcholine (34:2) | 758.6/184.1 |
| Phosphatidylcholine (34:1) | 760.6/184.1 |
| Phosphatidylcholine (36:3) | 784.6/184.1 |
| Phosphatidylcholine (36:2) | 786.6/184.1 |
| Phosphatidylcholine (38:6) | 806.6/184.1 |
| Phosphatidylcholine (38:5) | 808.6/184.1 |
| Phosphatidylcholine (38:4) | 810.6/184.1 |

Reference standard and internal standard (IS) stock solutions were provided by Altasciences (100 µg/mL FNN and NFNN in MeOH, 100 µg/mL FNN-D₅, and NFNN-D₆ in MeOH).

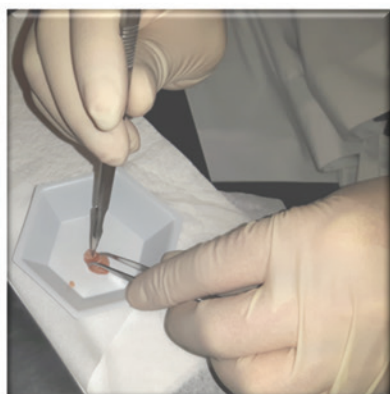
LC-MS/MS Instrumentation

Chromatographic separations were conducted on a C18 column (2.1 x 100 mm, 2.0 µm) with 10 mM NH₄HCO₃, pH 10.0 (MP-A), and MeOH (MP-B), delivered using an UPLC system under

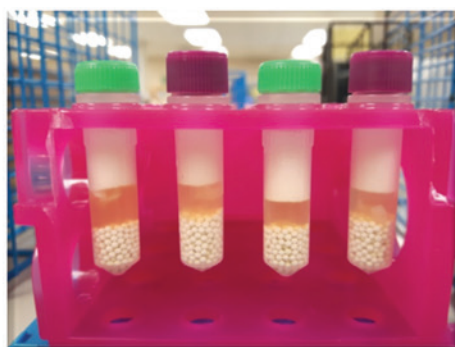
the following gradient: MP-B was ramped from 70 to 90% over 1.75 min and held isocratic for 0.5 min after which the column was re-equilibrated at 70% MP-B for 0.75 min. Column flow rate was 0.70 mL/min at a column temperature of 60°C; the LOQ was achieved using an injection volume of 8 µL. A triple quadrupole mass spectrometer was operated under positive electrospray ionisation (ESI) conditions with detection in multiple-reaction monitoring (MRM) mode for the transitions outlined in Table 1.

Sample Preparation

Mouse whole brain (CD-1 strain) was purchased from BIOIVT. Cerebellum was harvested from whole brain by dissection (Figure 3A). Either dissected cerebellum or whole brain were treated with water (10 µL per mg of tissue), after which ceramic beads (Matrix D, MP Biomedicals™) were added (Figure 3B) and the sample was homogenised (Figure 3C). An aliquot of homogenate (10 µL) was fortified with internal standard spiking solution (25 µL) and 5% NH₄OH (165 µL), then vortexed (1 min) and centrifuged (2 min, 738 g). The entire sample homogenate was loaded onto SLE plates using synthetic SLE sorbent (Chem Elut S 96-well plate, 200 mg, Agilent Technologies) for SLE extraction followed with the procedure shown in Figure 4. In order to prevent analyte loss during evaporation, a keeper consisting of HCl in MeOH was added in the collection plate prior to the elution step, which will create the analytes salt form. Consequently, this eliminated well to well variations previously noticed from the evaporation step.



(A)



(B)



(C)

Figure 3: Mouse brain pre-treatment, including A) dissection and weighing, B) Lysing buffer (water) and ceramic bead addition; C) homogenisation with MP Biomedicals FastPrep-96 homogeniser at 1,600 rpm for 40 seconds.

Results and Discussion

SLE Method Optimisation

The optimisation of SLE methodology involved the evaluation of analyte recovery, average reproducibility, and matrix effect as a function of elution solvent, solvent volume and sample soaking time. Elution solvents investigated included: MTBE, 1-Chlorobutane, DCM:EtOAc (1:1), MTBE:Chloroform (4:1) and MTBE:Hexane (4:1). A total volume of 1.2 mL was used for each elution solvent, applied in different aliquots: 3 x 400 μ L, as two aliquots of 600 μ L, 2 x 600 μ L and 1 x 1200 μ L. Results demonstrated in Figure 5 indicate that (a) the synthetic SLE sorbent provides higher analyte recovery, more consistent reproducibility, and reduced matrix effect when compared to DE, and (b) DCM:EtOAc (1:1) and EtOAc provide optimal recovery for each analyte, however elution with EtOAc exhibited higher matrix effect for NFNN. Consequently, DCM/EtOAc (1:1) was selected as the preferred elution solvent. Notably, when using the synthetic SLE sorbent, recovery was largely independent of the number of elution aliquots; 2 x 600 μ L provided marginally improved recovery and was therefore selected as the final elution scheme. However, when using DE SLE, changes in recovery were more significant with different elution steps.

Equilibration time following sample loading on the SLE sorbent was investigated for up

to 40 min. Results indicate no significant variation in recovery at different equilibrium time, and therefore 5 min was used to optimise sample throughput. Fact that recovery was not impacted with additional equilibration time ensures a level of robustness in the methodology, since analytes do not irreversibly bind to the sorbent substrate with extended soaking.

Phospholipid Depletion

Phospholipids (PPLs) have been identified as a major source of matrix effect in LC-MS/MS assays, leading to signal suppression under ESI conditions. As PPLs elute over a wide range of retention times, their removal via sample preparation is critical to minimise the likelihood of coelution with analyte, or accumulation within the LC/MS system. Within many different classes of phospholipids in biological matrix, phosphatidylcholine (PC) and lysophosphatidylcholine (Lyso-PC) are the two most abundant classes. Therefore, eight PC and four Lyso-PC compounds, together with total PPLs (184 > 184), were monitored for depletion following SLE on both synthetic

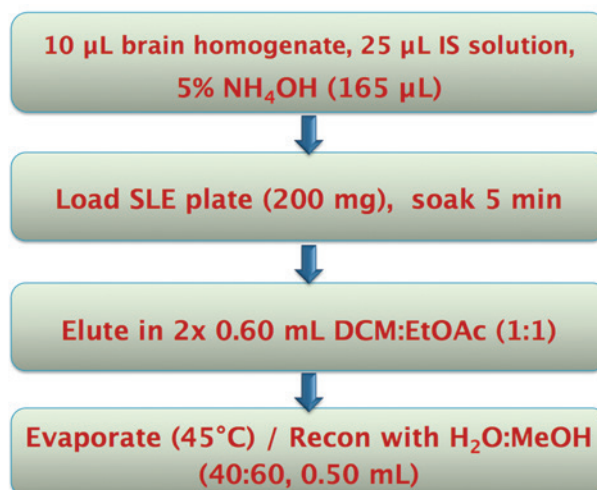


Figure 4: SLE procedure for mouse brain homogenate using synthetic SLE sorbent.

SLE sorbent and diatomaceous earth substrates. Results reported in Figure 6 demonstrate significant improvement for PPL depletion using the synthetic SLE sorbent, with > 50% of total PPL retained on the sorbent compared to diatomaceous earth.

With greater PPL depletion efficiency using the synthetic SLE sorbent, many benefits are conferred to the bio-analytical scientist, including (a) reduced likelihood for ionisation suppression, (b) the ability to develop faster chromatography without fear of PPL co-elution, and (c) increased assay robustness by elimination of PPL accumulation on-column and in the ion source, leading to extended column lifetime and reduced instrument downtime.

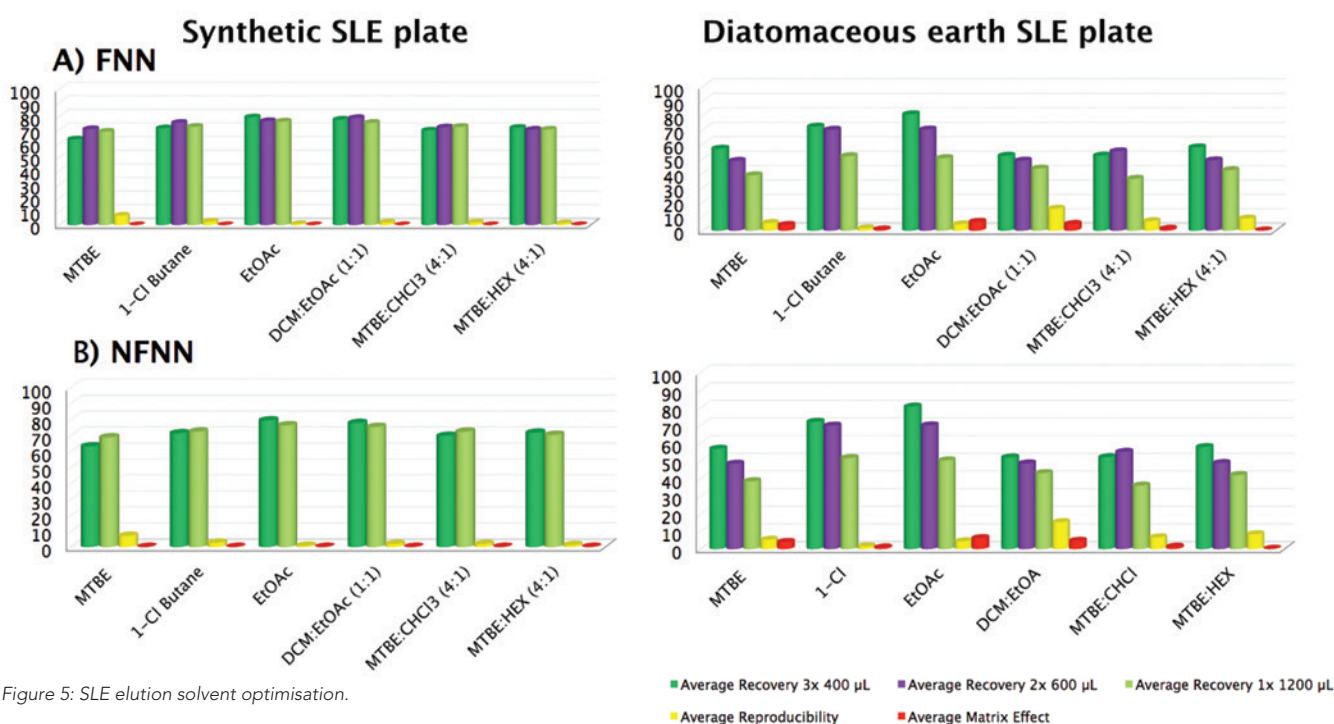


Figure 5: SLE elution solvent optimisation.

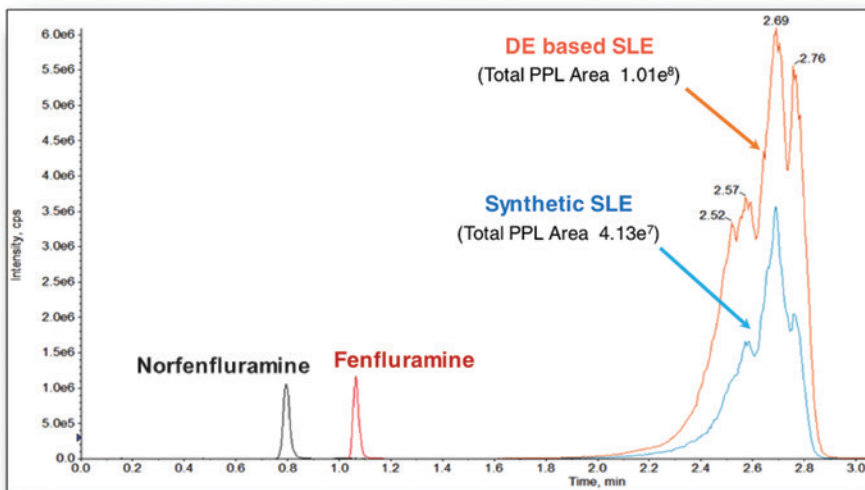


Figure 6: Phospholipid profile comparison for samples extracted using synthetic SLE sorbent (blue) vs. diatomaceous earth (red).

Method Validation

Following method optimisation, the synthetic SLE procedure was validated for the quantitative determination of FNN and NFNN in mouse brain from 0.05 – 5.0 µg/g (Figure 7), with subsequent cross validation in mouse cerebellum. Exemplary selectivity and sensitivity results are presented in Figure 8.

Method accuracy and precision derived from three separate batches using three different lots of synthetic SLE plates to prepare mouse brain extracts are summarised in Table 2. Four levels of QC samples were fortified into brain homogenate and extracted in replicates of six. Method accuracy and precision for QC samples prepared in mouse cerebellum whose concentrations were determined from a calibration curve prepared in whole brain are reported in Table 3. Matrix effect, as determined from eight lots of brain homogenate, met all acceptance criteria (Table 4).

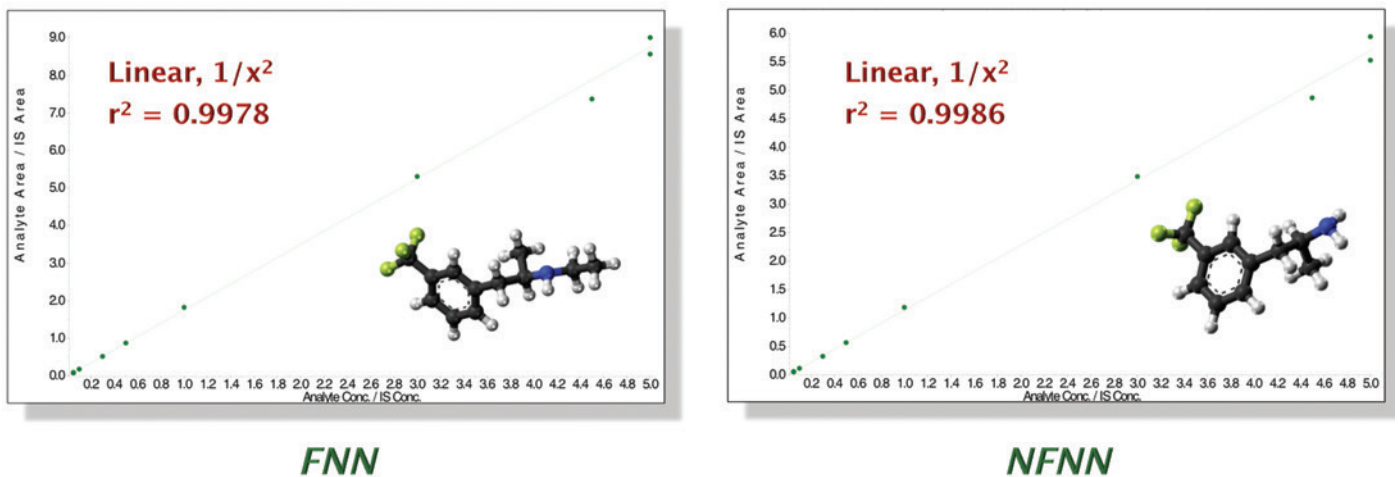


Figure 7: Method calibration curve linearity from 0.05 - 5.0 µg/g extracted from mouse brain. Responses of peak area ratio for analyte/IS were used for calibration curve plot.

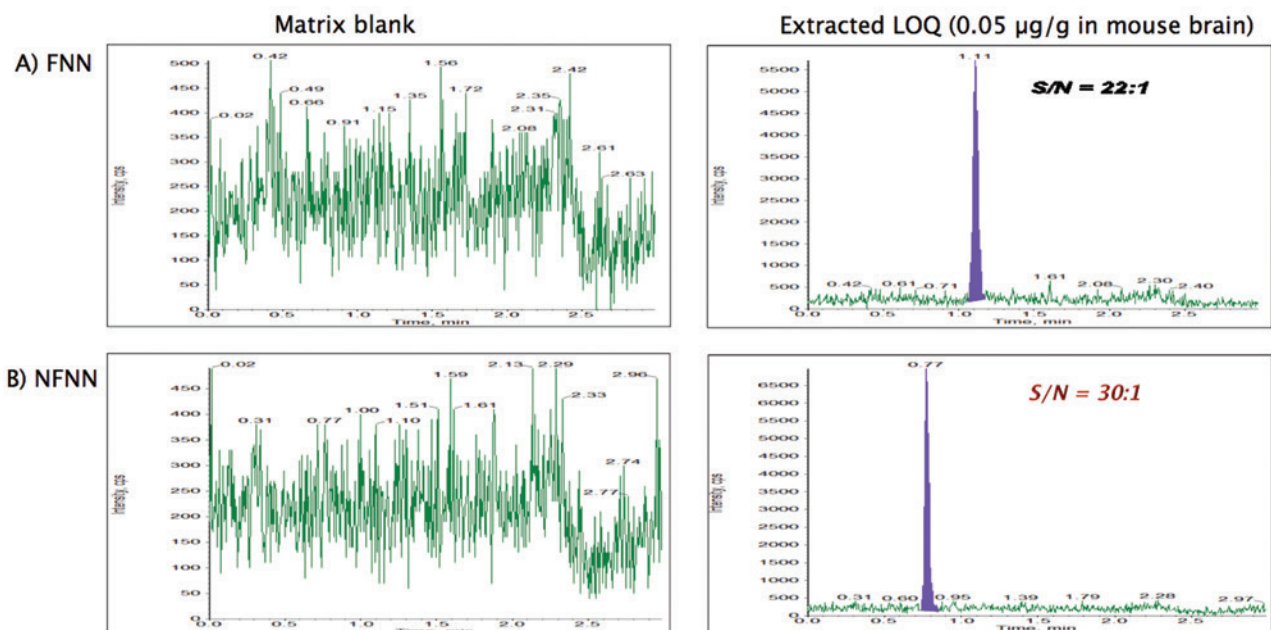


Figure 8: Method selectivity and sensitivity of FNN and NFNN extracted from mouse brain using validated method with synthetic SLE sorbent.

Table 2: Method accuracy and precision results for FNN and NFNN extracted from mouse brain for three extracted batches using three different lots of synthetic SLE plates. Six replicates were extracted for each QC concentration level.

| Statistics | LLOQ QC 0.050 µg/g | | Low QC 0.150 µg/g | | Mid QC 2.50 µg/g | | High QC 4.00 µg/g | |
|---------------------------------|-----------------------|-------|----------------------|-------|---------------------|-------|----------------------|-------|
| | FNN | NFNN | FNN | NFNN | FNN | NFNN | FNN | NFNN |
| Calculated concentration (µg/g) | 0.053 | 0.053 | 0.148 | 0.153 | 2.500 | 2.498 | 3.825 | 3.832 |
| SD | 0.003 | 0.003 | 0.007 | 0.007 | 0.067 | 0.100 | 0.109 | 0.090 |
| %CV | 5.8 | 5.0 | 4.5 | 4.9 | 2.7 | 4.0 | 4.2 | 2.4 |
| %Nominal | 105.4 | 106.2 | 98.5 | 102.3 | 100.0 | 99.9 | 95.6 | 95.5 |

Table 3: Method accuracy and precision results for three batches extracted from mouse cerebellum. Six replicates were extracted for each QC concentration level. Quality control samples were spiked in cerebellum homogenate, and then quantified against a calibration curve derived from brain homogenate.

| Statistics | Low QC 0.150 µg/g | | High QC 4.00 µg/g | |
|---------------------------------|----------------------|-------|----------------------|-------|
| | FNN | NFNN | FNN | NFNN |
| Calculated concentration (µg/g) | 0.161 | 0.163 | 4.051 | 4.044 |
| SD | 0.005 | 0.004 | 0.207 | 0.203 |
| CV[%] | 3.0 | 2.2 | 5.1 | 5.0 |
| Nominal [%] | 107.0 | 108.4 | 101.3 | 101.1 |

Table 4: Matrix effect evaluation from eight lots of brain homogenate. Three replicates were extracted at each QC concentration level.

| Statistics | Low QC 0.150 µg/g | | High QC 4.00 µg/g | |
|---------------------------------|----------------------|-------|----------------------|-------|
| | FNN | NFNN | FNN | NFNN |
| Calculated concentration (µg/g) | 0.161 | 0.163 | 4.051 | 4.044 |
| SD | 0.005 | 0.004 | 0.207 | 0.203 |
| CV[%] | 3.0 | 2.2 | 5.1 | 5.0 |
| Nominal [%] | 107.0 | 108.4 | 101.3 | 101.1 |

Conclusions

A sample preparation method using a synthetic SLE sorbent (96-well plate format, 200 mg) was developed and validated for the quantitative determination of fenfluramine and norfenfluramine in mouse brain, and cross-validated in mouse cerebellum. The SLE method was optimised for elution solvent, elution volume, number of elution aliquots, and sample equilibrium

time based on analyte recovery, method reproducibility and matrix effects. The developed SLE method using synthetic SLE sorbent was subsequently validated for method selectivity and sensitivity, calibration curve linearity, accuracy and precision, and matrix effect in multiple donor lots. All acceptance criteria were met for calibration curve linearity and intra- and inter-day accuracy and precision, the latter derived from three synthetic SLE sorbent lots. When

compared to diatomaceous earth SLE, the synthetic SLE sorbent provided higher overall analyte recoveries, improved assay reproducibility and greater phospholipid depletion for brain and cerebellum extracts. The developed SLE assay in the 96-well plate format is amenable to fast and automated sample preparation in high throughput laboratories.

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Vitamin D3 metabolites quantification; an example of where fast chromatography is needed to matrix effects.

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