

Conjugation of the Dark Quencher QSY 7 to Various Synthetic Cannabinoids for Use in Fluorescence-Based Detection Platforms

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14. ABSTRACT We have been able to synthesize and characterize several synthetic cannabinoid–dark quencher (DQ) conjugates for the use in fluorescence-based cannabinoid detection platforms. The carboxylic acid reactive QSY 7 amine was reacted with the carboxylated metabolites of JWH 018 and JWH 073 via a simple peptide conjugation reaction with the catalyst O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate to yield 3 new DQ conjugates. Thin layer chromatography and liquid chromatography coupled mass spectrometry show full conversion of the synthetic cannabinoid starting material to product with observed and expected masses of 1,097 g/mol for JWH 018 pentanoic acid–QSY 7 amine and 1,081.4 g/mol for JWH 073 butanoic acid–QSY 7 amine. These characterized conjugates are now ready for cannabinoid-receptor-based binding assays.					
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1. Introduction

Synthetic cannabinoids (SCs) are chemical compounds that were developed to bind to the cannabinoid (CB) receptors within the human body as either agonists or antagonists of receptor function.¹⁻⁶ There are 2 CB receptor subtypes in humans: cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). CB1, or the central cannabinoid receptor, is expressed mainly in the central nervous system while CB2 is known as the peripheral CB receptor and is mainly expressed in the peripheral nervous system by immune cells primarily in the thymus, bone marrow, and spleen.⁷ Originally developed to treat a wide variety of diseases from obesity to cancer therapeutics, SCs have more recently been marketed as recreational drugs in the form of herbal smoking mixtures.⁸⁻¹⁷ These drugs have names such as Spice Gold, Spice Silver, and Yucatan Fire and are legal in many countries and states (Fig. 1).¹⁸ The marketing of these SCs has led to disastrous effects, as these compounds tend to be much more potent than traditional cannabis.^{12,17} Cannabis sativa contains tetrahydrocannabinol (THC) as the active psychotropic ingredient and has an affinity of 10 nM for CB1 and 24 nM for CB2. As Spice and similar products contain compounds specifically developed to bind to the CB receptors, their affinity for the CB receptors is much greater. For example, the first-generation synthetic cannabinoid HU-210 has affinities of 0.061 nM and 0.52 nM for CB1 and CB2, respectively, which is approximately 164 and 46 times, respectively, tighter than THC for the same receptors.^{12,17} This increase in affinity is proportional to the dramatic increase in potency observed with these compounds.



Fig. 1 Varieties of herbal smoking mixtures laced with synthetic cannabinoids

Until recently, many of these herbal mixtures were perfectly legal and available for purchase at many gas stations, convenience stores, and through the Internet.^{1,19,20} Abuse rates have skyrocketed over the past few years: A 2010 poll by the US Drug Enforcement Administration showed that 35% of juveniles tested for drugs tested positive for SCs, and in 2011, 11% of high school seniors admitted to trying SC-laced herbal blends.²¹ Moreover, SC abuse is rampant within the armed forces, with more than half of the tested personnel showing a positive result in 2012.²² These data have led to a widespread push to make these mixtures illegal. This aim is not easily achieved, as the manufacturers are constantly modifying the compounds at various substituent points to avoid detection by the authorities (Fig. 2). Although it is possible to detect SCs in serum and oral fluid, a library of known compounds is needed to screen the tests for all of the known SCs.^{23,24} Therefore, this method does not keep pace with the ever-increasing variations of SCs, as the compounds must be known to be detected, leading to a long turnaround time, in turn leading to a backlog of tens of thousands of samples.

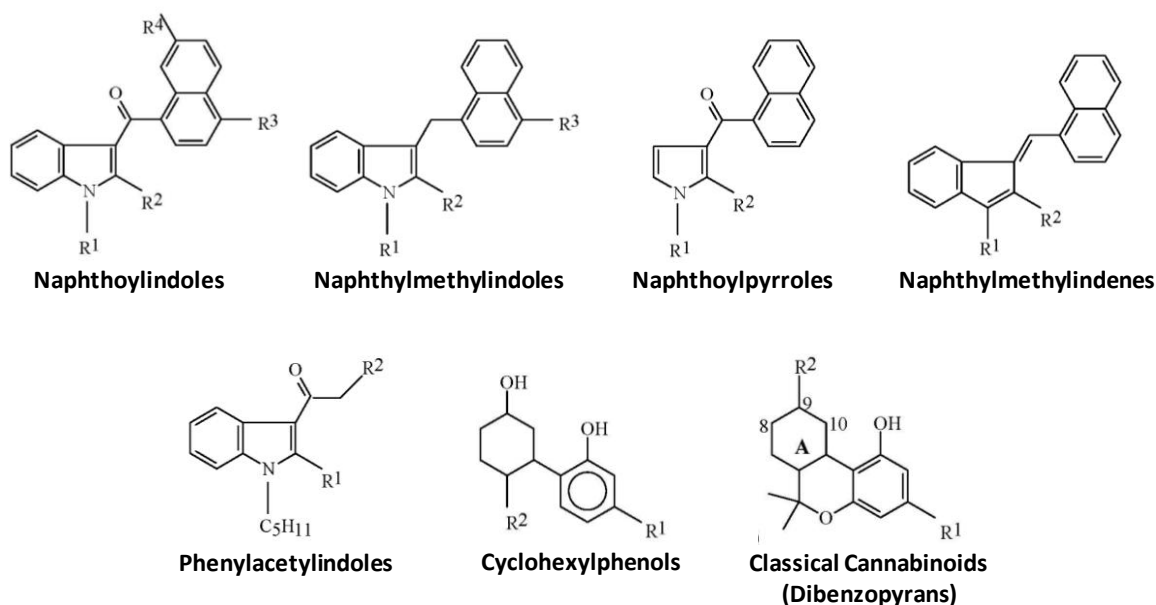


Fig. 2 Basic chemical structures of the 7 different synthetic cannabinoid groups. The R groups are positions at which substituent variants are possible.

To circumvent the previously described detection limitations, a new detection methodology must be developed. An attractive approach would be to harness the CB binding capabilities of the CB receptor proteins that would negate the need for a library of SC compounds as a reference for detection. Thus, this approach would enable enforcement to keep pace with the development of novel SCs by drug manufacturers.

Clearly, the development and characterization of such a detection system would provide numerous advantages over the currently available methods including 1) immediate turnaround time with a simple yes or no output, 2) no need to look for specific or known SCs, as any

compound that binds the receptor will give a positive hit, and 3) enforcement would be able to keep pace with the development of novel SCs for recreational use.

There has been recent success using receptor-protein-based fluorescent biosensors to measure ligand/protein interactions.²⁵ These sensors employ quantum dots (QDs) and dark quenchers (DQs) to monitor the binding of a ligand to a protein. The protein is conjugated to the QD while the DQ molecule is conjugated to a receptor ligand. In this sensor, the DQ/low-affinity ligand binds to the protein QD complex and the fluorescence of the QD is quenched. Subsequent binding of the higher affinity ligand of interest displaces the quencher complex and the fluorescence of the QD can be measured (Fig. 3). Medintz et al. constructed such a sensor in 2003 to monitor maltose binding to the maltose binding protein.²⁵

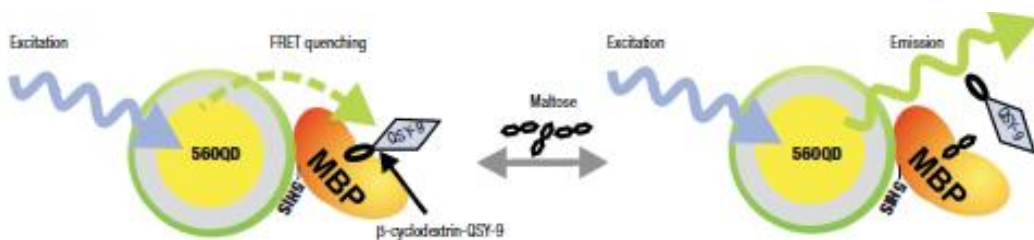


Fig. 3 QD/DQ-based biosensor developed for maltose detection by Medintz et al.²⁵

A properly designed ligand-DQ system that is highly robust and functional is critical to the development of both the fluoroanalytical and bionanoelectronic synthetic cannabinoid detection platforms. The primary design requirements of the ligand-DQ conjugate are 2-fold.

1. A ligand whose affinity for the CB2 receptor protein is in a range such that it is readily displaced by the majority of synthetic cannabinoids while not being competitively displaced by other molecules (noncannabinoids) in the system.
2. Transport a DQ molecule within fluorescence resonance energy transfer (FRET) coupling range of the fluorescent QD.

We have successfully synthesized and characterized several synthetic CB-DQ conjugates for the use in fluorescence-based cannabinoid detection platforms. Specifically, the DQ QSY 7 was reacted with the CB receptor ligand metabolites JWH 018 n-pentanoic acid and JWH 073 n-butanoic acid to create 2 different DQ conjugates for future use in a receptor-based SC detection assay. To our knowledge, these conjugates are the first of their kind to be successfully synthesized.

2. Materials and Methods

2.1 Chemicals

Dimethylformamide (DMF), O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU), triethylamine (TEA), acetonitrile, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO); JWH-018 pentanoic acid and JWH-073 butanoic acid were purchased from Cayman Chemical (Ann Arbor, MI); and QSY 7 amine was purchased from Life Technologies (Carlsbad, CA). All solvents were of high-performance liquid chromatography-grade or higher and used without further purification. Ultrapure milli-Q water was used for all experiments.

2.2 Synthesis and Purification of JWH-018 Pentanoic Acid and JWH-073 Butanoic Acid to QSY 7 Amine Conjugates

Synthesis: Molar equivalents and quantities of reagents used in the synthesis protocol are highlighted in Table 1. QSY 7 amine and JWH-018 or JWH-073 were each resuspended in 500 mL of DMF and added to a 5-mL vial. HBTU was added to the vial, then the mixture was stirred vigorously for 5 min, after which TEA was added. After stirring for 4 h, the reactant to product conversion was monitored via thin layer chromatography (TLC) in 100% ethyl acetate.

Purification: For product purification, the reaction was diluted 3× with ethyl acetate and filtered through celite. The excess solvent was removed under reduced pressure and dried under high vacuum.

Table 1 Molar equivalents and quantities of reagents used in the synthesis protocol

Compound	Molar Equivalents	Used (mmol)	Molecular Weight	Amount Added (mg)
JWH-018 pentanoic acid	1	0.013	371.4	5
JWH-073 butanoic acid	1	0.013	357.4	5
QSY 7 amine	1.2	0.0156	814.86	10
TEA	3	0.039	101.19	4

2.3 Liquid Chromatography Coupled Mass Spectrometry (LC-MS) Analysis of JWH-018 Pentanoic Acid and JWH-073 Butanoic Acid to QSY 7 Amine Conjugates

The overall purity of the Win55-212 extracts was analyzed via LC-MS. A single quadrupole Agilent 6130 mass spectrometer was used in conjunction with an Agilent 1200 series LC system (Agilent Technologies, Santa Clara, CA). The LC column was an Agilent Eclipse XDB C18 column (150- × 4.6-mm interior diameter, 5-μm particle size), maintained at 25 °C with a mobile

phase flow rate of 0.6 mL/min. Gradient elution mobile phases consisted of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at pH 3.6. The gradient initially began at 0% B and remained isocratic until 2 min. The gradient increased linearly to 100% B from 2 to 50 min. Any remaining compounds were eluted from the column during a wash with 100% B from 50 to 60 min. Detection wavelengths for are shown in Table 2 and are the maximum absorbance wavelengths given by the chemical suppliers. Quantification of the analytes was undertaken using positive scan mode with a molecular mass scan from 100 to 1,200 g/mol.

Table 2 Detection wavelengths and expected masses of compounds used in this study

Compound	Detection Wavelength (nm)	Expected Mass (g/mol)
JWH-018 pentanoic acid	218, 316	371.4
JWH-073 butanoic acid	218, 246, 315	357.4
QSY 7 amine	560	741.08
JWH-018 pentanoic acid: QSY 7 amine	218, 316, 560	1,095.42
JWH-073 butanoic acid: QSY 7 amine	218, 246, 315, 560	1,081.42

3. Results and Discussion

3.1 Characterization of SC to DQ Conjugation Efficiency Via LC-MS

Synthesizing an SC to DQ conjugate is the first step in creating a functional receptor-based sensor. Several considerations must be taken into account when developing a functional conjugate. First, the conjugate must be stable and allow the SC domain of the molecule to diffuse into the lipid for receptor binding. Second, the final conjugate needed to contain enough polarity to prevent it from remaining trapped in the lipid within FRET coupling distance of the receptor/QD complex.

The SC used in synthesis must have a chemical moiety that reacts with the functional group on the DQ. As we were using QSY 7 functionalized with an amine (Fig. 4A) for our DQ, we needed an SC that contained a carboxylic acid, aldehyde, or ketone in order of descending reactivity.

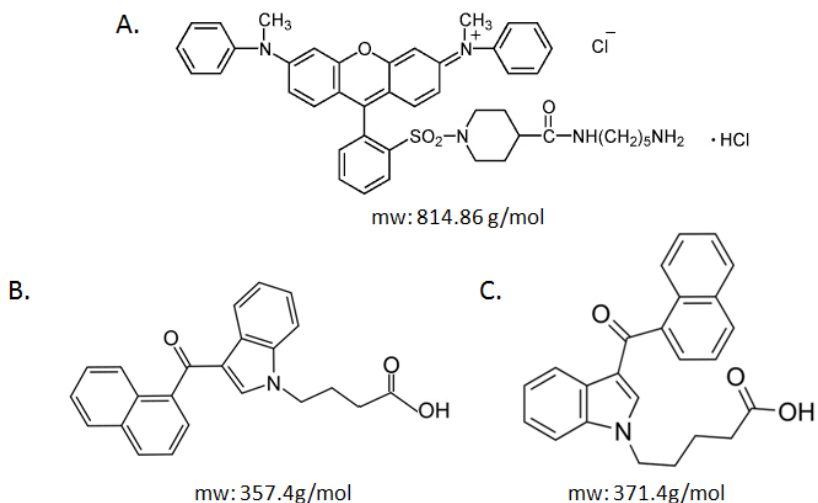


Fig. 4 Structures of QSY 7 amine (A), JWH-073 n-butanoic acid (B), and JWH-018 n-pentanoic acid (C)

There are no available SCs with carboxylic acid moieties; however, there are several phase 1 carboxylated metabolites of common SCs that have carboxylic acids in a variety of positions. JWH-073 n-butanoic acid and JWH-018 n-pentanoic acid are examples of phase 1 carboxylated metabolites and were the compounds chosen for conjugate synthesis (Fig. 4B and C). The SCs and QSY 7 amine were reacted in a 1:1 molar ratio under standard peptide coupling conditions (HBTU/TEA/DMF) to form a final product linked via a peptide bond (Fig. 5A and B). TLC analysis showed complete disappearance of the parent SC and appearance of product after 4 h.

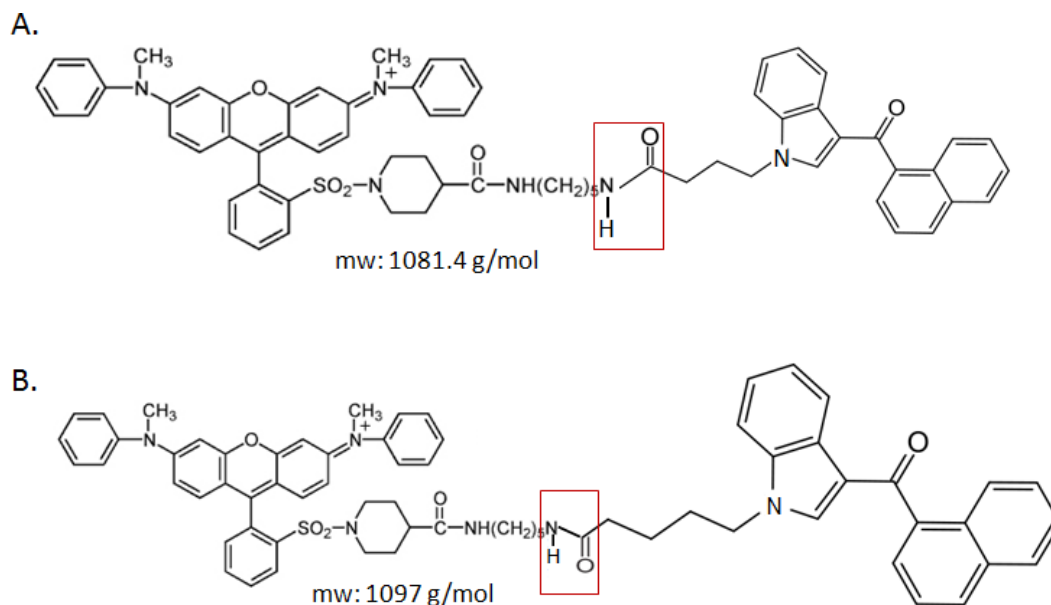


Fig. 5 Structure of QSY 7:JWH-073 n-butanoic acid conjugate (A) and QSY 7:JWH-018 n-pentanoic acid conjugates (B). The red boxes highlight the location of the peptide bond that links the SC to the DQ.

The reaction products were characterized with LC-MS (Figs. 6–8). Control runs were conducted with JWH-018 n-pentanoic acid, JWH-073 n-butanoic acid, and QSY 7 amine alone to determine retention time and separation and are shown in Figs. 6–8. The LC-MS of QSY 7 amine alone elutes at approximately 41 min with an observed protonated (from positive scan mode) mass of 784.4 g/mol. This observed mass is higher than the expected mass of QSY 7 amine without its conjugate salt (778.4 g/mol) but is consistently observed at this value ($n = 3$). One explanation could be that there are several protonation points throughout the molecule that become protonated in positive scan mode. The SC JWH-073 n-butanoic acid elutes at 34.9 min with an observed mass⁺ of 358.2 g/mol (expected: 357.4). The good separation in elution times of JWH-073 compared with that of QSY 7 amine (34.9 versus 41.5, respectively) enables simplified product analysis. JWH-018 n-pentanoic acid elutes at approximately 35 min, also giving good separation between the elution time of SC and DQ. The observed mass⁺ of 372.3 g/mol is in very good agreement with the expected, nonionized mass of 371.4 g/mol. In addition, both of the SCs and QSY 7 amine were very pure, as both the LC spectrum and mass analysis of the LC peak showed only the parent compound and the $m/2$ or $2/m$ mass for the compounds.

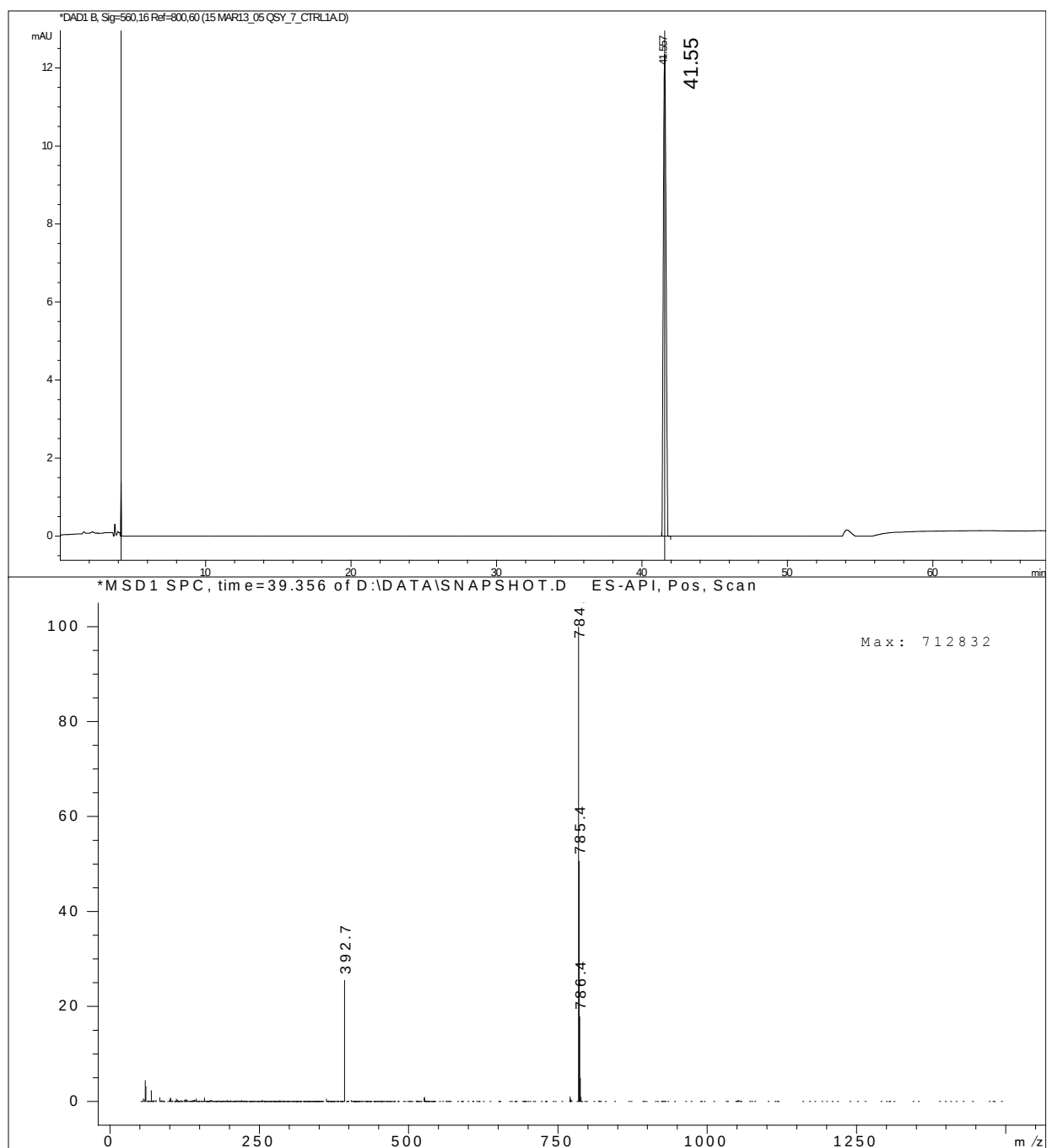


Fig. 6 LC chromatogram (top) and mass (bottom) of QSY 7 amine in DMF. QSY 7 amine has a retention time of 41.55 min and an observed mass⁺ of 784.4 g/mol (expected: 778.4 g/mol without hydrogen chloride salt).

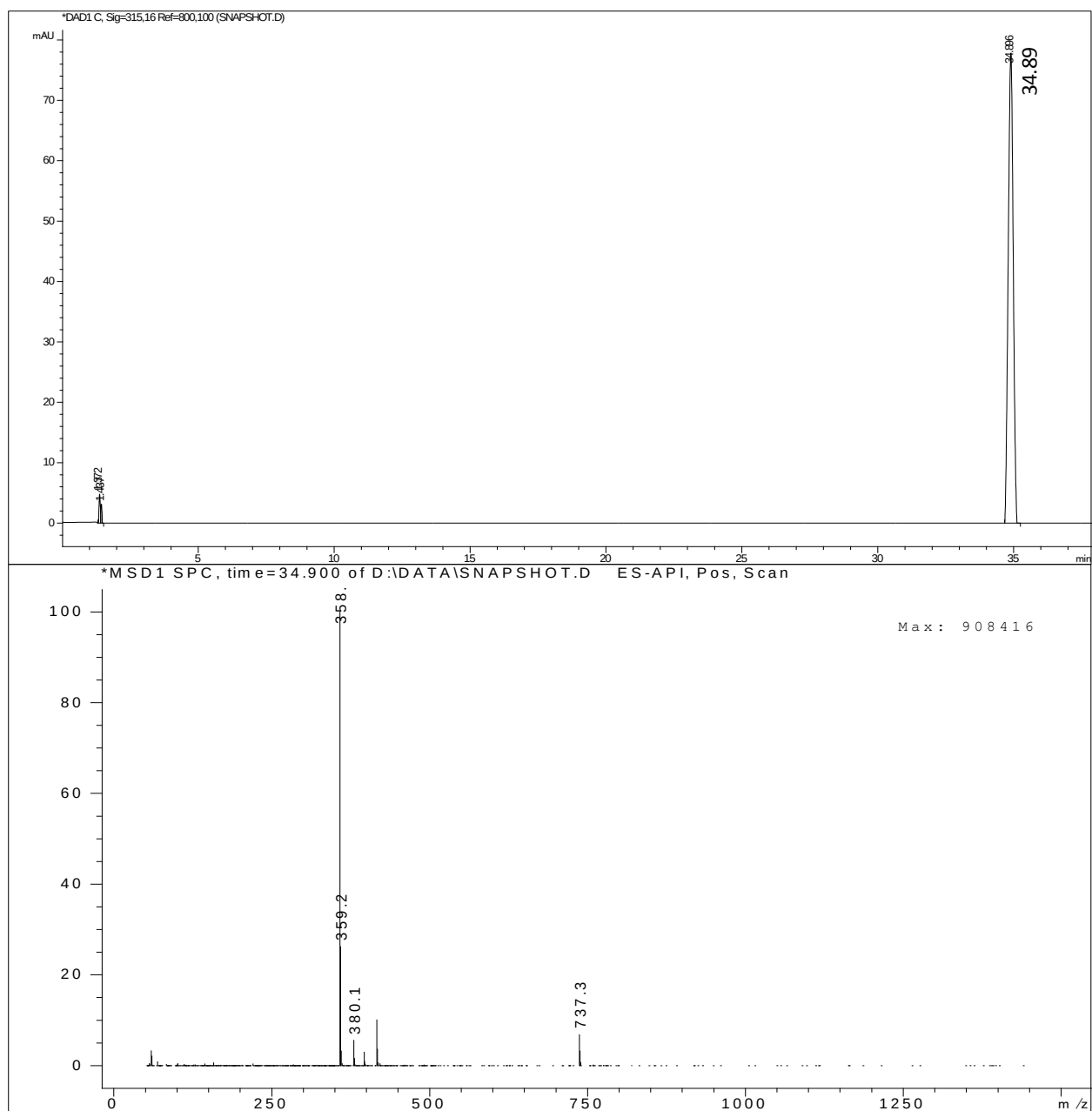


Fig. 7 LC chromatogram (top) and mass (bottom) of JWH-073 n-butanoic acid in DMF. JWH-018 n-pentanoic acid has a retention time of 34.9 min and an observed mass⁺ of 358.2 g/mol (expected: 357.4g/mol nonionized).

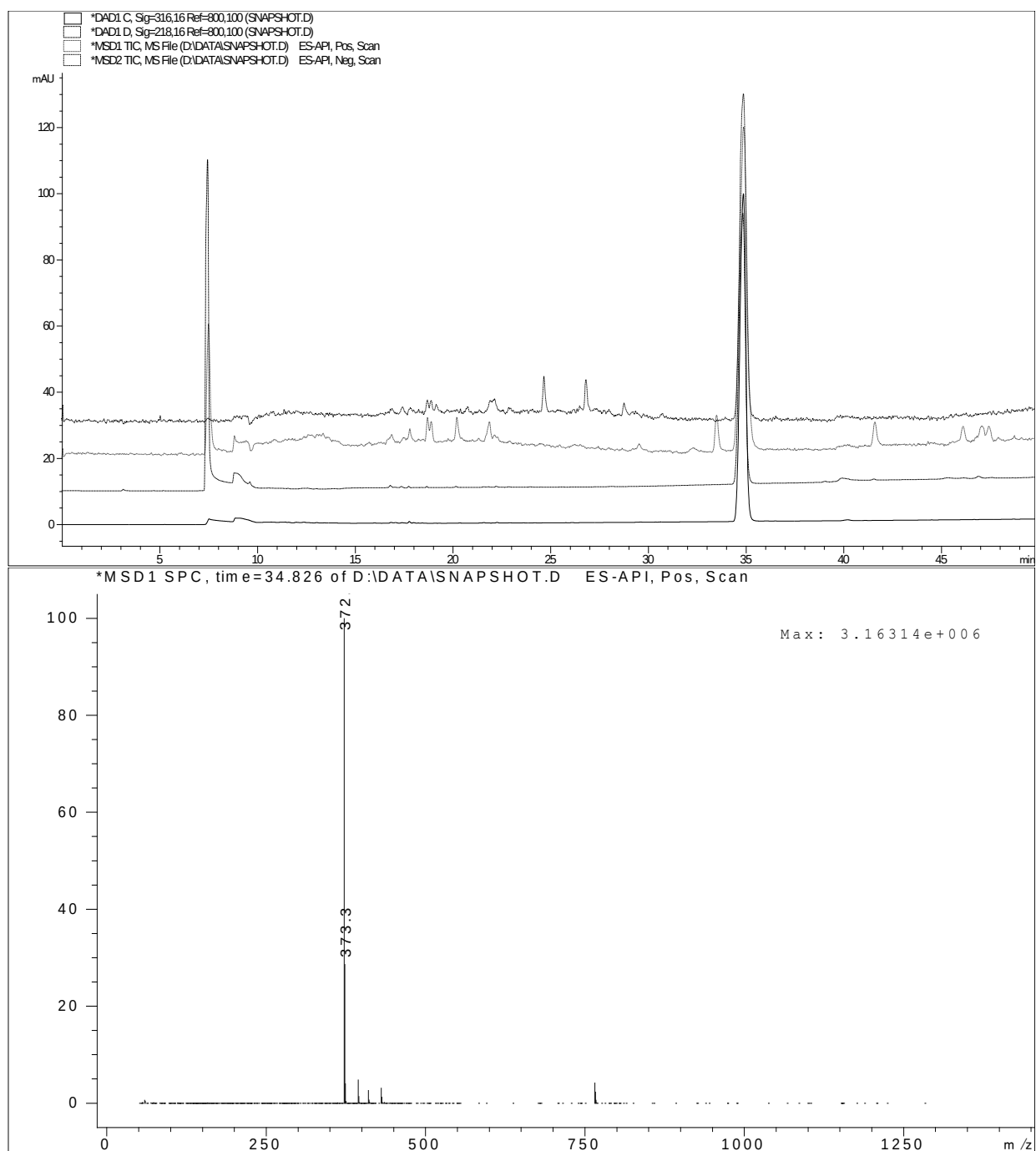


Fig. 8 LC chromatogram (top) and mass (bottom) of JWH-018 n-pentanoic acid in DMF. JWH-018 n-pentanoic acid has a retention time of 35 min and an observed mass⁺ of 372.3 g/mol (expected: 371.4 g/mol nonionized).

After full LC-MS characterization had been completed with the starting materials, the products were subjected to LC-MS and analyzed for purity (Fig. 9). The JWH-073 n-butanoic acid:QSY 7 amine conjugate eluted at 50 min with an observed mass of 1,081.5 g/mol. The predicted mass of the conjugate is 1,081.42 g/mol. Thus, JWH-073 n-butanoic acid:QSY 7 amine conjugate is not

protonated in the mass spectrometer. The peak for the conjugate was sharp and intense, which is indicative of a pure product. No residual JWH-073 n-butanoic acid was observed after the reaction; however, there was a small amount of QSY 7 amine (purple arrow, Fig. 7) that was not completely consumed. As the SC and the DQ should react in a 1:1 ratio, it is possible that the JWH-073 n-butanoic acid was slightly degraded over the course of the reaction or that the SC was slightly impure upon arrival.

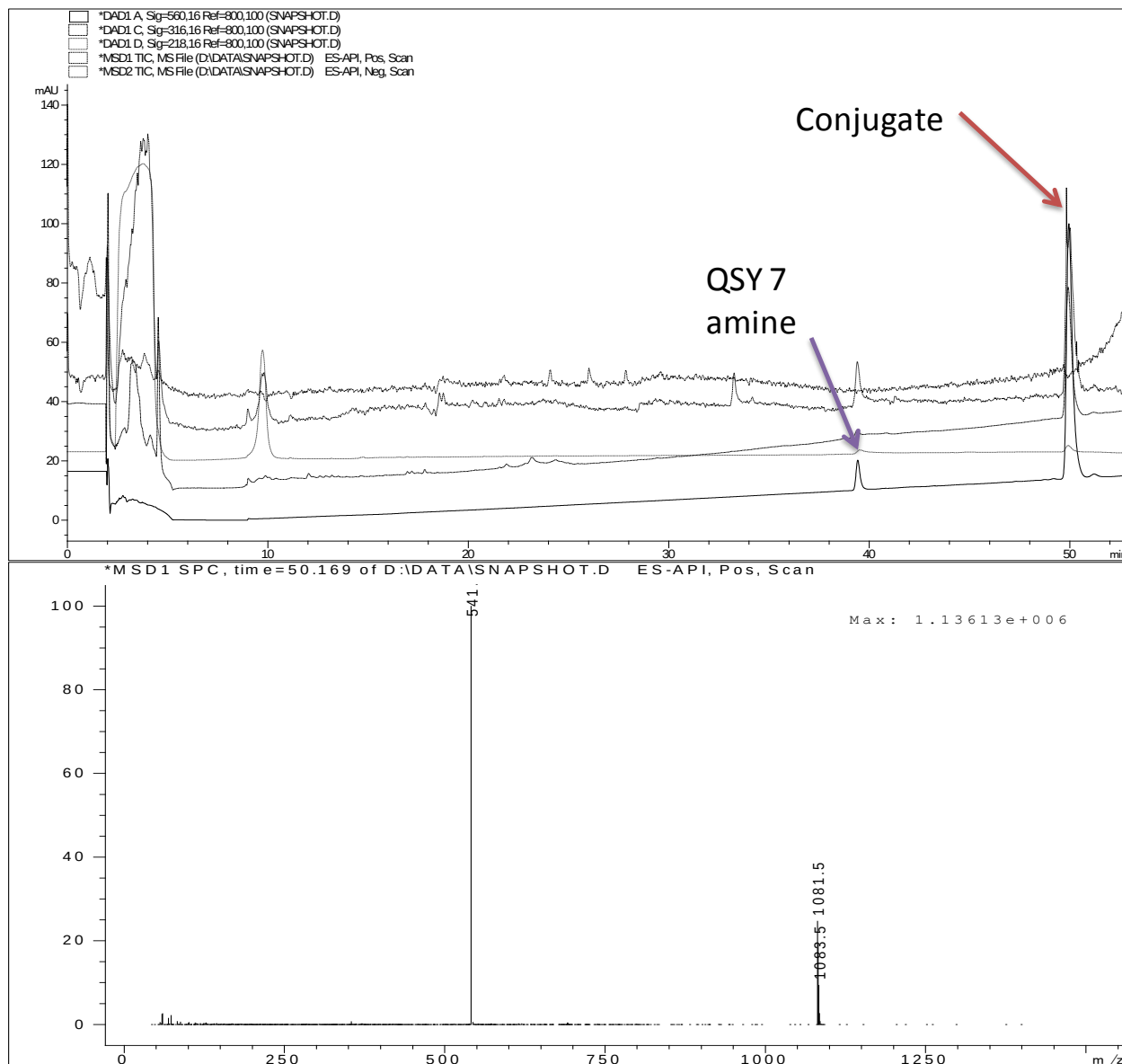


Fig. 9 LC chromatogram (top) and mass (bottom) of the JWH-073 n-butanoic acid: QSY 7 amine conjugate in DMF. JWH-018 n-pentanoic acid has a retention time of 35 min and an observed mass of 1,081.5 g/mol (expected: 1,081.42 g/mol nonionized).

LC-MS analysis of the JWH-018 n-pentanoic acid:QSY 7 amine conjugate showed a reasonably pure product that elutes at 51.5 min. The observed mass of the conjugate was 1,095.7 g/mol, which is in very good agreement with the expected mass of 1,095.42 g/mol. Similar to the JWH-073 conjugate, the JWH-018 conjugate also shows some residual QSY 7 amine remaining after the conjugation reaction. This observation lends further evidence to the possibility that the SCs were slightly impure or degraded upon arrival from the vendor. Moreover, some contamination can be seen in the mass spectrometer trace of JWH-018 n-pentanoic acid shown by the dashed lines in Fig. 10.

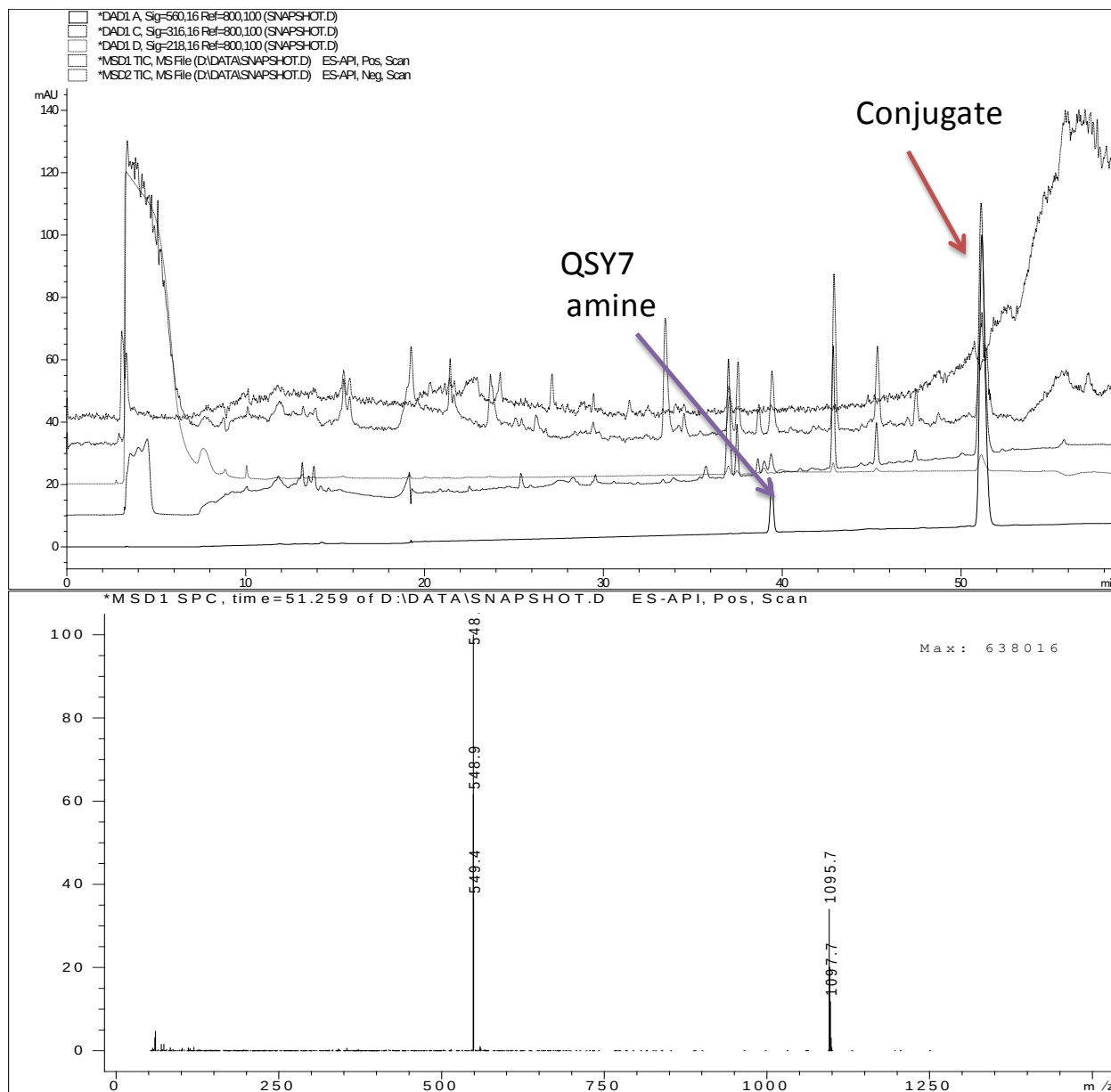


Fig. 10 LC chromatogram (top) and mass (bottom) of JWH-018 n-pentanoic acid:QSY 7 amine conjugate in DMF. JWH-018 n-pentanoic acid has a retention time of 35 min and an observed mass⁺ of 372.3 g/mol (expected: 371.4 g/mol nonionized).

4. Summary and Conclusions

We have successfully synthesized 2 SC-DQ conjugates for use in a receptor-based SC detection assay. To our knowledge, these conjugates are the first successful attempt at linking a dye to a CB compound. Both of the conjugates eluted as intense and pure peaks with good separation from any contaminants remaining from the reaction. Interestingly, both reactions had residual QSY 7 left over after the course of the reaction, which is indicative of low levels of impurities or degradation of the SCs received from Cayman Chemical. Further study is needed to resolve the CB receptor binding ability of the conjugates to determine if they can be effective in the detection platform.

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List of Symbols, Abbreviations, and Acronyms

CB1	cannabinoid receptor 1 (central)
CB 2	cannabinoid receptor 2 (peripheral)
DMF	dimethylformamide
DQ	dark quencher
FRET	fluorescence resonance energy transfer
HBTU	O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
LC-MS	liquid chromatography coupled mass spectrometry
QD	quantum dot
SC	synthetic cannabinoid
TEA	triethylamine
THC	tetrahydrocannabinol
TLC	thin layer chromatography

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