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Selective solid phase extraction of JWH synthetic cannabinoids by using computationally designed peptides

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ABSTRACT

The objective of the present work is to demonstrate a rational way to prepare selective sorbents able to extract simultaneously several structural analogs. For this purpose the binding specificity of two hexapeptides computationally designed (VYWLVW and YYIGGF) versus four synthetic cannabinoids Naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH 018), naphthalen-1-yl-(1-butylindol-3-yl)methanone (JWH 073), (R)-(1-((1-methylpiperidin-2-yl)methyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone (AM 1220) and (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN 55) was computationally studied and then experimentally tested by solid-phase extraction (SPE) clean-up and ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis. The two peptides were chosen using a semi combinatorial virtual technique by generating 4 cycles of peptide libraries (around 2.3×10^4 elements). To select the two peptides, the simulated binding scores between synthetic cannabinoids and peptides was used by maximizing the recognition properties of amino acid motif between the two JWH and the other synthetic cannabinoids. In particular, the peptide YYIGGF, having also affinity for AM 120, was selected as control because it was the only one without tryptophan residues within the best peptides obtained from simulation. Experimentally, the two hexapeptides were tested as SPE sorbent using nanomolar solutions of the four drugs. After optimization of best retentions the binding constants were calculated by loading synthetic cannabinoids solutions at different concentrations. The results indicated a strong interaction between hexapeptide VYWLVW and JWH 018 ($15.58 \pm 2.03\times10^6$ M^{-1}), 3-fold and 40-fold larger compared to the analog JWH 073 and both AM 1220 and the WIN 55. Similar trend was observed for the hexapeptide YYIGGF but the binding constants were at least three times lower highlighting the key role of the tryptophan. To demonstrate the hexapeptides specific interaction with only synthetic cannabinoids, a cross-reactivity study was carried out using other drugs (cocaine, morphine, phencyclidine and methamphetamine) in the same SPE condition. Finally the practical utility of these peptide modified sorbent materials was further demonstrated by detecting the synthetic cannabinoids in real samples using hair matrix.

1. Introduction

In the last years new synthetic cannabinoids were extensively studied in forensic science [1–5]. The importance of this type of drugs is well described by different works reporting that these new substances with cannabis-like effects are more and more frequently observed in the drug scene [6–9]. A fast and cheap detection of drugs of abuse can be carried out with immunochemical methods but, regrettably, those methods are unsuitable for a systematic toxicological analysis requested by these new molecules that continuously appear on the illicit market [10,11]. To have a determination of psychoactive substances and their metabolites with high mass accuracy, liquid chromatography can be coupled with high resolution mass spectrometry (MS) [12,13]. In modern illicit drug testing sample clean-up takes 50–75% of the total analysis time and remains one of the main bottlenecks [14]. Generally, to detect synthetic cannabinoids, commercial resins without

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any post functionalization are used to clean up samples like wastewater, human serum, whole blood, urine and hair [12,15–17]. Recently, new molecular recognition sorbents were proposed in SPE as aptamers, peptides and molecular imprinted polymers (MIPs) [18–21]. These new affinity media used in selective extraction, have similar performances of antibodies by overcoming several drawbacks as cost, variability in binding properties between lot-to-lot and chemical degradation [22,23]. Usually DNA aptamers and peptides are selected via combinatorial chemistry subjected to complex laboratory procedures that increase exponentially with the number of executed cycles [24,25]. The introduction of predictive computational models, instead of trial and error procedures, offers advantages in minimizing experimental problems like non-specific recognition, reagent stability and costs.

In the past years molecular modelling was used to rationally design synthetic receptors mostly in MIPs selection but only few times for peptides [26–29]. In this work we investigated the thermodynamic binding properties versus synthetic cannabinoids of hexapeptides used as stationary phase in SPE. The peptides were chosen after a virtual screening by applying a semi combinatorial strategy driven by maximizing the difference in binding within the synthetic cannabinoids studied. The peptides were tested as sorbent materials for retaining 4 different synthetic cannabinoids and the selectivity was proofed using other common drugs. Finally, the practical applicability of these sorbent materials functionalized with peptides were tested in real samples using hair matrix. This paper confirms that good results could be achieved without the use of only empirical methods such as combinatorial screening that require difficult and expensive procedures in terms of time and resources.

2. Materials and methods

2.1. Virtual screening

In a preliminary step, the zwitterionic form of all the considered peptides was generated using Hyperchem 8.0.5 software on a Microsoft Windows 7 laptop. All the other modelling steps were performed using Openeye Scientific Software tools (www.eyesopen.com) with free academic license. All molecular modelling experiments were performed on a in desktop 19 processors Intel Xeon X5690 at 3. 47 GHz each, with 94.5 GiB RAM, running Kernel Linux 2.6.32-642.1. 1el6.x86_64, GNOME 2.28.2. The main computational procedure can be resumed in the following steps:

1. Drawing ligands via LEXICHEM 2.1.0 package, by converting ligands standard IUPAC names into their corresponding structures [30].
2. Minimizing and optimizing molecular geometries of peptide libraries and ligands by means of SZYBKI 1.5.7 with default parameterization [31].
3. Creating conformers by exploring with OMEGA 2.4.6 the conformational space for both receptors and ligands [32–34].
4. Creating the binding box and docking peptide libraries to the synthetic cannabinoids using multi-conformer rigid body docking program OEDocking 3.0.0, also with default parameters [35].

For the molecular docking step, the peptides as a whole were considered as receptors, meaning that for each peptide conformer, the entire molecular surface was included in the active site box defining the area where ligands were expected to bind. The scoring function used in this step was Chemgauss4, a modification of Chemgauss3, with improved hydrogen bonding and metal chelator terms. The time elapsed for processing each peptide conformer was about 2 min per processor, covering the generation of initial 3D structures to final docking results. In the final step, the binding score average for each peptide was calculated over all the conformers. In the simulations, ten conformers per peptide and a maximum of 200 conformers for each of the four ligands were considered. This ensures a good compromise between calculation time and accuracy of the output data for this type of receptors [36]. In the docking process, a dedicated box (500–7500 $Å^3$) was generated for each receptor vs. the 4 synthetic cannabinoids used as ligands. The lower score values, calculated using Chemgauss4 scoring function, represented the higher ligand-receptor affinity.

For visualizing structures, generation of molecular surfaces and analysis of physicochemical properties it was used VIDA 4.2.1 [37]. The net charge of peptides at pH 7, and their isoelectric points were calculated by using an online tool for calculating peptide properties (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator.asp). The entire process was automated using a bash script and for post processing data analysis using a freeware BASIC-like scripting language (AutoIT V3) on a Microsoft Windows 7 laptop.

2.2. Experimental testing

2.2.1. Reagents

The standards of synthetic cannabinoids JWH 018, JWH 073, AM 1220 and WIN 55 were purchased from Cayman Chemical (Ann Arbor, MI, USA) at a concentration of 1 or 0.1 mg mL^{-1} based on availability. Standards of cocaine (COC), phencyclidine (PCP), morphine (MOR) and methamphetamine (MAMP) were purchased from LGC Standard (Italy). The purity of the reference compounds was > 99%. All standards were provided at a concentration of 3 mM. Individual stock solutions were prepared in methanol at 300 μM and working standard mixtures were prepared by appropriate dilution of the standard solutions in methanol. All solutions were stored at −20 °C in dark condition. All buffer reagents, methanol, acetonitrile and water were acquired from Fisher Scientific (Fair Lawn, NJ, USA). All solvents employed in the extraction were ultra-performance liquid chromatography (UPLC) grade, and LC–MS grade.

The solid phase extraction sorbent materials VYWLVW-resin (W) and YYIGGF-resin (F), with a peptide substitution level of 0.17 mmol g^{-1} and a synthesis reproducibility > 95%. were bought from EspiKem srl (Italy). The resin used to attach the hexapeptides was a Fmoc-PAL-AM. The C-18 cartridges (30 mg mL^{-1}) were from Phenomenex. SPE Isolute column (Empty 1 mL Reservoir) was from STEPBIO (Italy).

2.2.2. Solid phase extraction

The cartridges (volume 1 mL) were packed with 30 mg of resin (the blank) or modified hexapeptide resin dissolved in 5 mL of a methanol/water solution (80:20, v/v) and kept at room temperature for 6–8 h. This suspension was slowly loaded into the cartridge with a teflon frit on the bottom. During this procedure, the cartridge was continuously shaken in order to obtain a homogeneous packing. After loading, a second frit was used to cover the resin into the cartridge. Then the cartridge was conditioned and equilibrated by washing with methanol. All the experiments were carried out by means of a VISIPREP device and the solvent fractions collected were named progressively. The extraction procedure was performed in four steps:

1. Conditioning of the stationary phase with methanol/water solution (20:80, v/v).
2. Sample loading (1 mL).
3. Washing with 1 mL of phase with methanol/water solution (20:80, v/v).
4. Elution with 1 mL of methanol.

The same extraction procedure was applied to cartridges packed with the resin without hexapeptides (the blank) and the C-18 cartridge.

2.2.3. UHPLC-MS/MS analysis

The procedure was optimized and validated in a previous work [12]. Briefly, chromatographic separation was achieved with a Beta-Basic 18 column, 150×2.1 mm (Thermo Scientific, Bremen, Germany) held at a temperature of 40 °C and a flow rate of 0.6 mL min^{-1}. Mobile phases were 0.1% formic acid in water (Phase A) and 0.1% formic acid in acetonitrile (Phase B). The gradient elution was as follows: the initial composition (5% B) was increased from 5% to 50% B over 4.5 min, from 50% to 100% over 0.5 min, held at 100% for 2 min and returned to initial conditions over 1 min. A 2 min equilibration followed, yielding a total run time of 10 min.

The UHPLC equipment consisted of a Nexera LC20AD XR system, with autosampler, vacuum degasser and column oven, from Shimadzu (Tokyo, JA) coupled with a 4500 Qtrap from Sciex (Toronto, ON, Canada) equipped with a Turbo V electrospray ionization (ESI) source was used for multi-class drug analysis.

The analytes were detected in positive ionization (PI) with a capillary voltage of 5500 V, nebulizer gas (air) at 60 psi, turbo gas (nitrogen) at 50 psi at a temperature of 600 °C.

Two multi-reaction monitoring (MRM) transitions were chosen for each analyte. The ion currents were acquired in MRM mode and quantitation was performed by the IS method using Multiquant Software from Sciex. The selected MRM transitions and UHPLC–MS/MS parameters are reported in Table 1.

2.2.4. Hair extraction

For external decontamination cut hair was washed with water and twice methanol by vortex mixing for 2 min. Hair samples were fortified by soaking using a previously reported procedure [38]. Briefly, hair were cut and immersed in a flask containing a solution consisting of 1.5 mL of HCl (0.02 M in dimethyl sulfoxide (DMSO)) and 1.5 mL of water containing the analytes. The hair samples were then soaked for 21 days, filtered using a Büchner funnel and washed with water and methanol.

After removal of solvent, hair was air-dried and further cut into 1–2 mm segments. The sample was homogenized with diatomaceous earth (Sigma-Aldrich, Milan, Italy) using a mortar and pestle. The mixture was then placed in a 1 mL pressure resistant stainless steel cell that was sealed at both ends with cellulose filters. Void volumes in the cell were filled up with diatomaceous earth and 25 µL of an internal standard were added at a concentration of 50 nM in methanol. Hair incubation was performed by pressurized liquid extraction (PLE) using a Dionex ASE 200 (Sunnyvale, CA, USA) accelerated-solvent-extraction system. A single extraction cycle was performed using as extraction solvent a 70:30 (v/v) water–methanol mixture. The extraction conditions were as follows: pressure 100 bar; temperature 120 °C; preheat time 1 min; heat time 7 min; static time 5 min; flush volume 0%; purge time 60 s. The PLE extract (5–6 mL) was automatically collected in glass vial with caps solvent resistant (PTFE) septa. PLE extracts were transferred into a conical tube and centrifuged at 6000×*g* for 5 min at 25 °C.

Table 1
The selected MRM transitions and UHPLC–MS/MS parameters used for the 4 synthetic cannabinoids.

Analyte	t_R (min)	Q1 (amu)	DP (V)	EP (V)	Q3 (amu)	CE (V)	CXP (V)
JWH 018	3.25	341.90	37.00	12.00	155.00	34.00	6.00
					126.90	69.00	20.00
JWH 073	2.85	328.00	41.00	12.00	155.00	33.00	8.00
					126.90	62.50	22.00
WIN 55	2.25	426.90	45.00	10.00	154.90	40.00	25.00
					98.80	67.00	13.00
AM 1220	1.54	382.90	35.00	12.00	98.00	54.60	47.00
					112.00	35.00	33.00

3. Results and discussion

3.1. Virtual screening

An incremental construction approach was used by taking in every subsequent iteration, a focused library of peptides of increasing complexity, built on previous iteration results. The virtual screening procedure is resumed in Fig. 1. The approach aimed for higher ligand affinities by improving both the flexibility and chemical complementarity of peptides. In practical terms the simulated binding scores between synthetic cannabinoids and peptides were used to select in each screening stage 50 of the top average scoring peptides, 25 vs both JWH and 25 for the other two synthetic cannabinoids. These peptides had relevant aminoacid composition and structural features that allowed them to efficiently bind the ligands. In order to refine and improve their affinity for the ligands, these peptides were used as seeds for generating new combinatorial libraries by inserting each of the 20 natural aminoacids in every position of the sequence.

With this approach nearly 2.3×10^4 peptides were screened during the entire computational phase. However, this is a very small number compared to a full scale combinatorial screening of all 6.4×10^7 possible hexapeptides. Actually, with the strategy used, it was possible to identify good receptors for the ligands analyzed by exploring a minimum fraction (0.01% approximately) of the entire hexapeptides sample space. As shown in Table 2, each screening stage successfully narrowed the minimum – maximum binding scores variability, as well as the average binding scores, indicating that the peptides complexity increased together with their affinity for the ligands. It should be noted that positive binding scores indicated a virtually no positive interaction between ligand and receptor molecules, thus, association complexes were not favorable. The statistical behavior of peptides libraries confirms a symmetry in the distribution with average and median very similar in all cases.

These results highlighted a steady and significant trend to improve binding scores in each following stage. Libraries were more focused after each iteration, so it was possible to find better receptors for the ligands studied. The average scores toward the fourth and final stage show a tendency to stabilize, indicating that further iterations of the process may not give significant improvement over the results obtained at that point. For example, the difference of binding score averages between first and second stages, and second to third is approximately 1.27 units, while third to fourth iteration average binding score difference is just 0.72 units.

The weakest interactions were found with WIN 55, probably because of its size and high polarity when compared to the other ligands. Ligands AM 1220 and JWH 073 yielded similar results and slightly better affinities than JWH 018, in general all three had the best association complexes.

Once the final screening stage was completed, top scoring hexapeptides were evaluated. This results analysis was finalized to maximize the recognition properties of amino acid motif between the two JWH and the other two synthetic cannabinoids. The underlying idea was to find peptides with different affinities for the drugs studied, so that when they are used as sorbent materials, they could retain selectively functional analogs from the others drugs. In Table 3 an example of the average binding scores vs. the four synthetic cannabinoids obtained for the 20 top scoring hexapeptides vs JWH 018. The data analysis showed that top peptides had the same behavior in binding both JWH with also strong interaction with AM 1220 but less for WIN 55. The presence of aromatic residues in top peptides were relevant for an effective receptor-ligand association. Particularly, tryptophan was present in almost all the top hexapeptides vs JWH. Also, phenylalanine residue was well represented among the top ranked peptides.

In order to have in experimental trials a selective recognition between the synthetic cannabinoids, two peptides VYWLVW and YYIGGF were chosen, the first able to differentiate both JWH from the rest and the later with a good interaction also for AM 1220.

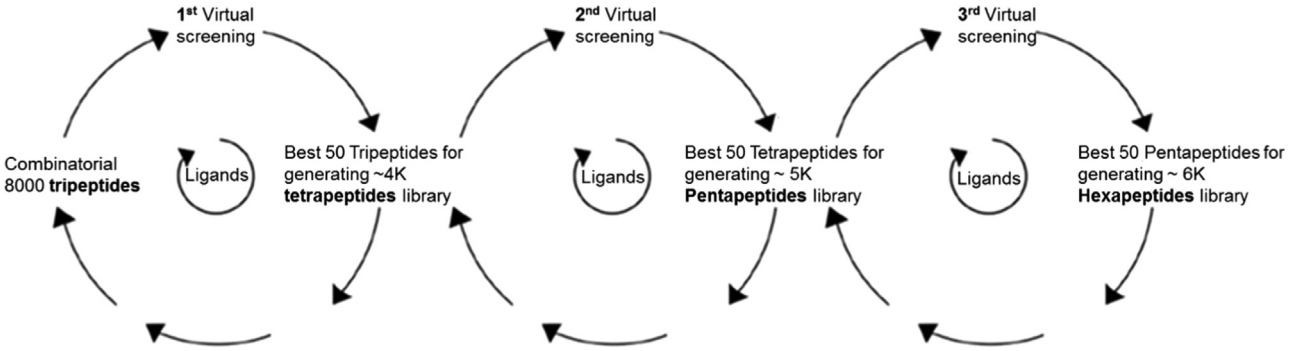

Fig. 1. The 4 steps virtual screening procedure. In the first three stages, starting with a library of 8000 tripeptides, peptides complexity was selectively increased in successive stages until reaching the hexapeptide length. The fourth and final stage aimed to identify the synthesis candidates. An approximate grand total of 23k peptides were screened in the entire process.

Table 2
Statistical parameters of the scores behavior obtained using 4 peptides libraries versus the 4 synthetic cannabinoids.

	JWH 018	JWH 073	AM 1220	WIN 55
		Tripeptides		
Min	−0.51	−0.55	−1.20	0.46
Max	5.93	5.35	5.63	7.74
Av	3.22	2.70	2.74	4.63
Median	3.31	2.77	2.82	4.67
		Tetrapeptides		
Min	−1.31	−1.35	−1.33	0.16
Max	4.73	4.35	4.13	7.34
Av	2.01	1.70	1.34	3.35
Median	2.16	1.78	1.30	3.35
		Pentapeptides		
Min	−1.92	−2.02	−2.27	−0.77
Max	2.65	2.55	2.62	3.54
Av	0.65	0.35	0.24	1.90
Median	0.6	0.36	0.15	1.79
		Hexapeptides		
Min	−2.54	−2.76	−2.95	−1.39
Max	2.18	1.89	1.84	2.89
Av	−0.10	−0.35	−0.42	1.14
Median	0.01	−0.30	−0.37	1.16

Table 3
The 20 hexapeptides with best binding score averages vs. JWH 018.

	JWH 018	JWH 073	AM 1220	WIN 55
TEAWWF	−2.54	−1.68	−1.04	−0.67
WWFYAF	−2.52	−2.75	−1.85	−0.51
VYWLVW	*−2.47*	*−2.28*	*−0.25*	*0.04*
WCNWFV	−2.46	−2.02	−2.04	0.15
KWWADF	−2.44	−2.12	−1.18	0.41
EWWAFM	−2.4	−1.90	−1.75	−1.01
EAAWWF	−2.37	−2.65	−1.23	−0.34
WWFAHF	−2.37	−2.28	−2.67	−1.19
CWWCWA	−2.32	−2.74	−2.73	−1.07
WWFAFL	−2.3	−2.74	−2.09	−1.34
WAHEWF	−2.29	−2.54	−1.96	−0.64
EAWQWF	−2.29	−2.21	−2.28	−0.94
AEAWWH	−2.26	−2.46	−1.45	−1.08
AEFWWH	−2.22	−2.5	−0.88	−0.31
IWWFAF	−2.2	−2.52	−1.05	−0.13
ELAWWF	−2.2	−2.27	−1.05	−0.11
MWHCFL	−2.19	−1.90	−2.47	0.12
YYIGGF	*−2.18*	*−2.23*	*−2.07*	*−0.05*
EAGWWH	−2.17	−2.49	−1.00	−1.01
VEAWWF	−2.15	−2.35	−1.01	−0.16

Moreover the peptide YYIGGF was chosen as control to study the interaction of peptide-synthetic drugs without tryptophan. In fact, this peptide was the only one without tryptophan residues within the top peptides vs JWH 018.

In Table 4 are presented some structural and physicochemical parameters calculated for each structure, both ligands and receptors. Peptide YYIGGF was significantly more polar than VYWLVW, because of the relatively high content of tyrosine and absence of tryptophan. Glycine residues could also contribute since they are small and their backbone has lower steric hindrance thus, is more exposed.

Regarding the ligands, the parameters calculated for JWH 018 and JWH 073 were similar since the only difference between them is a methyl group. They are hydrophobic molecules with few possibilities of forming H-bonds. However, WIN 55 is, in comparison, very polar and with higher propensity to form H-bonds. The differences observed are in agreement with simulations and suggest that both JWH ligands can form better association complexes with peptide VYWLVW, whereas peptide YYIGGF can also bind AM 1220. Both peptides had very weak interaction with WIN 55 because of strong polar surface area, therefore this ligand was used as control in experimental part.

The Interaction zones and type of contacts are shown in Fig. 2. The receptor-ligand interface in the association complexes were very similar in each peptide. Peptide VYWLVW formed an aromatic rich convex interaction surface with residues 2, 3 and 6. Ligands displayed a tendency to bury the indole system inside that hull, with nitrogen pointing to its bottom. On the other hand, the interaction zone of YYIGGF acquired a saddle-like configuration where almost all residues had a direct contribution except for isoleucine in position 3 which was facing outwards. Both cavities were highly hydrophobic, however, in YYIGGF the peptide backbone was closer to the surface granting a slight polar character to it. This would explain why AM 1220 and WIN 55 had better binding scores with this peptide than with VYWLVW. It is also important to note that the interaction zone of both hexapeptides with WIN 55 was displaced with respect to the other three ligands.

3.2. Experimental testing

The SPE protocol included conditioning, sample loading, washing, and analyte elution steps. In a first pilot test the volume ratio of methanol/water solution was studied. In both washing and condition steps, three different volume ratio of respectively 10:90, 20:80 and 30:70 were tested. For all the four synthetic cannabinoids loaded at the 100 nM an increase in retention was observed using methanol/water solution 20:80 v/v; when the methanol was over 30% a drastic decrease in retention was obtained for all the four drugs. These data drove to use methanol as elution solvent. In fact, after washing the cartridges with methanol all molecules were eluted and, in terms of reusability all experimental trials (more than 100 tests) could be carried out using the same cartridge for the duration of one month without any loose of performance. To evaluate no-specific interactions between drugs and resin, a cartridge was packed with the stationary phases without hexapeptides (blank) showing no significant retentions for all the four drugs (less than 10%) at the optimized SPE conditions (methanol/

Table 4
Calculated structural and physicochemical parameters of molecules selected in the experimental part. N confs=number of conformers; MW=molecular weight; PSA=polar surface area; RB=rotatable bond; Acc=Lipinski acceptors; Don=Lipinski donors.

Peptide	Net charge pH 7	Iso point (pH)	MW	LogP	PSA	RB	Acc	Don
F: YYIGGF	1	9.59	719	0.44	253	76	15	8
W: VYWLVW	1	9.84	865	4.02	265	101	16	9

Compound	Chemical formula	N Conf	MW	LogP	PSA	RB	Acc	Don
JWH 018	C24H23NO	200	341	6.54	22	46	2	0
JWH 073	C23H21NO	200	327	6.04	22	44	2	0
AM 1220	C26H27N2O	200	384	5.7	26	56	3	1
WIN 55	C27H27N2O3	85	428	3.49	44	60	5	1

water solution 20:80 v/v). It should be noted that before their application, the cartridges were swelled and dried several times because higher retention was observed after 10 cycles of conditioning and washing.

After this preliminary optimization, the pH effect on the hexapeptides retention was studied. The AM 1220 and WIN 55 can be slightly deprotonated by changing the pH and the hexapeptides were attached to the resin via the carboxyl-terminus leaving free to deprotonate the N-terminus group. Three buffer solutions, formate buffer (pH 3.5), phosphate buffer (pH 7.0) and ammonia buffer (pH 8.5) were prepared for loading the drugs. Drugs were loaded at different pH at 100 nM, the cartridges were then washed with 1 mL of methanol/water solution 20:80 v/v and the analyte was eluted using 1 mL of methanol.

Table 5 shows the SPE experimental results using the two hexapeptides as sorbent retaining 100 nM solutions of the four drugs at pH 3.5, 7.0 and 8.5. At pH 7.0 the results reflected those obtained in preliminary tests, with the highest retention (85%) showed by VYWLVW-resin for JWH 018 and a clear difference in retention between the two JWH and the other synthetic cannabinoids (AM 1220 and WIN 55). This behavior was predicted by the simulation for the hexapeptide VYWLVW but not for the hexapeptide YYIGGF that showed a significant simulated binding for the AM 1220. The two JWH, having a similar molecular structure, were retained by both hexapeptides in similar manner having a decrease in retention using ammonia buffer at pH 8.5 and no significant change in retention for pH 3.5. Only the YYIGGF-resin had a loss in retention particularly for JWH 073. A low retention was observed while using both AM 1220 and WIN 55 having a molecular structure rather different than the two JWH. Particularly AM 1220 at low pH presented the lowest retention for both hexapeptide sorbent materials (W and F).

Table 5
SPE experimental results using the selected hexapeptides as sorbent versus 100 nM solutions of the four drugs at pH 3.5, 7.0 and 8.5. F: YYIGGF; W: VYWLVW. The results were expressed as percentage in retention.

		pH 3.5 (%)	pH 7.0 (%)	pH 8.5 (%)
JWH 018	**F**	59 ± 3	72 ± 3	45 ± 2
	W	83 ± 7	85 ± 5	73 ± 6
JWH 073	**F**	50 ± 3	69 ± 6	48 ± 4
	W	76 ± 7	78 ± 5	76 ± 6
AM 1220	**F**	30 ± 2	51 ± 3	41 ± 4
	W	35 ± 2	55 ± 4	50 ± 3
WIN 55	**F**	45 ± 4	54 ± 5	44 ± 4
	W	52 ± 4	62 ± 3	61 ± 6

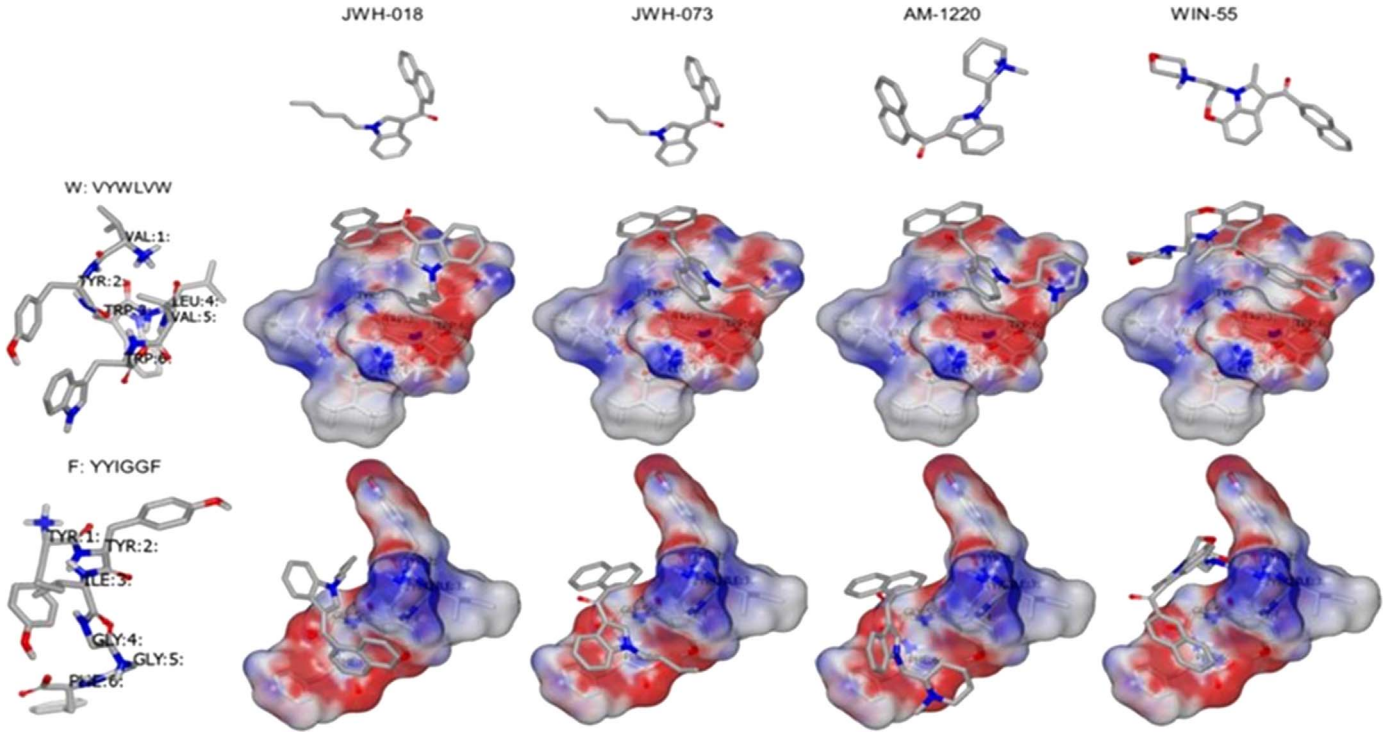

Fig. 2. Three-dimensional structures of the two hexapeptides and the 4 synthetic cannabinoids used in this work, as well as the best association complexes formed between them.

Table 6
Assessment between the virtual binding scores and the experimentally binding constants of hexapeptide-drug complexes, all calculated at pH 7.0. Binding score in percentage was calculated using the entire hexapeptides library results having the top and bottom score values respectively of −2.95 and 2.89.

	Experimental K binding $\times 10^6$ (M^{-1})		Virtual binding score %	
	F	W	F	W
JWH 018	2.27 ± 0.30	15.58 ± 2.03	87	92
JWH 073	0.98 ± 0.13	5.84 ± 0.41	88	89
AM 1220	0.18 ± 0.01	0.37 ± 0.05	85	54
WIN 55	0.21 ± 0.02	0.41 ± 0.04	50	49

The best retention condition for all synthetic cannabinoids was at pH 7.0 and this pH was used for calculating the binding constants of the hexapeptides-synthetic cannabinoids complexes. The binding constants were calculated by loading synthetic cannabinoids solutions at different concentrations ranging from 3.0×10^{-8} to 3.0×10^{-5} M. A saturation effect was observed at micromolar concentration. After subtracting the unspecific bound contribution given by blank cartridge retention, the bound drug was determined by subtracting the free analyte from the total loaded. Considering 1:1 complexation stoichiometry, the ratio between bound and free drug versus the bound one was plotted and the binding constant was calculated by fitting a linear regression through these data [39].

The results, reported in Table 6, indicated a strong interaction between hexapeptide VYWLVW and JWH 018 that was around 3-fold and 40-fold larger compared to the analog JWH 073 and both AM 1220 and the WIN 55. Similar trend was observed for the hexapeptide YYIGGF but the binding constants for the two JWH were at least three times lower. This behavior was predicted in simulation for the hexapeptide VYWLVW highlighting the key role of tryptophan in binding JWH, but not for hexapeptide YYIGGF showing, in these experimental conditions, no selectivity in binding also AM 1220. One of the factor influencing peptide-drug binding constant was the solvent effect. In experimental assay carried out in 20% of methanol, the solvent interference was relevant and receptor-ligand interactions could be more hindered for AM 1220 and the WIN 55 than for the two JWH, resulting in much lower affinity. In fact both AM 1220 and WIN 55 are richer in hydrogen donor/acceptor centers than the JWH counterparts.

The molecular docking functions used in this work ignored solvent-related terms (i.e., hydrogen bonding interactions with implicit solvent). The rigid body docking approach attempted to simplify the experimental conditions in order to process large amount of data in reasonable time. Considering this, it is well known that simulated and experimental results not necessarily always match. In this case, molecular docking provided the prediction of grossly wrong electrostatic properties thus allowing for careful experimental assays on a relatively small number of database compounds.

The binding properties of peptide sorbent materials were compared to the blank and C-18 SPE cartridges using the same extraction protocol. Moreover to demonstrate that in these SPE conditions the observed retention response by the hexapeptides had specific interaction with only synthetic cannabinoids and not with other drugs, we compared the specificity of the hexapeptide sorbent materials using 100 nM of other common drugs. For this purpose, cocaine, morphine, PCP and MAMP were chosen to study the cross-reactivity of the hexapeptides. Fig. 3A illustrates the selectivity of the hexapeptide sorbent materials (W and F), the blank and the C18 vs the four synthetic drugs. The Fig. 3B depicted the cross-reactivity performances of those cartridges in retaining the other drugs (cocaine, morphine, PCP and MAMP).

As expected, the C-18 resin having a wide interaction range showed a similar response for the four synthetic cannabinoids and the other

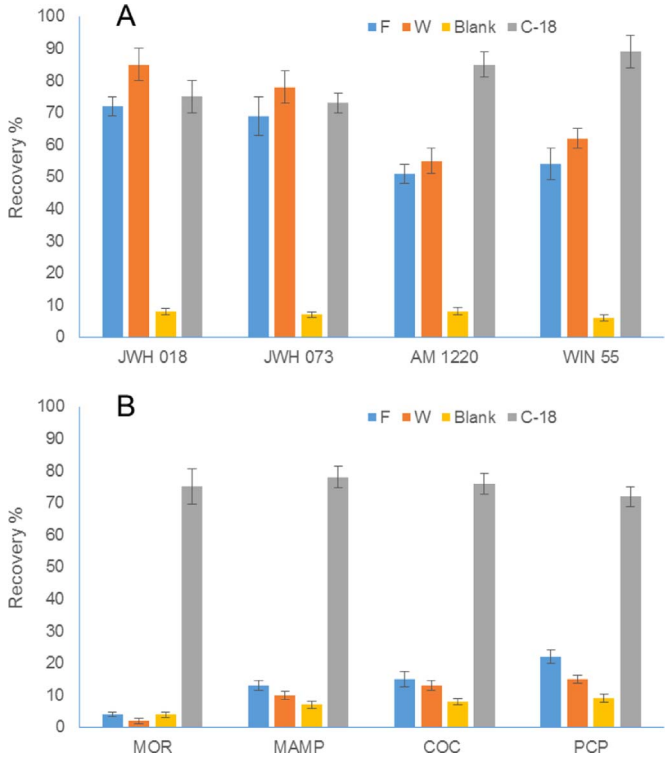

Fig. 3. Selectivity (A) and cross-reactivity tests (B) using the resins W, F, blank and C-18 versus: A) the 4 synthetic cannabinoids; B) Morphine (MOR), Methamphetamine (MAMP), cocaine (COC) and Phencyclidine (PCP). All drugs at the concentration of 100 nM. The drugs recovery was reported in percentage.

drugs. The blank in all cases had a poor retention demonstrating no interference in studying the peptides binding. The response in retention of the peptides cartridges, obtained under the same experimental conditions and using the same drugs concentration (100 nM) demonstrated clearly that both peptides resins retained only the synthetic cannabinoids with at the least six fold less retention for the other drugs. These results are consistent with previous and confirm the possibility to use these peptides as selective sorbent materials for JWH synthetic cannabinoids.

The practical utility of these modified sorbent materials was further demonstrated by detecting the synthetic cannabinoids in real samples using hair matrix. Hair matrix is often used in forensic laboratories because it offers wider time window, non-invasive sampling and good stability of the analytes over time. Moreover, On the basis of literature data, hair sample is quite more challenging than urine in terms of complexity, especially in synthetic cannabinoids detection [12,40–42].

Hair extraction procedure involved a preliminary washing step, hair incubation by pressure PLE followed by SPE clean-up. All steps were optimized in a previous work to obtain best recoveries and low matrix effect [12]. The hair samples were fortified by soaking that is considered a good alternative for drug users' hair in analytical purposes [43]. In the present study, the first experiments were focused on an optimization of extraction solvent using two different buffer/methanol 80/20 v/v mixtures one more acidic using acetate and the other with phosphate at pH 7.0. In acidic condition poor recovery was obtained, but it was observed an increase of drugs elution with using phosphate buffer thus a better SPE recovery.

Tests were carried out using 50 mg of hair sample fortified with a mix of the four synthetic cannabinoids each at 100 nM. After PLE procedure, the extracts were processed with the SPE parameters previously optimized, using VYWLVW-resin (W) and C-18 cartridges. As illustrated in Fig. 4 the W resin had a good retention of both JWH decreasing for the other two synthetic cannabinoids as also reported by the data obtained in standard solutions. The C-18 resin gave an

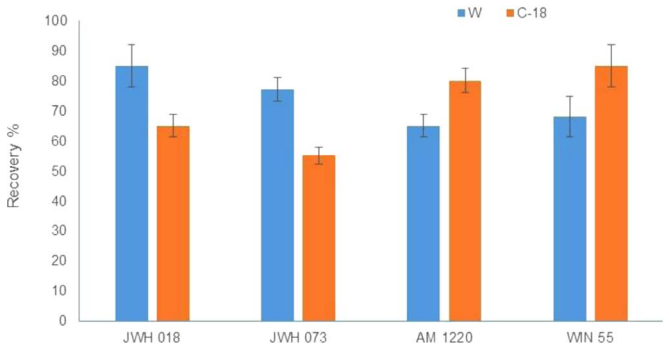

Fig. 4. Hair matrix analysis. The recovery of resin W and C-18 vs the 4 synthetic cannabinoids was reported in percentage.

opposite response in retention the synthetic cannabinoids with poor recovery for both JWH and more than 80% for AM 1220 and WIN 55. Such behavior should be explained by steric effects more than by hydrophobic properties. In fact, the JWH logP, the hydrophobic parameter reported in Table 4, was higher than the one calculated for the other two synthetic cannabinoids.

The selectivity performances of resin W towards C-18 sorbent was proved by the evaluation of matrix effect: in hair matrix usually significant matrix effect often occurs with a strong suppression of ion signal in ESI; the extracts that have been submitted to resin W clean-up showed very low ion suppression for both JWH (< 5%), 10% for WIN 55 and 18% for AM 1220. The ion suppression for the same analytes recorded using C18 cartridges was quite higher (> 30% for all analytes).

Despite the difference between standards and real samples, the use of hair samples confirmed a good selectivity within the four synthetic cannabinoids studied and this can be useful when selective separation is necessary.

4. Conclusions

Avoiding very large procedures like combinatorial work, we obtained a focused peptides with high affinity properties vs the two JWH synthetic cannabinoids. The approach was based on the concept that recognition properties of amino acid motif can be increased by an incremental construction approach by taking in every subsequent iteration, a focused library of more complex peptides showing greater binding properties. The work can be repeated for as long as necessary to identify a ligand with sufficient affinity.

Binding constants calculated in experimental step confirmed the purpose to have a selective response between synthetic cannabinoids, supporting the semi combinatorial virtual procedure that was carried out maximizing the differences of the peptides affinity vs the synthetic cannabinoids. This fact was also confirmed in SPE by the two hexapeptides having specific interaction with only JWH synthetic cannabinoids and not with other drugs (cocaine, morphine, phencyclidine and methamphetamine). Moreover the hexapeptide sorbent materials showed completely differ retention with drugs compared with a commercial C-18 cartridge. These differences were also highlighted using hair matrix showing the practical applicability of these kind of pre analytical selective clean up tools in real samples analysis.

However, the experimental results confirmed only in part the feasibility of virtual screening to produce peptides with selective properties. In fact using the virtual designed peptides YYIGGF it was not possible to have a good experimental binding affinity for AM 1220 as predicted in virtual screening. In this case simulation and experiments did not match probably because the molecular docking functions used in this work ignored solvent-related terms (i.e., hydrogen bonding interactions with implicit solvent). In both virtual and experimental data the weakest interactions were found with WIN 55. This was due to its size and high polarity when compared to the other ligands. For those kind of compounds other dedicated virtual procedure has to be carried out. This methodology can be extended to other new drugs that are more and more frequently observed in the illicit market.

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