ARL-TR-7570 ● JAN 2016

US Army Research Laboratory

Purification Efficacy of Synthetic Cannabinoid Conjugates Using High-Pressure Liquid Chromatography

by Alexis M Fakner, Abby L West, Shashi P Karna, and Mark H Griep

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Purification Efficacy of Synthetic Cannabinoid Conjugates Using High-Pressure Liquid Chromatography

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED (From - To)
January 2016	Final	May 2014–August 2014

4. TITLE AND SUBTITLE
Purification Efficacy of Synthetic Cannabinoid Conjugates Using High-Pressure Liquid Chromatography

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Alexis M Fakner, Abby L West, Shashi P Karna, and Mark H Griep

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
US Army Research Laboratory
ATTN: RDRL-WMM-A
Aberdeen Proving Ground, MD 21005-5069

8. PERFORMING ORGANIZATION REPORT NUMBER
ARL-TR-7570

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In the current study, we successfully purified several synthetic cannabinoid (SC):dark quencher conjugates essential for the success of the synthetic cannabinoid detection platform developed at the US Army Research Laboratory's Weapons and Materials Research Directorate. The purification was conducted using high-pressure liquid chromatography and gradient screens to determine the most effective means of purifying the SC:dark quencher conjugates to obtain the highest yields and purity. The purity was verified using liquid chromatography–coupled mass spectroscopy and nuclear magnetic resonance.

15. SUBJECT TERMS
synthetic cannabinoids, biosensor, LC-MS, chemical purification, dark quencher conjugation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Abby West
Unclassified	Unclassified	Unclassified	UU	28	19b. TELEPHONE NUMBER (Include area code) 410-306-4952

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Acknowledgments

This research was supported in part by an appointment to the Research Participation Program at the US Army Research Laboratory (ARL) administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and ARL. The authors would like to thank Rose Pesce-Rodriguez for allowing use of the liquid chromatography coupled mass spectrometry and for her help with the setup and running of the instrument. The authors would also like to thank Michael Sellers for his aid and input on this work.

1. Introduction and Background

The effects associated with using marijuana are due to the active ingredient, a compound named tetrahydrocannabinol (THC), binding to and partially activating the cannabinoid receptor (CB) in the brain.[1,2] As THC only partially activates the receptor, or acts as a partial agonist, the effects are usually transient and comparatively minor compared with other illegal drugs such as heroin, which acts as a full agonist on opioid receptors.[3] Recently, laboratory-developed synthetic cannabinoids (SCs) have been developed that act as tight binding, full agonists of the CB receptors.[4–8] The effects of these drugs can range from increased heart rate and vomiting to panic, hallucinations, and psychosis. [2,6,9,10] SCs may mimic some of the effects of using marijuana; however, because they bind to the receptors with much higher affinity and act as full agonists, the effects are prolonged and often lead to permanent brain damage.[10–12] SCs are often added to dried plant material and sold as incense, potpourri, or smoking mixtures in gas stations and smoke shops.[13] These drugs have names such as Spice, K2, and Yucatan Fire.[4,7,10]

The use of these substances is increasing across the United States and many other countries. In August 2014, the governor of New Hampshire declared a state of emergency after more than 40 overdoses were reported in just 72 hours from smoking a synthetic cannabinoid substance that was sold in convenience stores.[14] SC usage has also become a major problem among the US military.[15,16] A 2012 study of military personnel showed that of those tested, more than half tested positive for SCs.[17] Studies have shown that one primary reason for the popularity of SCs among the civilian and military populations is that they are assumed to be difficult to detect in standard drug screens and they are often available over the counter.[15,18]

Due to the pronounced deleterious effects of SC usage, there has been a push to make these substances illegal, yet the scheduling of these drugs makes it difficult. Generally, as soon a particular SC compound is scheduled, the manufacturers create several new compounds with slight changes to the inactive parts of the backbone to circumvent the laws and avoid detection, as shown in Fig. 1.[4,7,19]

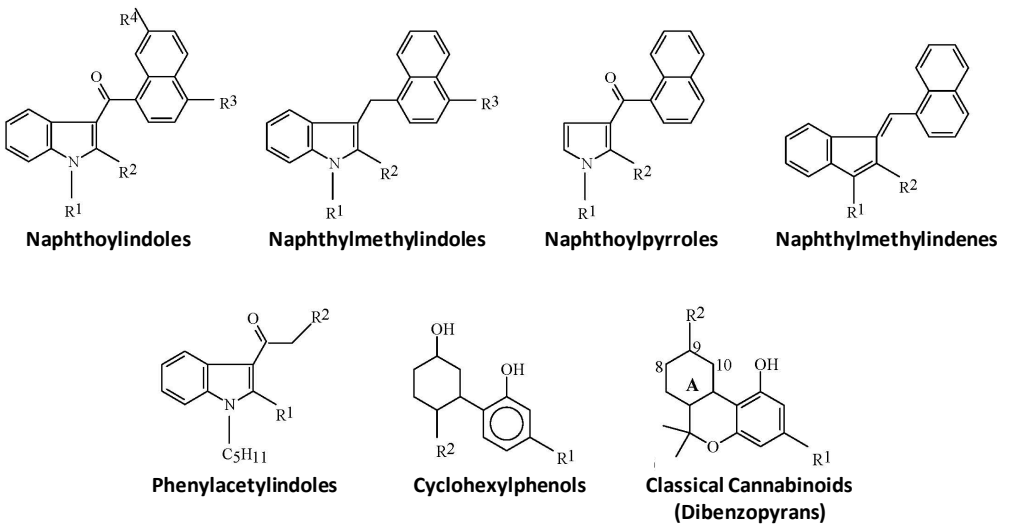

Fig. 1 Basic chemical structures of the 7 different synthetic cannabinoid groups. The R groups are positions at which substituent variants are possible.

Adding to the difficult nature of prosecution and detection of those using SCs is the manner by which these compounds are detected. Typically, detection of SCs is conducted using liquid chromatography–coupled mass spectrometry (LC-MS) or gas chromatography coupled mass spectrometry. These methods require comparison to a known compound, which means that SCs must be fully characterized prior to one being able to detect them in samples.[12,20] There are hundreds of different forms of synthetic cannabinoids and with more being continuously created, this method of detection cannot keep up with the ever-changing drug. This leads to ultra-long turnaround times (6–8 months) and backlogs of thousands of samples in toxicology laboratories.[9]

Clearly, a new detection method must be developed that does not require previous characterization of SCs for success. The method should be able to detect all SCs independent of chemical structure and without the need for prior characterization. Such a method would greatly reduce the backlog at testing facilities, as it would act as a first line test where the non-SC compounds can be removed from the sample inventory. One approach to create such a sensor would be to utilize the CB receptor as a basis for detection. If a compound is an SC, then it will only bind to the CB receptor. Thus, the system would be highly specific and robust in recognizing all different iterations of SCs.

Recently, receptor-protein-based fluorescent biosensors have been used to measure ligand/protein interactions. In this method, a receptor protein is conjugated to a quantum dot and a dark quencher molecule is conjugated to a receptor ligand. The dark quencher/low-affinity ligand binds to the protein-quantum dot compound and

the fluorescence of the quantum dot is quenched. When a test ligand with a higher affinity binds the protein, the dark quencher/low-affinity ligand is displaced and fluorescence is observed. Medintz et al. constructed such a sensor in 2003 to monitor maltose binding to the maltose binding protein.[21]

This type of sensor would be advantageous for use as an SC detector as it would give a quick result and be capable of detecting any substance binding the CB receptor. This would allow the technology to keep up with the ever-changing compounds being manufactured. In ARL-TR-7188, *Conjugation of the Dark Quencher QSY 7 to Various Synthetic Cannabinoids for Use in Fluorescence-Based Detection Platforms*, 2 SC:dark quencher conjugates were synthesized and characterized for the use in fluorescence-based cannabinoid detection platforms.[22] However, the conjugates synthesized in ARL-TR-7188 were not sufficiently pure to test the binding affinity of the conjugate to the receptor. It is imperative that the low-affinity dark quencher conjugates be pure, otherwise binding of the parent compound may be detected. This may lead to a very high background in the assay as the parent compound has a higher affinity for the receptor than the conjugated compound. Thus, to get an accurate measurement of the affinity of the dark quencher conjugate for the CB receptor, purification of the conjugates must be optimized to yield samples 100% pure of parent compound.

Five different SC:dark quencher conjugates were purified for future use in a receptor-based SC detection assay, as shown in Fig. 2. The associated predicted masses are shown in Table 1. The purity was verified using LC-MS. Results demonstrated that 3 of the compounds were successfully synthesized and purified (JWH018:QSY 7 amine, JWH018:$(PEG)_4$:QSY7 amine, and JWH073:QSY 7 amine), whereas 2 of the compounds were either not correctly synthesized or very quickly degraded to nonuseful products (JWH368:QSY 7 amine and JWH368:$(PEG)_4$:QSY 7 amine).

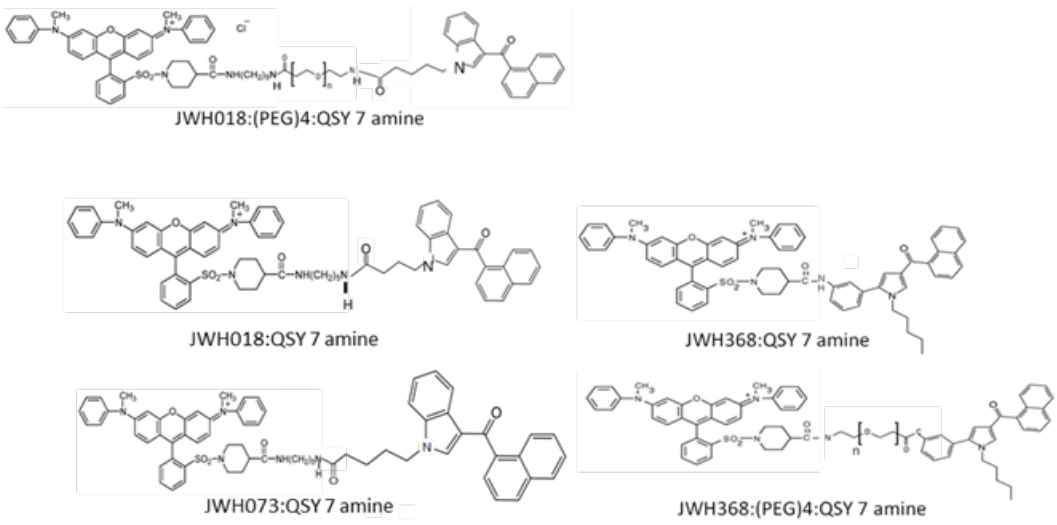

Fig. 2 Structures of the 5 synthesized SC conjugates

Table 1 Expected masses of compounds used in this study

Compound	Expected mass (g/mol)
JWH018 pentanoic acid	371.4
JWH368	385.5
QSY 7 amine	814.86
JWH018 pentanoic acid:$(PEG)_4$:QSY 7 amine	1,095.42
JWH368:$(PEG)_4$: QSY 7 amine	1,337.4
JWH073 butanoic acid: QSY 7 amine	1,081.42

2. Synthesis of Materials

2.1 Chemicals

Dimethylformamide (DMF), acetonitrile, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). JWH018 pentanoic acid, JWH073 butanoic acid, and JWH368 were purchased from Cayman Chemical (Ann Arbor, MI). QSY 7 amine was purchased from Life Technologies (Carlsbad, CA). All solvents were of high-pressure liquid chromatography (HPLC)–grade or higher and used without further purification. Ultrapure milli-Q water was used for all experiments.

2.2 Purification of SC:Dark Quencher Conjugates

The conjugates were synthesized as previously described in ARL-TR-7188.[22] Briefly, molar equivalents of the dark quencher and the SC were mixed with DMF. O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate was added and stirred for 5 min. Then triethylamine was added and the reaction was incubated for 4 h. In addition to the 2 compounds QSY 7:JWH073 n-butanoic acid conjugate and QSY 7:JWH018 n-pentanoic acid conjugate synthesized previously, the dark quencher QSY 7 amine was reacted with the CB receptor ligand JWH368 via the same procedure. A polyethyleneglycol (PEG) linker was added between SCs, JWH018 n-pentanoic acid and JWH368, and the dark quencher to allow for more flexibility.

The JWH018:$(PEG)_4$:QSY 7 amine, JWH368:$(PEG)_4$:QSY 7 amine, and JWH073:QSY 7 amine conjugates were purified using HPLC. The LC column was an Agilent Eclipse XDB C18 column (250- × 9.4-mm ID, 5-μm particle size), maintained at 25 °C with a mobile phase flow rate of 2.00 mL/min. Gradient elution mobile phases consisted of A (5 mM ammonium formate in water) and B (acetonitrile). The gradients used for the purification of each conjugate are shown in Tables 2–4. The detection wavelengths used in this study were 218, 316, and 560 nm. The expected mass of each compound can be seen in Table 1.

Table 2 Gradients of solvents used in this study for the HPLC purification of JWH368:$(PEG)_4$:QSY 7 amine and JWH368:QSY 7 amine

Time (min)	%B
5	10
10	45
15	50
20	55
27	58
30	100

Table 3 Gradients of solvents used in this study for the HPLC purification of JWH018:$(PEG)_4$:QSY 7 amine and JWH018:QSY 7 amine

Time (min)	%B
5	10
10	50
15	65
20	70
25	75
30	80
35	85
40	90
45	100

Table 4 Gradients of solvents used in this study for the HPLC purification of JWH073:QSY 7 amine

Time (min)	%B
5	15
10	30
15	50
20	70
30	80
40	90
50	100

2.3 LC-MS Analysis of SC:Dark Quencher Conjugates

The overall purity of the JWH018, JWH073, and JWH368 conjugates 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 ID, 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 (5 mM ammonium formate in water) and B (acetonitrile). The same elution gradients shown previously were used. Quantification of the analytes was undertaken using positive scan mode with a molecular mass scan from 100 to 1,200 g/mol.

3. Results and Discussion

3.1 LC-MS Gradient Studies for Purification of Conjugates

Liquid chromatography was run on the conjugates, JWH018:$(PEG)_4$:QSY 7 amine, JWH368:$(PEG)_4$:QSY 7 amine, and JWH073:QSY 7 amine. The gradients for each conjugate were optimized to allow for the most efficient separation. After the gradient was optimized and the peaks were collected, LC-MS was performed on the peaks to determine which peaks were of value. The elution peaks from the LC-MS are shown in Figs. 3–5.

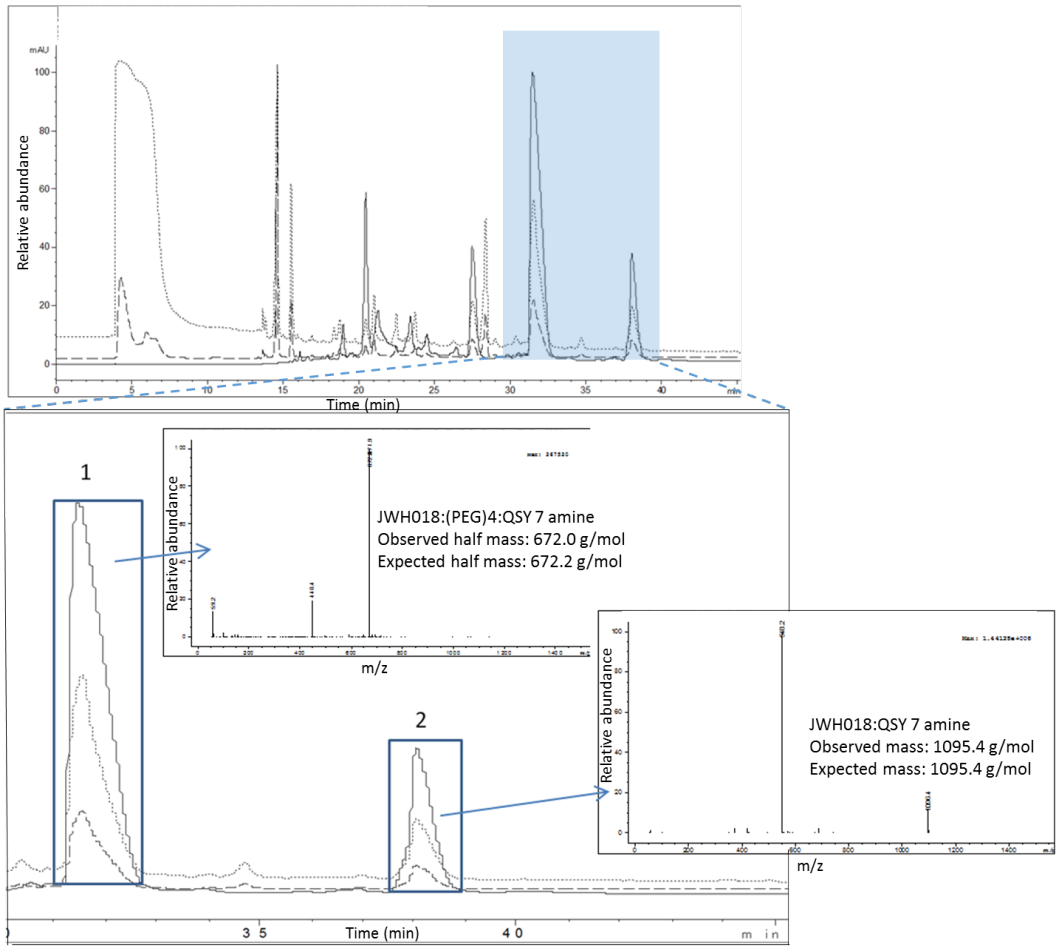

Fig. 3 The peaks observed during the purification of JWH018:(PEG)$_4$:QSY 7 amine using the optimized gradient (top). A zoomed-in view of the top figure showing the peaks collected during that run and the associated mass spec data (bottom). The peak indicated with a number 1 represents the JWH018:(PEG)$_4$:QSY 7 amine conjugate. The peak indicated with a number 2 represents the JWH018:QSY 7 amine conjugate.

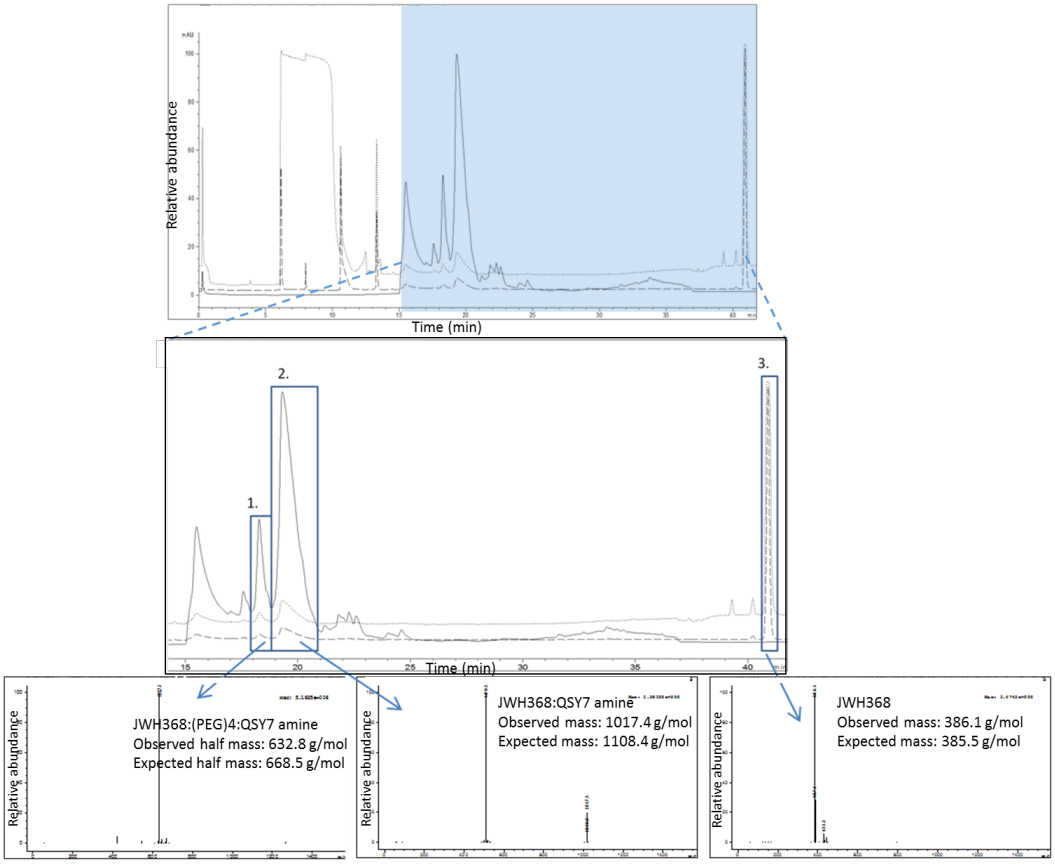

Fig. 4 The peaks observed during the purification of JWH368:(PEG)$_4$:QSY 7 amine using the optimized gradient (top). A zoomed-in view of the top figure showing the peaks collected during that run (middle). The peak indicated with a number 1 represents the JWH368:(PEG)$_4$:QSY 7 amine conjugate. The peak indicated with a number 2 represents the JWH368:QSY 7 amine conjugate. The peak indicated with a number 3 represents the JWH368 compound. The mass spectra of each of the 3 peaks collected (bottom).

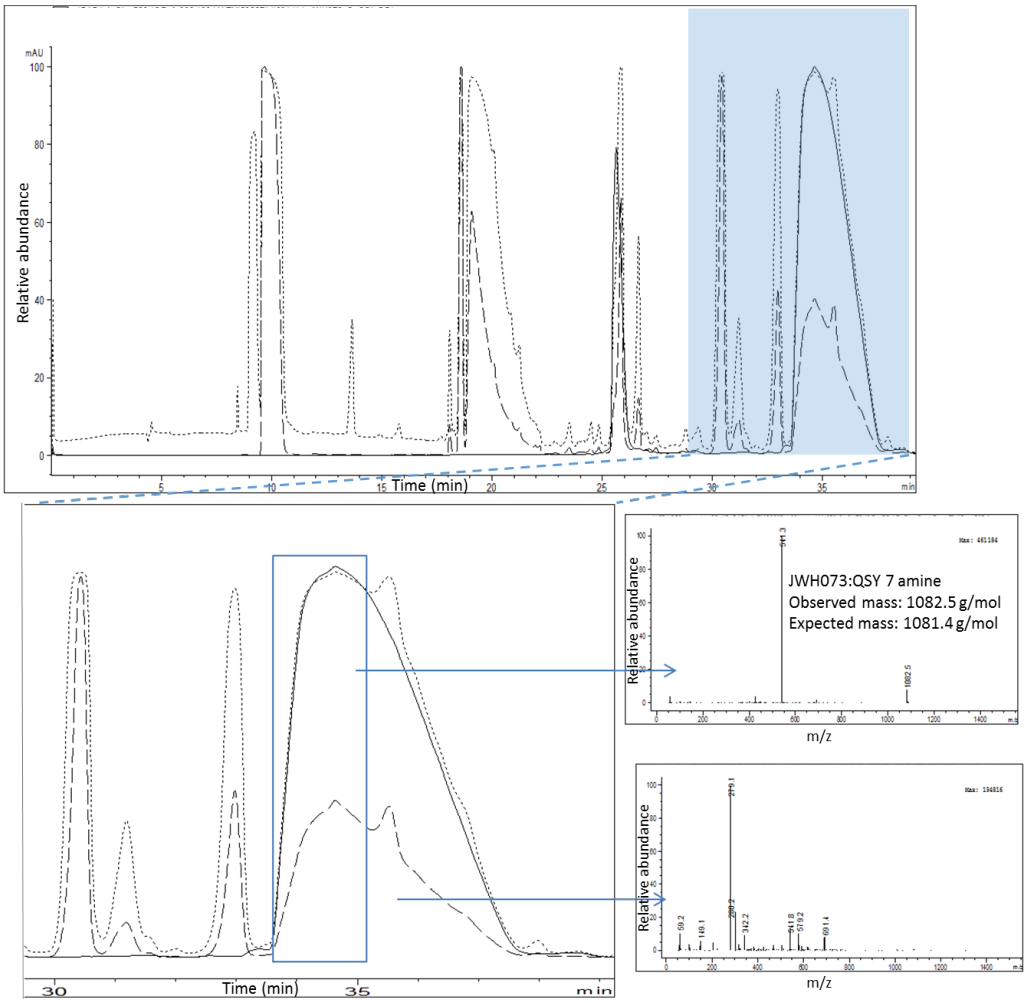

Fig. 5 The peaks observed during the purification of JWH073:QSY 7 amine using the optimized gradient (top). A zoomed-in view of the top figure showing the peaks collected during that run (bottom) and the associated mass spec data. The peak indicated with a box represents the JWH073:QSY 7 amine conjugate. The second portion of the peak, shown on the bottom, indicates impurities.

During the separation of these conjugates, it was discovered that during synthesis of JWH368:$(PEG)_4$:QSY 7 amine and JWH018:$(PEG)_4$:QSY 7 amine, 2 part conjugates of the JWH compound and the dark quencher without the PEG linker were also formed (JWH368:QSY 7 amine and JWH018:QSY 7 amine). These conjugates were verified with mass spectrometry. The PEG spacer and JWH compound were added first and allowed to react. The reaction must have been inefficient and some JWH compound remained. This allowed the JWH compound to react with the dark quencher when it was added to the reaction. Many additional peaks can be seen in the top portion of Figs. 3–5. These other peaks are impurities and unreacted compounds that can interfere with future binding affinity studies. It

is therefore necessary to separate all of the synthesized conjugates for downstream testing. All of the peaks identified in Figs. 3–5 were collected, lyophilized, and the purity was verified through mass spectrometry.

The structures of the SC metabolites JWH073 n-butanoic acid and JWH018 n-pentanoic acid are very similar and differ only by one carbon, as seen previously in Fig. 2. Due to the similarity in structure, it would be expected that the conjugates of these structures with the dark quencher, QSY 7 amine, would elute from the HPLC column at similar times. JWH073:QSY 7 amine elutes at 33.5 min at approximately 83.5% solvent B. JWH018:QSY 7 amine elutes at 37.5 min at approximately 87.5% solvent B. These are similar elution times given that the JWH368:QSY 7 amine conjugate elutes at around 19 min at approximately 54% solvent B.

3.2 LC-MS Analysis of SC:Dark Quencher Conjugates

After HPLC separation had been completed with the conjugates, the separated products were subjected to LC-MS and analyzed for purity.

The LC-MS analysis of the JWH368:$(PEG)_4$:QSY 7 amine conjugate showed a 52% pure product that elutes at 28.019 min (Fig. 6). No parent compound was detected. The observed half mass of the conjugate was 632.8 g/mol. The expected mass was 1,337 g/mol (half mass of 668.5 g/mol). The observed mass was in slight discordance with the expected mass. There is also an additional mass of 422.3 g/mol observed. This mass is inconsistent with any compounds added to the reaction. It is likely due to degradation of the conjugate. The final product yield was 8.1 μg.

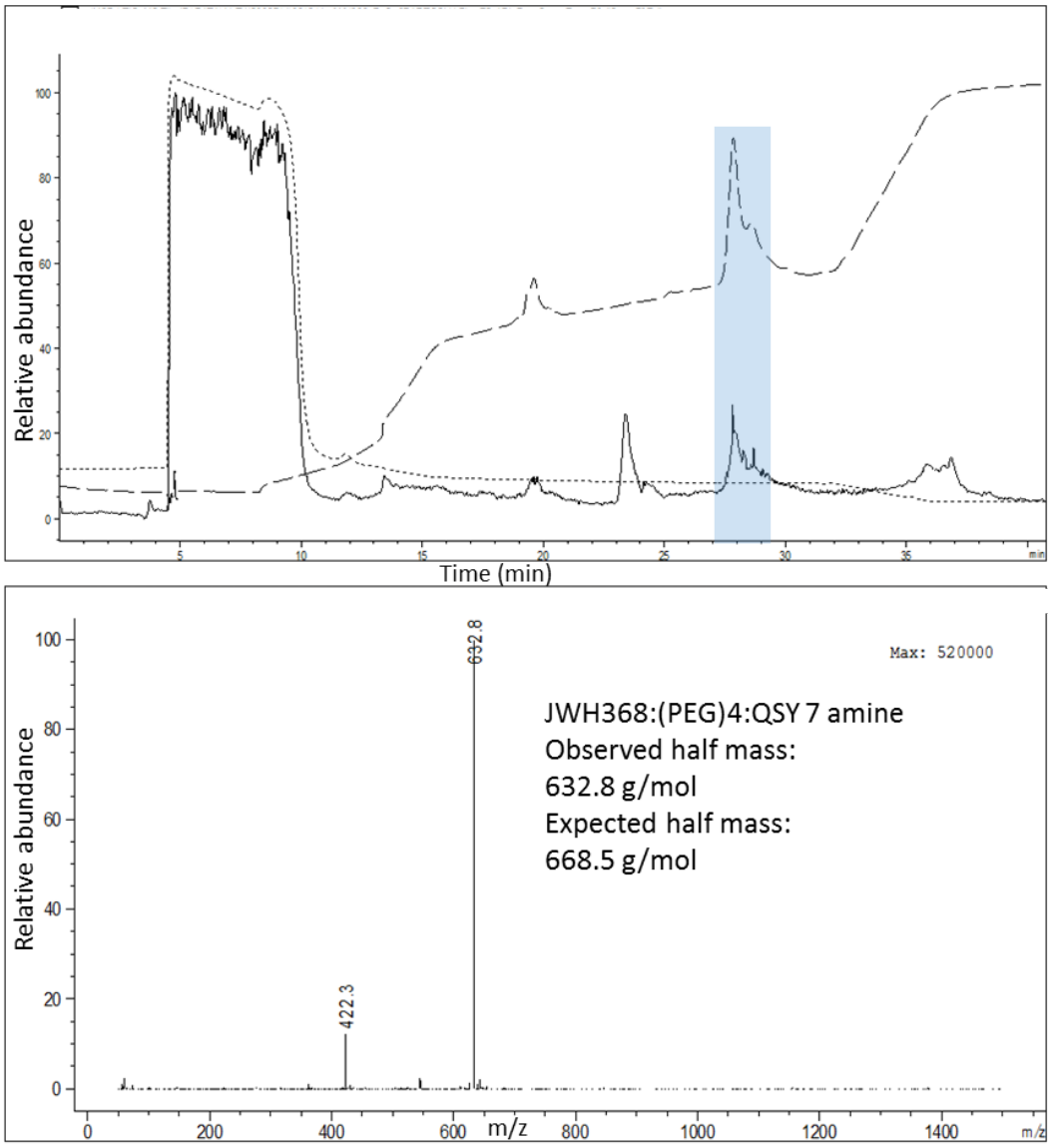

Fig. 6 LC chromatogram (top) and mass (bottom) of purified the 3-part conjugate, JWH368:$(PEG)_4$:QSY 7 amine. The conjugate has a retention time of 28.019 min and an observed half mass$^+$ of 632.8 g/mol (expected: 1,337 g/mol nonionized).

Analysis of the JWH368:QSY 7 amine conjugate with LCMS showed a 98% pure product that elutes at 34.3 min (Fig. 7). The observed mass of the conjugate was 1,017.4 g/mol, which is inconsistent with the expected mass of 1,108.4 g/mol. This may indicate an issue during synthesis or degradation of the compound after synthesis. Future nuclear magnetic resonance studies of both compounds will help to explain these discrepancies. The final product yield was 20.0 µg.

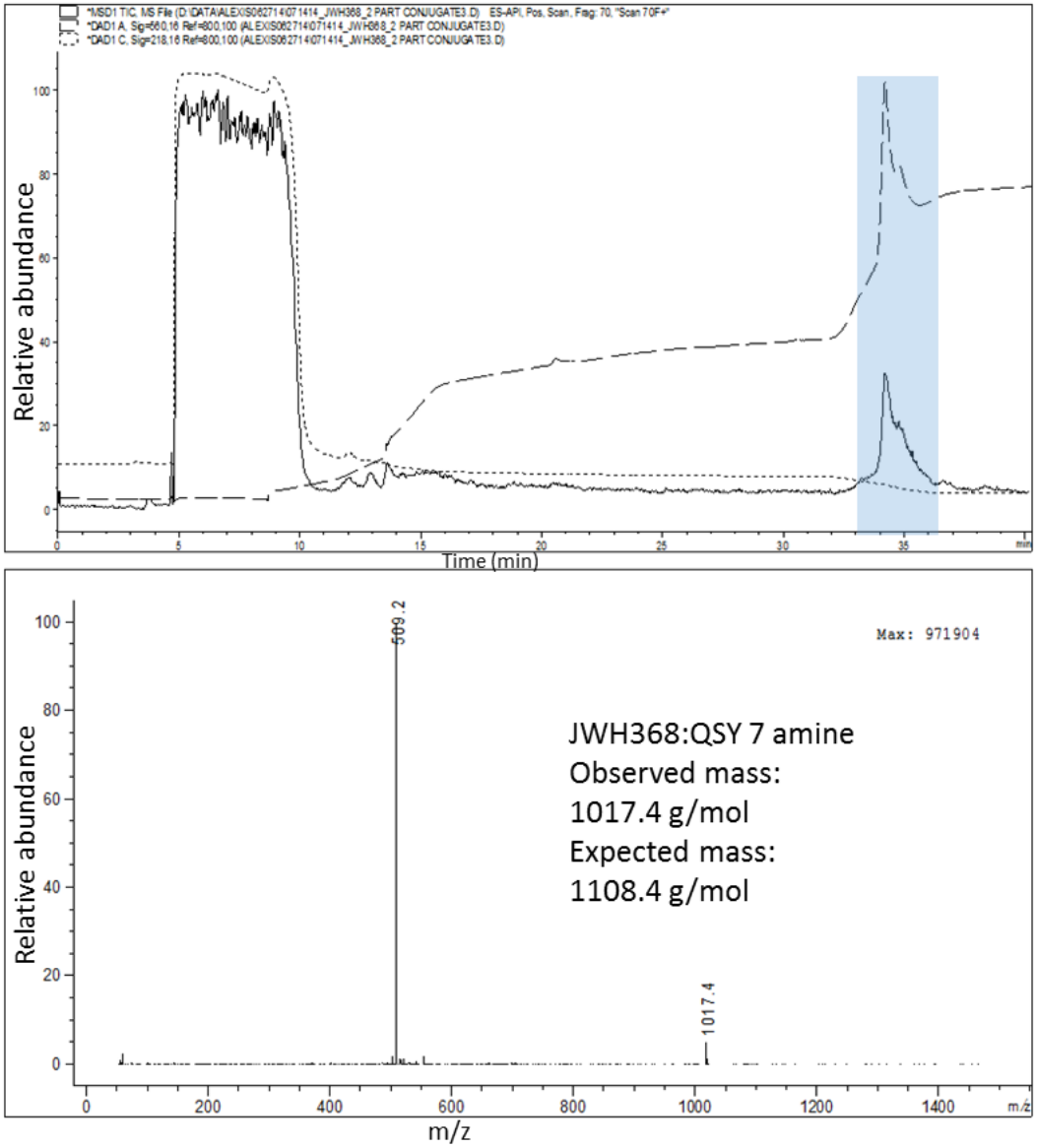

Fig. 7 LC chromatogram (top) and mass (bottom) of the purified 2-part conjugate, JWH368:QSY 7 amine. JWH368:QSY 7 amine has a retention time of 34.3 min and an observed mass^{+} of 1,017.4 g/mol (expected: 1,108.4 g/mol nonionized).

The LC-MS analysis of the JWH018 n-pentanoic acid:$(PEG)_4$:QSY 7 amine conjugate showed a 90% pure product that elutes at 35.602 min (Fig. 8). The observed half mass of the conjugate was 672.0 g/mol, which is in very good agreement to the expected mass of 1,344.3 g/mol (half mass of 672.2 g/mol). There is an additional mass of 448.5 g/mol. This mass is inconsistent with any compounds added to the reaction. It is likely due to degradation of the conjugate. The final product yield was 55.4 µg.

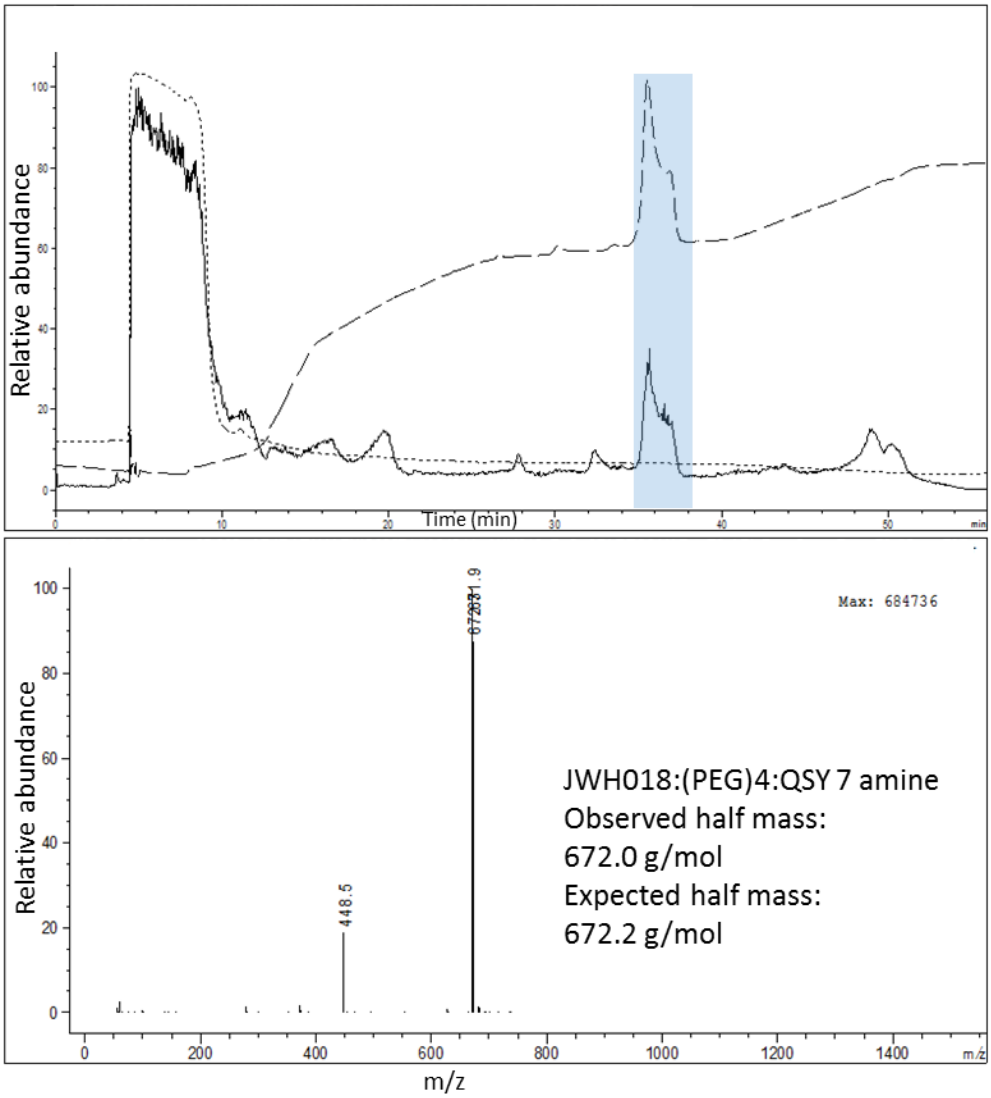

Fig. 8 LC chromatogram (top) and mass (bottom) of the purified 3-part conjugate JWH018:$(PEG)_4$:QSY 7 amine. JWH018:$(PEG)_4$:QSY 7 amine has a retention time of 35.602 min and an observed half mass$^+$ of 672.0 g/mol (expected: 1,344.3 g/mol nonionized).

Analysis of the JWH018 n-pentanoic acid:QSY 7 amine conjugate with LC-MS showed a 96% product that elutes at 44.494 min (Fig. 9). The observed mass of the conjugate was 1,095.4 g/mol, which is in very good agreement to the expected mass of 1,095.42 g/mol. The final product yield was 10.3 µg.

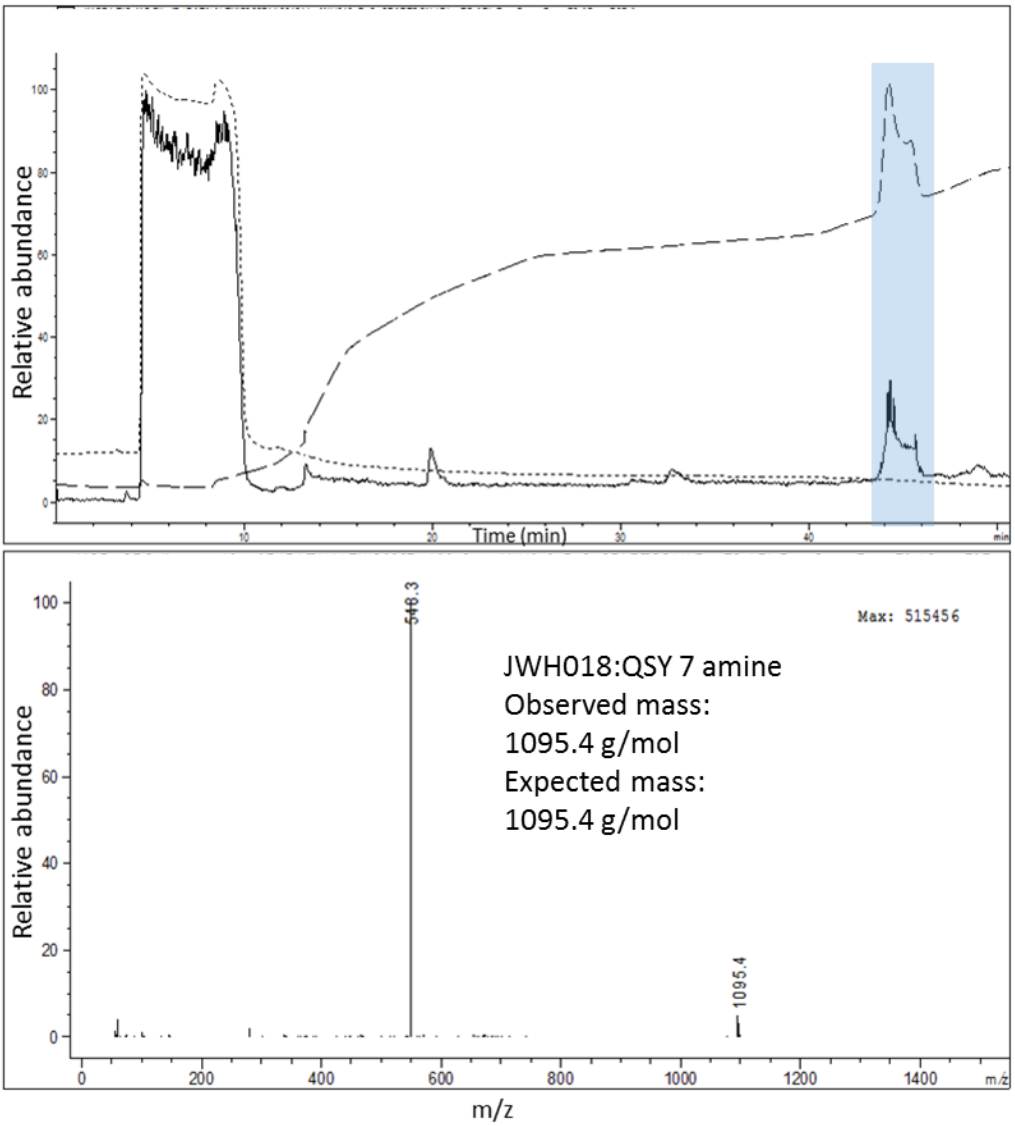

Fig. 9 LC chromatogram (top) and mass (bottom) of the purified 2-part conjugate JWH018:QSY 7 amine. JWH018:QSY 7 amine has a retention time of 44.494 min and an observed mass^{+} of 1,095.4 g/mol (expected: 1,095.4 g/mol nonionized).

Finally, the LC-MS analysis of the JWH073 butanoic acid:QSY 7 amine conjugate showed a 98% pure product that elutes at 44 min (Fig. 10). The observed mass of the conjugate was 1,081.3 g/mol, which is in very good agreement to the expected mass of 1,081.42 g/mol. The final product yield was 43.8 µg.

The yields for all of the compounds were lower than expected. There was significant loss during the purification methods. In the future, better collection techniques can be put in place to decrease the product loss.

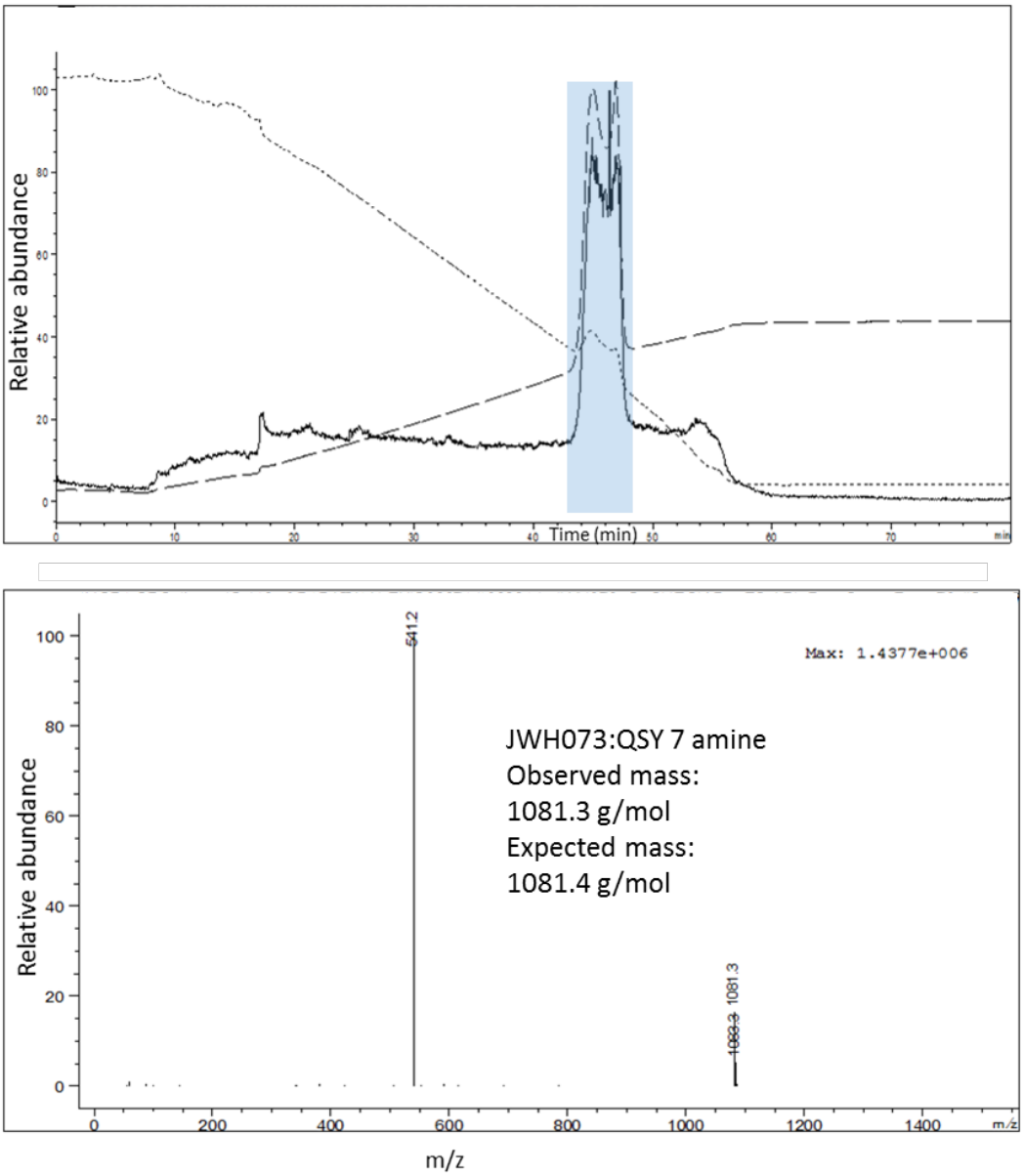

Fig. 10 LC chromatogram (top) and mass (bottom) of the purified JWH073 n-butanoic acid:QSY 7 amine conjugate. JWH073:QSY 7 amine has a retention time of 44 min and an observed mass^{+} of 1,081.3 g/mol (expected: 1,081.4 g/mol nonionized).

4. Summary and Conclusions

We have successfully purified 3 SC:dark quencher conjugates, which were synthesized according to ARL-TR-7188 for use in a receptor-based SC detection assay. All of the conjugates eluted as strong peaks with minimal impurities and no detection of parent compounds. These SC:dark quencher conjugates are sufficiently pure of parent compound to allow for CB-receptor binding studies that are necessary to determine if these conjugates can be used in the cannabinoid detection platform in development here at ARL.

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List of Symbols, Abbreviations, and Acronyms

CB	cannabinoid receptor
DMF	dimethylformamide
HPLC	high-pressure liquid chromatography
LC-MS	liquid chromatography–coupled mass spectrometry
PEG	polyethyleneglycol
SC	synthetic cannabinoid
THC	tetrahydrocannabinol

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