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Suitability testing of commercial solid-phase extraction sorbents for sample clean-up in systematic toxicological analysis using liquid chromatography–(tandem) mass spectrometry

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Abstract

An entire series of SPE sorbents, classified into three different categories (apolar, mixed-mode and polymeric) was evaluated for sample preparation of a data-dependent LC–MS–MS ''general unknown'' screening procedure. An extraction procedure was formulated for each individual column, in agreement with the enclosed instructions, according to the characteristics of each packing. For conciseness, only neutral and basic compounds were chosen for this sorbent suitability test. Thus, the goal of our research was to select the best sorbent with regard to extraction yield and cleanliness of the extracts, all with respect to data-dependent acquisition (DDA) mediated LC–MS–MS general unknown screening. We conclude that for that purpose an Isolute™ C_8 sorbent performs best in terms of extraction yield and clean-up potential.

Keywords: Solid-phase extraction; Systematic toxicological analysis; Sorbents; Data-dependent acquisition

1. Introduction

Systematic toxicological analysis (STA), i.e. screening for drugs and poisons, is one of the most challenging tasks clinical and forensic toxicologists are facing today [1]. While in most laboratories, GC–MS is still the most commonly used procedure for STA [2,3], LC–MS ''general unknown'' screening procedures using electrospray are gradually gaining acceptance [4–6]. Convinced of the suitability of LC–MS in STA, our research team has successfully evaluated the added value of data-dependent acquisition (DDA) [7]. The use of DDA in LC–MS has the major advantage that ''clean'' product-ion mass spectra can be obtained without prior knowledge of the precursor ion to be selected in MS_1. Our approach simultaneously provides qualitative (molecular mass and library searchable diagnostic product ions) as well as quantitative information. Nevertheless, even with the best LC–MS method and the most sophisticated instrumentation, a substance cannot be unambiguously identified in a biological matrix without a suitable extraction procedure. For a long time, liquid–liquid extraction (LLE) was known as the golden standard for sample work-up. Nonetheless, solid-phase extraction (SPE) is becoming more popular for sample pre-treatment [8,9], not only because of the possi-

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bility of high throughput automation, but also because of the increased commercial availability of innovative SPE sorbents during the last few years [10–12]. Indeed, a whole series of packings is now being marketed, from strongly apolar to polar, beyond mixed-mode, ion exchange, polymeric and combinations of ion exchange and polymeric. Intelligent application-goal directed selection affords a powerful extraction tool which can be adapted to the particular needs of the analysis. Accordingly, to complement our DDA mediated LC–MS procedure, the suitability of 12 different SPE sorbents was assessed: seven apolar, three mixed-mode and two polymeric packings. In a first phase of this study, water was fortified with 18 basic and neutral compounds, chosen to represent a wide variety of compound classes as well as a broad spectrum of physicochemical characteristics. These samples were extracted according to a sorbent-specific SPE procedure, tailored to our needs, with varying conditions for critical procedural steps. Thereafter, these samples were LC–MS analysed in multiple-reaction monitoring (MRM) mode and the results used for evaluation of the extraction yields of the various compounds. In light of the obtained results, we then continued the evaluation study with the two SPE sorbents which scored best as regards overall extraction yield. In a second phase, these two sorbents were evaluated for their clean-up potential by fortifying blank blood with the same set of compounds and analysing the ensuing extracts in DDA mode. Indeed, for overall evaluation of various SPE sorbents and methods, there has to be a compromise between an acceptable recovery of the compounds and adequate clean-up potential of the sorbents. STA requires a wide approach, aiming for a range of compounds as broad as possible, without impairing matrix related analysis too much.

2. Experimental

2.1. Materials

Drug standards were available from the collection at the Laboratory of Toxicology (Ghent University, Belgium). The compounds studied were morphine, benzoylecgonine, XTC, codeine, strychnine, ethylmorphine, nalorphine, cocaine, butorphanol (candidate internal standard), lidocaine, bromazepam, methaqualone, diazepam, triazolam, methadone, trazodone, haloperidol and oxazepam. Methanol, acetonitrile, water and hexane were all of HPLC grade (Merck-Eurolab, Leuven, Belgium). Acetic acid (purity minimum 99.7%) and ammonium acetate (purity minimum 98%) were supplied by Sigma–Aldrich (Steinheim, Germany), while ammonia solution 25% and hydrochloric acid were purchased from Merck-Eurolab. The apolar and mixed-mode columns were provided by International Sorbent Technology (IST; Hengoed, Mid Glamorgan, UK), while the polymeric columns were supplied by Waters (Milford, MA, USA).

2.2. Analyte preparation

Two mixtures of the drug standards were prepared in acetonitrile: one at a concentration of 160 ng/ml of each compound, and the other at a concentration of 4 μg/ml of each compound. The first was used for the experiments performed in MRM mode, the second for those in DDA mode. The concentration of butorphanol used in MRM mode (80 ng/ml) was different from that used in DDA mode (1 μg/ml).

2.3. SPE sorbents studied

As mentioned above, SPE sorbents of three different categories were studied. For the apolar sorbents, Isolute™ C_2, C_4, C_8, C_{18}, C_{18}MF, PH and CN were chosen. In addition, for the mixed-mode packings, we opted for Isolute™ HCX, HCX3 and HCX5 sorbents (all supplied by IST), while the organic polymers were represented by the OASIS™ HLB and MCX sorbents (Waters). A sorbent mass of 100 mg was used for the apolar and mixed-mode packings, whereas a sorbent mass of 30 mg was preferred for the polymer group. In all cases, 1-ml solid-phase cartridges were used.

2.4. Instrumentation

Reproducible, automated SPE was performed on a Zymark RapidTrace™ Solid Phase Extraction Workstation (Zymark, Hopkington, MA, USA) equipped with one single extraction module. For the MRM based experiments, liquid chromatography was conducted with an Xterra MS C_{18} column (2.5 μm

particle size, 20×2.1 mm; Waters) using a Waters Alliance 2695 separation module and a Quattro Ultima system (Micromass, Manchester, UK). DDA experiments were performed with the same column on a Waters Alliance 2790 separation module integrated with a Q-TOF instrument (Micromass, Manchester, UK). All samples were analysed (extracted and injected) in triplicate and injections of the samples were regularly interspaced by injections of pure standards for recovery calculation purposes.

2.5. Chromatographic conditions

For both the MRM- and DDA-based experiments, the following chromatographic conditions were used. Flow rate was set to 0.2 ml/min. Gradient elution was performed, starting at 100% of a mixture of water/methanol/acetonitrile (80:10:10, v/v) containing 5 m*M* ammonium acetate (solvent A), programmed linearly, within 7 min, to 40% of a mixture of water/methanol/acetonitrile (20:40:40, v/v), again containing 5 m*M* ammonium acetate (solvent B). In order to remove late eluting substances, a step gradient to 100% solvent B was included for 1.5 min. Subsequently, the system was programmed to regain its initial conditions over 0.5 min, followed by 8-min equilibration prior to the next injection. The injection volume was 25 μl and the entire column effluent was directed into the mass spectrometer.

2.6. Mass spectrometry

In both modes, nitrogen acted as nebulising and desolvation gas. Argon was used as collision gas.

2.6.1. MRM mode

The cone voltage and the collision energy (CE) were optimised for each compound using QuanOptimize, one of the features supplied by the MassLynx software. Using these CE, a substantial loss of sensitivity was registered for morphine, codeine, strychnine, ethylmorphine, nalorphine and bromazepam. This can be ascribed to the absence of prominent fragment ions in the MS–MS spectra of these compounds, which show either no or exhaustive fragmentation. Therefore, the CE was set to 1 for these compounds and MRM transitions of the protonated molecular ions were monitored, despite the loss of selectivity.

2.6.2. DDA mode

Optimisation of the mass spectrometer parameters has been described in detail in a previous publication [7]. However, for this study, several parameters were readjusted because of the use of real blood samples. The number of components eventually fragmented simultaneously was reduced to three from four, since the third channel is rarely used. Therefore, the blind spot [7], created in the MS trace by switching to the MS–MS mode, could be reduced to 9 instead of 12 s. The influence of the MS to MS–MS switching threshold has also been evaluated.

2.7. Sample preparation

An individual extraction procedure was formulated for each packing material in accordance with the enclosed instructions, and with respect to the particular characteristics of the packing material. In addition, several variations of the critical procedural steps were drawn up, including varying conditioning and elution volumes.

2.7.1. Water samples

In each case 900 μl water was spiked with 50 μl of the mixture of 17 compounds and 50 μl of the candidate internal standard.

2.7.1.1. Apolar sorbents. A 1 ml sample of 50 m*M* ammonium acetate buffer (pH 9.0) was added to the water samples. Before application of the samples (2 ml), the SPE columns were conditioned with 1 ml methanol and 1 ml 50 m*M* ammonium acetate buffer (pH 9.0). The columns were washed with 2 ml ammonium acetate buffer containing 5% methanol and were dried. The drying time varied depending on the carbon chain length. Elution was performed with 2 ml methanol/ammonia (90:10, v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen, and the residue was redissolved in 200 μl of solvent A, 25 μl of which was injected into the LC–MS system.

2.7.1.2. Mixed-mode sorbents. A 1 ml sample 50 m*M* ammonium acetate buffer (pH 6.0) was added to the water samples. For conditioning, the column was treated with 1 ml methanol and 1 ml 50 m*M* ammonium acetate buffer (pH 6.0). After extraction of the sample, the column was washed

with 1 ml 50 m*M* ammonium acetate buffer (pH 6.0), 1 ml acetic acid and 1 ml ammonium acetate buffer, containing 5% methanol. Again, the drying time varied depending on the carbon chain length. Thereafter, an additional washing step with 1 ml hexane was included to remove strong apolar interfering substances. The compounds were eluted with 2 ml methanol/ammonia (90:10, v/v). The solvent was removed with a stream of nitrogen, and the residue was dissolved in 200 μl of solvent A as described above.

2.7.1.3. Polymeric sorbents. The SPE procedure for the OASIS™ HLB sorbents was very similar to those for the apolar sorbents, except for the elution step, where 2 ml methanol was used. For the OASIS™ MCX sorbents 1 ml water (instead of buffer) was added to the samples. The columns were conditioned with 1 ml methanol and 1 ml water before application of the samples. Hydrochloric acid (0.1 N) was used to remove interferences and 2 ml methanol/ammonia (95:5, v/v) to elute the compounds.

2.7.2. Blood samples

Prior to the SPE clean-up procedure, the whole blood was pre-treated as follows. After fortifying the blood sample with the standards' mix, it was mixed on a vortex mixer for 30 s, equilibrated and ultrasonicated for 15 min. The blood sample was then diluted with either 1 ml water or 1 ml ammonium acetate buffer, depending on the sorbent used, whereupon a fixed period of mixing, ultrasonicating and centrifugation ($2000\times g$) followed. The resulting supernatant was then applied on the SPE extraction columns.

2.8. Transformation function for extraction yield evaluation

In order to evaluate the overall extraction efficiency of the different SPE sorbents, i.e. the first part of this study, we used a transformation function *F*. As shown in Fig. 1, a value *S* was calculated for each sorbent from the extraction yield (EY) of every compound as calculated by comparison to a pure standards' mix (average of flanking injections). To this end, each individual EY for each of the 18 compounds is transformed to a new value, designated V_1 to V_{18}, by a particular function *F*, and *S* is the summation of these new values. Therefore, comparison of the individual EYs of the sorbents is now reduced to a comparison of the value *S* of the sorbent. The transformation function *F* has the following characteristics: for EYs below 50% a more than linear penalty is assigned, while for EYs above 50% a more than linear reward is assigned.

3. Results and discussion

The split approach of our suitability assessment is dictated by the aim of the experiment: find the SPE sorbent and an accompanying procedure to provide extraction which is selective enough to allow uncomplicated LC–MS analysis, but at the same time allows adequate extraction recovery for a compound range as wide as possible. The experimental procedure chosen was not only divided as regards recovery versus clean-up potential, but also entailed a hierarchical approach, starting with many sorbents to evaluate extraction capability and continuing with only the most promising to assess clean-up potential. Concomitantly, the optimal LC–MS procedure was applied to each experimental section: MRM which is more sensitive and quantitatively correct but at the same time largely ''blind'' for interferences, to evaluate extraction recovery; and DDA which is universal in its detection potential but clearly less sensitive and quantitative (reproducible), to evaluate cleanliness of the extracts and thus suitability for DDA mediated STA. Certainly, a major criterion which governs the applicability of DDA in STA, is the lack of interferents which initiate, and thus ''occupy'', the MS–MS channels, effectively blinding the method to the compounds of real toxicological interest.

The extended sorbent evaluation (many sorbents, many compounds) with respect to extraction efficiency (part 1 of this study) resulted in a large two-dimensional data matrix. To aid its interpretation and provide an overall recovery index suitable for comparing sorbents, the transformation function, as illustrated in Fig. 1, was introduced. It disproportionally penalises extraction recoveries under 50%, while increasing recoveries up to 80% are exponentially rewarded. As an extraction recovery of over 80%

For sorbent$_x$	EY	
Compound 1	A%	Value **V1** = F(A%)
Compound 2	B%	Value **V2** = F(B%)
Compound 3	C%	Value **V3** = F(C%)
Compound 4	D%	Value **V4** = F(D%)
Compound 5	E%	Value **V5** = F(E%)
Compound 6	F%	Value **V6** = F(F%)
Compound 7	G%	Value **V7** = F(G%)
Compound 8	H%	Value **V8** = F(H%)
Compound 9	I%	Value **V9** = F(I%)
Compound 10	J%	Value **V10** = F(J%)
Compound 11	K%	Value **V11** = F(K%)
Compound 12	L%	Value **V12** = F(L%)
Compound 13	M%	Value **V13** = F(M%)
Compound 14	N%	Value **V14** = F(N%)
Compound 15	O%	Value **V15** = F(O%)
Compound 16	P%	Value **V16** = F(P%)
Compound 17	Q%	Value **V17** = F(Q%)
Compound 18	R%	Value **V18** = F(R%)

S = ∑ Value V1…18

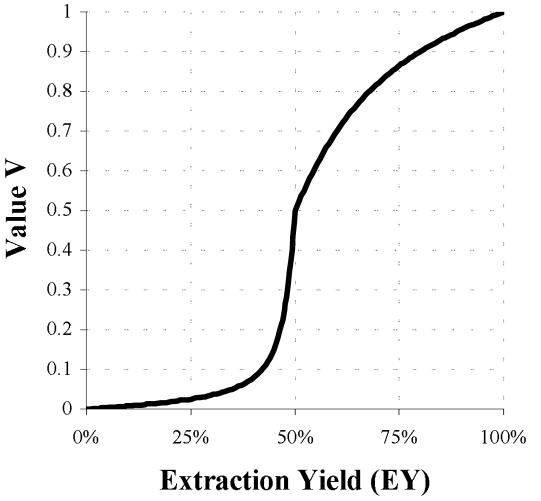

Fig. 1. Transformation function for extraction yield evaluation.

suits the needs of our method, the exponentiality of the function advantageously reduces the impact of single, very high recoveries. Fig. 2 gives an overview of the values *S* as calculated for each SPE sorbent, by using the transformation function *F*, for the fortified water samples. The *Y*-axis depicts the value *S* averaged over three extractions, since every SPE procedure was performed in triplicate. Obviously, the higher the value, the better the overall extraction yield. Based upon this, it can be concluded

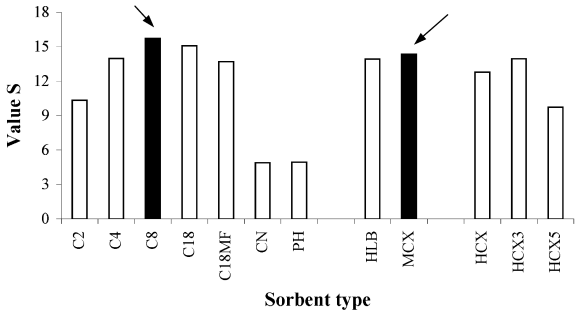

Fig. 2. Calculated *S* values for every SPE sorbent (fortified water samples, triplicate extractions, MRM based detection).

that the C_8 packing material demonstrated the best overall extraction yield. Therefore, this apolar sorbent was withheld for the second part of this study, i.e. the evaluation of its clean-up potential. Fig. 3 gives an overview of all MRM chromatograms of a water sample extracted according to this C_8 procedure. However, in addition to this apolar packing, we have opted to also select a second type of column for further evaluation, namely the OASIS™ MCX sorbent type. After all, as representative of the mixed-mode category, considering both the silica-based and polymeric based sorbents, the best extraction yields were obtained with this type of sorbent, extraction yields which were, moreover, only marginally lower than those of the C_8 packing. The fact that the mixed-mode principle affords more degrees of freedom to optimise clean-up strength or selectivity of the extraction also led to a mixed-mode sorbent being included in the evaluation of clean-up potential. In addition, the SPE procedure applied on this polymeric column was the least time-consuming. In this way, two packing materials were further evaluated for their clean-up potential.

To that end, whole blood was fortified with the

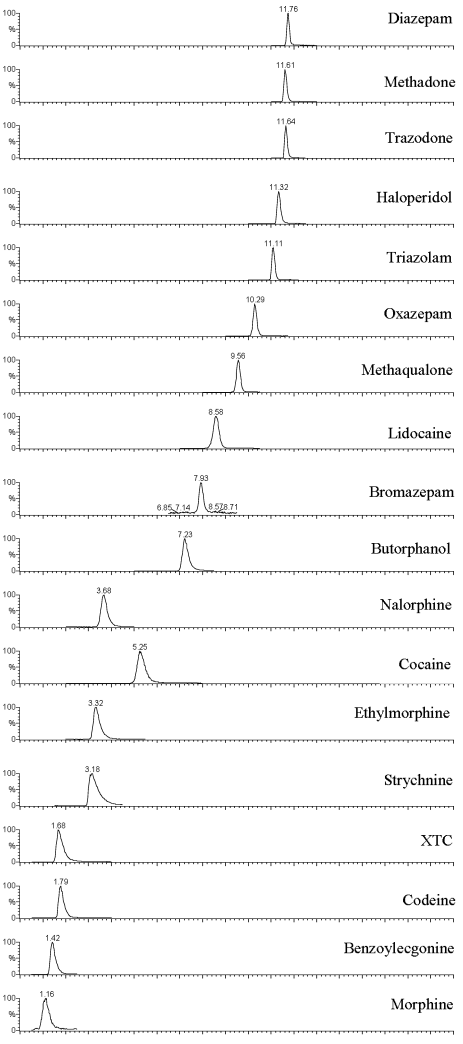

Fig. 3. MRM chromatograms of an extract of water fortified with 18 basic and neutral compounds. Extraction was performed using C_8 SPE columns.

same compounds and applied to both the Isolute™ C_8 and OASIS™ MCX SPE columns. The obtained extracts were analysed in DDA mode. For both sorbent types, a dirty matrix such as whole blood could successfully be purified in such a way that DDA mediated LC–MS–MS proved possible. In both cases, 14 of the 17 spiked compounds (in the second experiment, the candidate internal standard was effectively used as an internal standard) were fragmented by DDA. Moreover, in none of the cases were any MS–MS channels continuously occupied, proving the interference-free nature of the extracts. However, no MS–MS spectra were obtained for morphine, bromazepam or diazepam in either Isolute™ C_8 or OASIS™ MCX extract. In the case of morphine and diazepam, i.e. the first and last eluting compounds, respectively, in the LC run, this is probably due to the high number of interfering ions, temporarily crowding the MS–MS channels. At the beginning of the run, these interferences originate from the bulk of the non- or poorly retained, LC front associated constituents. At the end of the gradient run, the baseline, and the background ion intensity, gradually rise to such an extend that these ion intensities start to exceed the switching threshold. Finally, for bromazepam, poor ionisation efficiency was observed, resulting in the disappearance of the protonated molecular ion into the background noise.

Based on the obtained DDA data, the clean-up potentials of the apolar and polymeric mixed-mode packings were similar. As, at first sight, they seemed to perform equally well, the coefficients of variation of the EYs were calculated for each compound from the data obtained by DDA and compared, as illustrated in Fig. 4. This graph clearly shows that the coefficients of variation of the EYs obtained with the Isolute™ C_8 column are markedly better. In addition, the influence of a lower and higher MS to MS–MS threshold on these C.V.% has also been evaluated. Setting this parameter to a value of 400 affects the coefficients of variation in a positive way, as clearly illustrated in the graph. This can be attributed to the better defined peak shapes, obtained at this higher threshold value. The higher the MS to MS–MS threshold, the less switching to the MS–MS mode will occur. So, the peaks, recorded in MS mode and used as quantitative indication, consist of more data points, and are, as such, better described.

4. Conclusion

In conclusion, based on the presented results, the Isolute™ apolar C_8 columns have been chosen for further use in combination with DDA. However, the SPE procedure is still needs to be more extensively optimised. Our hierarchical approach has allowed a

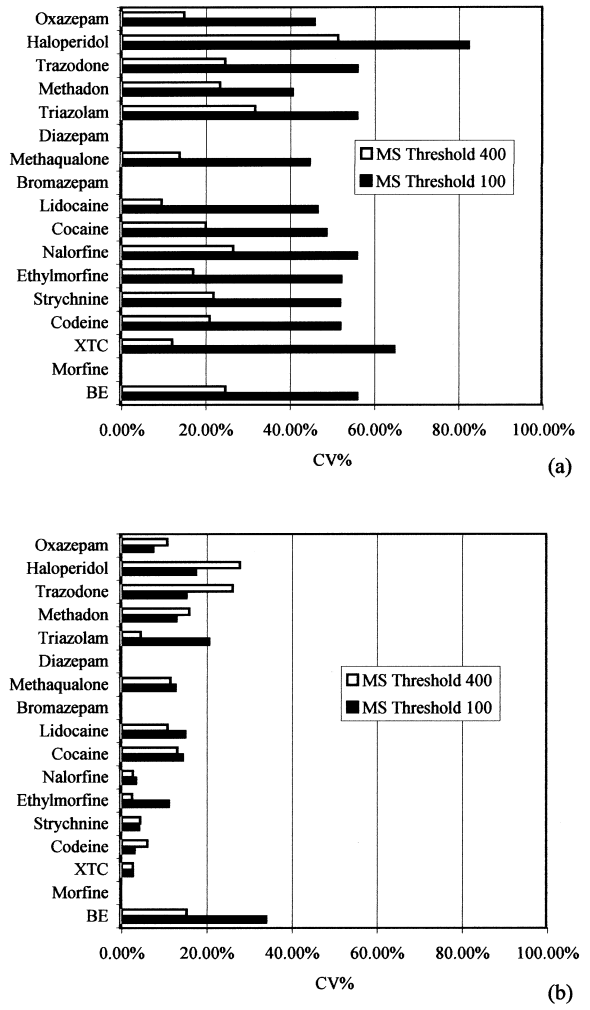

Fig. 4. Comparison of coefficients of variation for both evaluated SPE sorbents: (a) OASIS MCX versus (b) Isolute™ C_8 (fortified whole blood samples, triplicate extractions, DDA based detection).

reduction in the number of sorbents, the procedure for which can now be optimised in detail in an experimental design of realistic dimensions. At the same time, since three of the 17 spiked compounds were not fragmented in the DDA mode, certain modifications to the chromatographic conditions, especially to the k' value of the early eluting compounds, are to be implemented, contributing to the overall potential of the DDA approach to LC–MS–MS in STA. We conclude that the latter approach is a valid option even for a dirty matrix such as whole blood, provided a suitable SPE procedure is applied.

Acknowledgements

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