Journal of Chromatography A, 1232 (2012) 77–83

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# A multi-fiber handling device for *in vivo* solid phase microextraction–liquid chromatography–mass spectrometry applications

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ARTICLE INFO

*Article history:*
Available online 19 October 2011

*Keywords:*
Solid phase microextraction
Multi-fiber handling device
High sample throughput
Liquid chromatography
Mass spectrometry
Bioanalytical applications

ABSTRACT

Solid phase microextraction, an *in vivo* and *ex vivo* sample preparation method, continues to capture growing interest among researchers for bioanalytical applications. When coupled with liquid chromatography mass spectrometry, the procedure often involves large numbers of fibers in, for example, both pharmacokinetic and pharmadynamic studies as well as other bioapplications. In this regard, appropriate and adequate precaution will be critical in preventing the fibers firstly from any possible external contamination and damage to maintain high analytical data integrity. In addition, improving the offline desorption of fibers specifically for *in vivo* SPME will not only help in improving data quality, but will also significantly decrease the overall analysis time. This article introduces a prototype multi-fiber handling device capable of simultaneous extraction/desorption of multiple solid phase microextraction (SPME) fibers on a 96-deep well plate format. This device thus provides an alternative approach to improving higher sample throughput for *in vivo* SPME liquid chromatography mass spectrometry applications. The portable design of the device ensures effective protection and prevention of fibers against damage and possible contamination and thus maintains analytical data reliability. To ensure its suitability for parallel extraction/desorption, the device was carefully evaluated using four benzodiazepines (diazepam, nordiazepam, oxazepam and lorazepam) as model drugs by monitoring inter- and intra-well variability. The effect of agitation speed on data precision and accuracy, effect of device weight on data precision, and comparison of the overall performance of the device with traditional manual desorption approach were also assessed. Results obtained from evaluation of the device with particular focus on the desorption process indicated that the weight of the device has no effect on the reliability and reproducibility of data acquired using the device. The average amount of diazepam obtained for 20 selected wells with and without device was 48.8 pg and 49.4 pg, respectively. Intra-, inter-well, and inter fiber variations recorded were all ≤13% indicating an excellent precision and reproducibility can be attained with the device.



## 1. Introduction

Sample preparation forms a key procedure in modern chemical analysis of any biochemical system. It provides analyte enrichment for good detection and removes matrix or contaminants. Today, a relatively higher percentage of work activity and operating cost in most analytical laboratories is spent preparing samples for further analysis by any device. This is largely due to the "garbage in-garbage out" effects; the results obtained from any analytical technique are only as good as the sample preparation method. Therefore, appropriate sampling procedure, sample preservation and preparation are needed to ensure the quality of the data obtained from any chemical analysis.

The evolution of sample preparation methods is captured in the design of rapid simple and effective extraction procedures: convenience for on-site implementation, easy coupling to separation–quantification, and/or possible automation. It can be argued that "Green" and relatively simple methods such as solid phase extraction (SPE), dried blood spots (DBS), and solid phase microextraction (SPME) are replacing laborious and environmentally unfriendly traditional methods such as liquid–liquid extraction (LLE). In addition to the upsurge of new methods, automation of analytical procedures has gained remarkable interest. Apart from automated sample preparation methods, the developments in analytical hyphenated techniques such as the high performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) have stimulated major advancements especially in quantitative bioanalysis. The applications of faster chromatographic runtimes through the use of modern columns and the sensitivity and specificity of the mass spectrometer have

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doi:10.1016/j.chroma.2011.10.032

generally improved analysis throughput in clinical diagnostics [1,2], drug discovery and screening [3–7], environmental application [8], and toxicological studies [9].

Automated sample preparation methods with the capability of performing parallel extractions have contributed significantly to reducing timelines in quantitative bioanalysis. For example, since its introduction the 96-well plate format has become the most common platform for high throughput bioanalysis. Parallel extraction methods involving SPE [10–13] and even LLE [14–17] on a 96-well plate format have consequently been established. Sean et al. demonstrated the ability to perform high throughput automated SPE analysis on a 96-well format with precision less than 10% for blank plasma and supernatant cartilage tissue samples [18]. In comparison of manual and automated methods for plasma samples, Jemal et al. demonstrated the overwhelmingly higher throughput achieved using automated SPE and LLE [19]. Recently, the first ever high throughput SPME method coupled with LC–MS/MS on a 96-well plate format has been developed and even applied to the analyses of whole blood without the need for sample pre-treatment [20–22].

SPME method has successfully been coupled with LC–MS/MS for the analysis of drugs in biological matrices for both *ex vivo* and *in vivo* applications [23–25]. In recent metabolomic studies, *in vivo* SPME method was used to capture unstable and short-lived metabolites in freely moving mice [26]. Typical of SPME method development for LC–MS/MS applications, the two most critical and time-consuming processes are extraction and desorption. In addition, depending on the analyte-fiber partition constant, large numbers of samples (fibers) are often generated in pharmacokinetic (PK) and pharmacodynamic (PD) studies. Although SPME kinetic calibration methods provide high temporal resolution for the extraction stage (in some cases less than a minute of extraction), desorption processes are often completed manually especially for *in vivo* applications. The currently used traditional or manual desorption process is often tedious, characterized with low sample throughput and not cost effective. In brief, the method requires all fibers loaded with the analytes after an extraction process to be placed individually into an appropriate desorption solvent system inside a small vial or inserts, and later agitated on a commercial shaker/agitator for further analysis. Owing to the increasing applications of *in vivo* SPME to bioanalysis, shortened analysis time, and the desire to obtain very good reliable and accurate information, it is extremely critical that adequate precaution is taken so as not to compromise analytical data. Therefore, a multi-purpose SPME fiber handling device for *in vivo* and *in vitro* LC–MS applications that will prevent any possible external contamination and also improve overall analysis time will certainly be a valuable tool.

This work introduces a prototype handling device capable firstly of simultaneous desorption of multiple fibers for SPME–LC–MS *in vivo* drug analysis on a 96-well format. This prototype device has been the first ever that fits any commercial 96-well plate agitator to facilitate desorption process. In addition to providing higher throughput, the portable device effectively protects and prevents fibers against any possible damage and contamination, and therefore helps to maintain sample integrity. The article briefly describes the handling device and the evaluation using selected benzodiazepines (diazepam, nordiazepam, lorazepam and oxazepam) as model drugs. Despite the fact that the device was primarily designed to facilitate *in vivo* SPME bioanalytical analyses as demonstrated in the article, the device can successfully be applied to batch *in vitro* SPME methods for the analyses of contaminants in environmental and biological samples where the use of an automated robotic system is not available.

## 2. Experimental

### 2.1. Reagents and materials

All chromatographic solvents were high quality HPLC grade. Acetonitrile solvents were obtained from EMD Chemicals Inc. (Darmstadt, Germany) and HPLC grade acetic acid was obtained from Supelco (Bellefonte, PA, U.S.A.). Benzodiazepines (diazepam; nordiazepam; oxazepam; and lorazepam) were obtained from Radian International (Austin, TX, U.S.A.) as 1 mg/mL standard in methanol with the exception of lorazepam, which was in acetonitrile. The drugs were stored at 4 °C in a refrigerator. A mixed standard (1000 ng/mL) of the drugs was prepared in 1:1 (v/v) acetonitrile–water mixture and always stored in the fridge. The mixed standard was used as the stock solution for all subsequent experiments. Phosphate buffer solutions (PBS) were prepared in the laboratory using analytical grade chemicals by mixing 8.0 g of NaCl, 0.2 g of KCl, 144 g of $Na_2HPO_4$, and 0.24 g of $KH_2PO_4$ in deionized water and the pH adjusted to 7.4. *In vivo* SPME fibers with $C_{18}$ particles as extraction phase used for this model study were obtained from Supelco (Bellefonte, PA, U.S.A.). Deionized water used in part for dilution of stock solutions was from a Barnstead/Thermodyne NANO-pure ultra water system (Dubuque, IA, U.S.A.). The VWR DVX-2500 Multi-tube vortexer was used for vial agitations for all extractions while the PAS Concept 96 was used during desorption experiments.

### 2.2. HPLC and mass spectrometry conditions

HPLC analyses were performed on the Accela™ instrument from Thermo Scientific® equipped with a binary pump. Chromatographic separation of analytes was achieved with a Waters® reverse phase $C_{18}$ column (Symmetry Shield; 5 μm, 2.1 mm × 50 mm) in 5 min using gradient elution at a flow rate of 0.5 mL/min. Mobile phase A consisted of 90% aqueous and 10% acetonitrile and mobile phase B was 90% acetonitrile and 10% aqueous. Both mobile phases contained 0.1% acetic acid to enhance ionization in the ion source. The Accela™ autosampler from Thermo Scientific was used for sample introduction into the HPLC system. A sample volume of 10 μL was injected and analyzed by a triple quadrupole mass spectrometer (TSQ Vantage™).

The TSQ Vantage™ had the HESI probe installed for effective nebulization and ionization. All ions were monitored in the positive ionization mode. The mass ion transitions monitored were 271.1 → 140.1, 285.1 → 193.1, 287.1 → 241.1 and 321.0 → 275.1 for nordiazepam, diazepam, oxazepam and lorazepam, respectively. The source voltage, vapourizer and capillary temperature were 2000 V, 350 °C and 350 °C, respectively. The optimized sheath and auxiliary gases were set at 55 and 25, respectively. All data analyses were performed with the Xcalibur® software version 2.0.7.

### 2.3. Optimization of extraction and desorption processes

Prior to evaluation of the multiple fiber desorption device, the extraction and desorption processes were optimized. Optimization of the extraction process was achieved by generating an extraction–time profile for each analyte. From the plots, the equilibration time for each analyte was determined. The equilibration times for all the analytes during the extraction process were determined with the VWR DVX-2500 multi-tube vortexer at a preset agitation speed of 1200 rpm. Selected fibers were placed in a 2.0 mL amber vial containing 100 ng/mL of the drugs dissolved in PBS. Extractions were carried out at different preset times (5, 10, 15, 30, 45 and 60 min) under the same agitation conditions. After the extraction process, the analytes were desorbed from the fiber using 70% acetonitrile–water solution.

**Table 1**
Optimized SPME conditions for analysis of benzodiazepines.

| Parameter | Condition |
|---|---|
| Agitation speed | 1200 rpm |
| Desorption solvent | 70% acetonitrile solution |
| Equilibration time | 25 min |
| Desorption time | 25 min |

In SPME method development for liquid chromatographic applications, optimization of the desorption process entails choosing an appropriate solvent system for which the analytes selectively have better affinity and the least time to effectively remove almost all or all of the analytes from the fiber. The rational is to reduce the amount of carryover to negligible limits to ensure accurate quantitation of analytes in the sample. Desorption of analytes from the SPME fiber was achieved by placing the fibers in a 1 mL desorption solvent inside the well of a 96-well plate. The 1 mL desorption volume was chosen since that was the maximum solvent that could be contained in each well. In addition, it offers an opportunity to evaluate any possible cross-contamination that may occur as a result of agitation of the well plate. Lower desorption volumes can be used in cases where sensitivity is an issue. But care must be taken to ensure that the fiber are fully immersed in the desorption solution to prevent any variability. The Concept 96® was used to provide effective agitation for enhanced desorption kinetics. The amount of carryover was determined to be less than 0.4% for all four analytes. Details of the optimized parameters are presented in Table 1. Subsequent experiments were all carried out using the optimized experimental conditions.

### 2.4. The multi-fiber handling desorption device

The evaluation of the multiple SPME fiber handling device was achieved using commercially available SPME *in vivo* blood samplers/fibers, comprised of a hypodermic needle and a medical grade stainless steel wire as a plunger with one end coated with the extraction phase while the other fixed into a cylindrical rubber-like material as shown elsewhere. Depressing the plunger exposes the extraction phase for subsequent extraction and desorption processes in a given matrix.

The multiple-fiber handling device was primarily designed as a portable tool for simultaneous/parallel desorption of multiple SPME *in vivo* blood samplers by simply fitting it on top of a regular 96-deep well plate. In addition, the entire unit was designed with the following criteria in mind:

a. Allow easy loading and setting of fibers to a fixed position inside each individual well of the 96-well plate.
b. All loaded fibers must be firmly stationary to prevent fiber damage during transport and agitation.
c. The weight of the device should not affect effectiveness of mass transfer inside any of the wells.
d. Capable of preventing any external fiber contamination during the experiment and transport.

As a brief description, the device consists of a base, which supports and allows *in vivo* fibers to be directly fitted into each well of a 96-deep well plate immediately following an extraction. A flat plate with 96 holes that are aligned with the wells of the plate is placed on top of the base to serve as the guide for the SPME fibers and there is a stopper to ensure the same position of each fiber inside the well. Fig. 1a displays the guide on the base part of the device while Fig. 1b is the unit place on a commercially available 96-deep well plate loaded with the *in vivo* SPME fibers. In order to ensure that the loaded *in vivo* fibers are well protected against damage and possible contamination after the extraction process, the entire device is placed in a protective case or cover serving as housing (Fig. 1c). The protective cover with four clips is used to secure the device with the well-plate together for easier transportation to the laboratory for further analysis as shown in Fig. 1d.

The device was evaluated by focusing on factors likely to affect data reproducibility for parallel desorption on a 96-well format. The results were also compared with data obtained for conventional desorption approach. Benzodiazepines (diazepam, lorazepam, oxazepam and nordiazepam) were used as model drugs in this study by preparing 100 ng/mL samples in artificial physiological fluid. Extraction of the drugs was attained with selected $C_{18}$ coated surface fibers. After pre-conditioning in 50% methanol–water solution, each fiber was placed in a 1.8 mL sample volume in a vial through the septum screw cap. All extractions were done using the optimized method conditions described in Table 1. After the extraction process, all the fibers were loaded into the desorption device and placed directly unto a 96-deep well plate containing 1 mL of desorption solution. The SPME fiber guide was then removed while the base part with the deep-well plate was later transferred to a commercial agitator (Fig. 2) for simultaneous desorption of the analytes from all the fibers using the optimized desorption conditions.

## 3. Results and discussion

One of the contributing factors, which make *in vivo* research studies very critical, is that data obtained offers a better indication of what will or does occur in a real organism. However, *in vivo* sampling often poses significant challenges because the system under study undergoes continual dynamic chemical processes. Therefore, any model *in vivo* sampling requiring off-line sample treatment should be able to preserve sample integrity especially in cases when the samples could not be analyzed immediately and must be transported to the lab. SPME as a portable sampling technique that integrates sampling and sample preparation has seen remarkable application to various *in vivo* studies in recent times. In most *in vivo* SPME–LC–MS applications the fibers must be transported to the lab after sampling for further off-site analysis. Thus, critical measures are required to prevent fiber contamination and protection from damage after sampling and during transport. Typically, these fibers are kept in small vials and trays, which sometimes present handling challenges to the researcher. This study presents a handling device, which not only offers a solution to possible fiber contamination and damage but also provides an alternative approach for parallel desorption of all *in vivo* SPME fibers for increased throughput without compromising data integrity. For desorption processes, the handling device accommodates a maximum of 96 fibers, directly fits into a deep well plate and can be placed on a commercial agitator for enhanced analyte desorption process. To ensure the efficacy and reliability of the device for parallel desorption of multiple SPME fibers, thorough evaluation was carried out, as this is critical for maintaining data integrity.

### 3.1. Evaluation of multi-fiber handling device

#### 3.1.1. Investigating effect of the device on the uniformity of agitation during fiber desorption

As part of our objectives, the handling device was also designed for simultaneous/parallel desorption of multiple *in vivo* SPME fibers for LC–MS applications using commercially available agitators on a 96-well plate format. Since the device was to be manually placed on the agitator after the loading process, it was paramount that each fiber was kept in steady position during agitation to prevent sudden movement, which could result in differences in the amount of

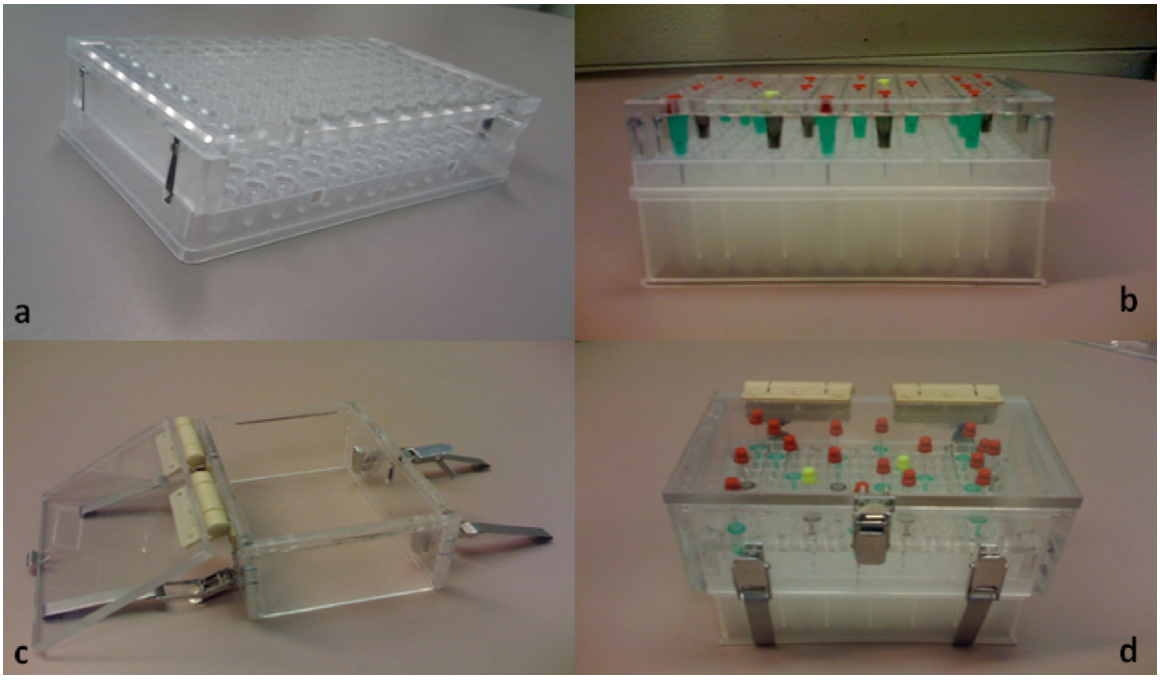


**Fig. 1.** Prototype desorption multiple SPME fiber desorption device (a); *in vivo* SPME sampler guide placed on the base part of the device (b); device holding *in vivo* SPME samplers fitted on a regular 96-deep well plate (c); and protective housing case with 4 clips as lockers (d). Entire unit placed on a 96-deep well plate with clips locking the various parts as a single unit.

analyte desorbed inside each well. Typically, for a multi-well plate during agitation, all the fibers must be firmly secured inside each well to ensure very effective desorption and thus reproducible data. This is because movement of the fibers inside the well may result in variation of the amount of analytes desorbed from each coating. Secondly, unlike other automated systems where the fibers are suspended by a solid support and then placed in a solution inside the well, in this study it is possible that the weight of the device could also affect the agitation process and the mass transport properties in each well, as the entire weight of the device would be brought to bear on the agitator. In this particular study, the weight included a regular 96-deep well plate containing the desorption solution, the total number of *in vivo* SPME fibers, and part of the device holding the fibers in place during the agitation process. The overall force exerted on the agitator during desorption could therefore be significant and thus affect the uniformity of the agitation inside each well. If the agitation and therefore mass transport properties within the wells were not uniform, this led to errors in the amount of analyte desorbed in each well, and affected overall data accuracy. The effect of the weight of the device was therefore evaluated by comparing the amount of each drug extracted/desorbed in each of the selected well positions over five independent desorption processes with/without the device.

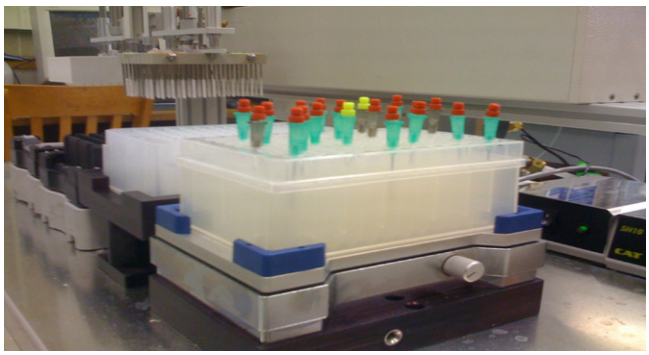

**Fig. 2.** Prototype multi-fiber device used for desorption of SPME fibers on a regular commercial agitator for a 96-deep well plate.

For desorption of SPME fibers in a well plate format, differences in agitation between the wells would lead to significant differences in the overall analyte mass transfer from each fiber in each well into the desorption solution. This would obviously lead to very poor data reproducibility. However, from the results obtained (Fig. 3), the weight of the desorption device did not affect the uniformity of agitation in the individual wells as there were no significant differences in the amount of analyte obtained from each well with/without the device. In addition, the results indicate that any relative movement of the fibers/coatings during the agitation process did not lead to significant differences in the amount of the analyte obtained from each well.

As a proof of concept, we also demonstrated the importance of higher agitation speeds for parallel desorption of multiple fibers on a well plate format, as lower agitation speeds are characterized by slow mass transfer from the fiber into the solution. This results in non-uniformity of the mass transport properties between the wells. The variation in the amount desorbed from each fiber as a function of the agitation speed was therefore monitored. This was achieved by determining the amount desorbed at two different agitation speeds, 500 rpm and 1200 rpm, respectively, for five successive experiments. The relative standard deviations (RSD%) at higher agitation speed were lower (RSD% $\leq$ 7.0; $n = 5$) compared to that obtained (RSD% $\geq$ 15; $n = 5$) with lower speed for the selected wells, i.e., for excellent precision, reproducible and reliable data higher agitation (~1200 rpm) of the 96-well plate was necessary provided it did not lead to a possible cross-well contamination. This implies that when using the optimized experimental conditions, any variation therefore will result from factors other than the positions of each fiber, weight of the device, and speed of agitation.

### *3.1.2. Monitoring well variations during multiple fiber desorption process*

One of the critical factors in SPME–LC–MS applications that could affect data reproducibility apart from inter fiber variations

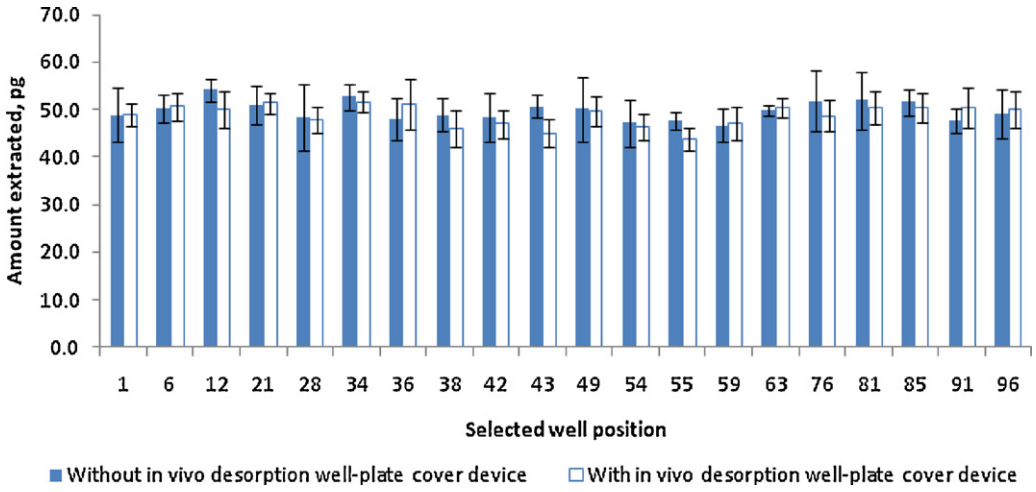


**Fig. 3.** Comparison of the amount of diazepam extracted/desorbed from the fibers at selected well positions with/without the desorption device ($n = 5$).

is the lack of consistency in the bulk well conditions during agitation of the well plate. Irreproducible well conditions affect the extraction/desorption rate and would alter the amount of analyte extracted by or desorbed from the fiber in different wells for same and different batch experiments. This leads to poor data precision and reproducibility, and often gross inaccuracies in data among batches. This implies that for a 96-well plate where conditions inside the individual wells could vary from well to well, it is therefore paramount to ensure that the bulk movement of desorption solution remains nearly constant. With the agitation speed at optimum condition, we first investigated overall inter- and intra-well variations for selected wells. This was carried out by preparing a 100 ng/mL of the benzodiazepines in a physiological fluid (PBS) and used as a sample. A 1-mL sample was placed in each selected well, extracted, and further desorbed using 70% acetonitrile with 30% water system as desorption solution at the same well positions.

The calculated inter-well variation (RSD%) for 20 selected wells ($n = 20$) for all benzodiazepines ranged from 8 to 12% with oxazepam and nordiazepam being the least and highest, respectively. The experiment was carried out assuming that variations that may result from the use of different fibers were negligible. Intra-well variations were investigated by considering reproducibility of the amount extracted from the same wells using the same set of fibers for five successive experiments, ensuring that each fiber was pre-conditioned prior to each experiment. The contributions to the variations obtained from the same well using the same set of fibers at specific well locations were each $\leq$10%. This implies that with the optimized conditions during the desorption process, variability within and between wells were negligible. Therefore, any possible variations could be due to the different fibers.

To eliminate variability from the fibers, further to the above results, we determined the variability from a single fiber at different well positions for five independent extractions and desorptions for the same concentration of benzodiazepines in PBS. We also determined the amount of analyte obtained by a single fiber for multiple desorptions ($n = 5$) from the same well. As shown in Fig. 4, similarly there were no significant variations in the amount obtained from the same fiber for multiple desorptions at different well positions and at the same well position for all the analytes. The RSD% calculated for all benzodiazepines was $\leq$9% in both cases. This implies that by carefully eliminating any variations that may result from the fiber and within the limits of experimental errors, the multi-fiber desorption device is capable of giving very reproducible and reliable.

### 3.1.3. *Investigating inter fiber variations*

Inter fiber variation was determined by comparing the amount of benzodiazepines obtained from the same well for different numbers of fibers. The RSD% for seven independent experiments for each analyte was then calculated using a different fiber in each case. It was carried out in this manner to ensure that any variability from the well was avoided. The calculated RSD% for the benzodiazepines ranged from 10 to 13% with oxazepam and nordiazepam having the highest and least variations, respectively (Fig. 5).

By comparing results obtained from inter-well and inter fiber variations, precision and reproducibility of the data were largely determined by the variability from the fibers (extraction coatings)

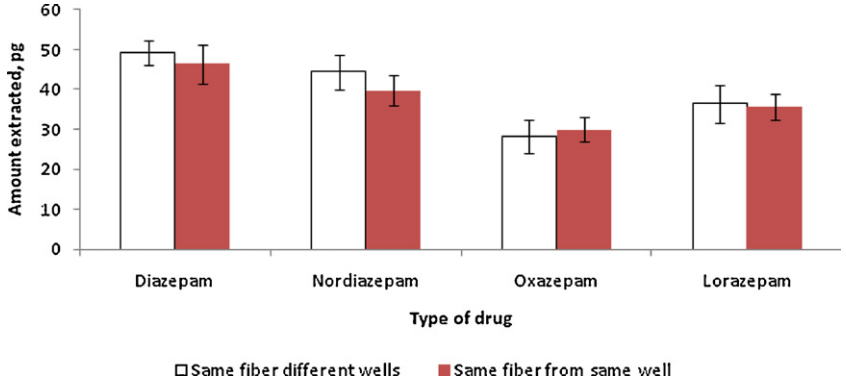


**Fig. 4.** Comparison of variations obtained from same well using same fiber with same fiber at different well positions ($n = 5$).

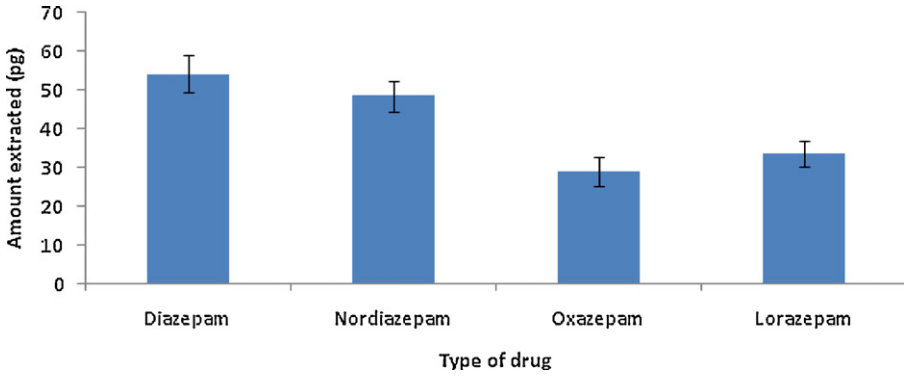


**Fig. 5.** Monitoring inter fiber variability for multiple desorptions of benzodiazepines from different numbers of fibers ($n = 7$).

**Table 2**
Comparison of the performance of the multiple fiber desorption device with conventional SPME desorption process.

| Parameter | Multi-fiber handling desorption device method | Conventional fiber desorption method |
|---|---|---|
| Total analysis time after sampling (min) | 30 | 65 |
| Reproducibility (CV%); $n = 20$ | 6–9 | 11–14 |

and not from the well. This implies that the handling device does not in any way significantly affect the data precision, reproducibility and reliability.

### 3.2. Comparison of the multi-fiber handling device with conventional SPME fiber desorption method

To further demonstrate the efficiency and advantage of the device as a tool for parallel desorption of multiple *in vivo* SPME fibers, data obtained with the device was compared to the conventional SPME desorption process for the same set of drugs. To facilitate good comparison, the same optimized conditions were used for both approaches and the total analyses time in addition to the overall method precision were determined after extraction of the drugs from a 50 ng/mL PBS solution of benzodiazepines. Another set of 20 SPME fibers were used for this experiment.

As shown in Table 2, after the sampling process, the multiple fiber desorption device offered a much shorter time (30 min) and therefore a higher throughput compared to the traditional SPME desorption approach, which took 65 min. In addition, the handling desorption device recorded lower RSD% compared to the conventional SPME approach for offline desorption process. The significant difference in time between the two methods was basically due to the time taken to prepare each set of fibers prior to desorption with the agitators. The entire approach offers less human contact time to physically handle the fibers and thus reduces any possible contamination during preparation. In addition, results obtained were similar to previous work reported elsewhere [22] using the automated robotic autosamplers for parallel desorption of SPME probes (blades) on a 96-well plate format.

## 4. Conclusion

The results of this study outline the major advantages of using the multi-fiber handling device to enhance the desorption process in SPME applications to bioanalysis, especially for *in vivo* analysis. Evaluation of the device showed excellent precision and reproducible data with relative standard deviations $\leq$13% in all experiments. In comparison with conventional SPME desorption process for *in vivo* fibers, the handling device offered a higher throughput with results similar to that obtained using automated desorption systems. In addition, the device offers an alternative approach to effectively minimize any possible external contamination of SPME *in vivo* fibers post sampling processes. It is anticipated that the *in vivo* SPME probes will be provided to scientists in packages of 96 for easy handing as described here especially to ensure convenient use of sterile probes. Although the device was primarily introduced for *in vivo* SPME–LC–MS bioanalytical applications, it can also be applied effectively for batch analyses of contaminants in environmental water samples and biological matrices such as urine, plasma and whole blood for situations where automated robotic systems are not available for parallel analyses on a 96-well plate format.

## Acknowledgements

Authors would like to express their sincere gratitude to the Natural Sciences and Engineering Research Council of Canada (NSERC), PAS Technologies and Supelco for financing this project. Thanks also to the University of Waterloo science machine shop for assistance in developing the prototype device and finally to Supelco Inc. for providing the fibers used. Authors are grateful for every form of support received in various ways.

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