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Confirmation and Analysis of Fentanyl and Its Analogues Without the Standards

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Abstract: In recent years, the number of controlled fentanyl species has increased gradually, and up to now, the number of controlled fentanyl species increased to twenty-seven. Since fentanyl standards are difficult to obtain, there are only several kinds of controlled fentanyl species involved in the literature, and the analysis of all 27 controlled fentanyl species have not been reported in the literature. Therefore, it is of great significance to establish a method for the rapid screening and confirmation of all 27 controlled fentanyl species without the use of standards. In this paper, an analytical method for the rapid screening and confirmation of 27 controlled fentanyl substances without the use of standards was established. Suspected samples were rapidly screened by ultra-high performance liquid chromatography/Orbitrap high resolution mass spectrometry (UPLC/Orbitrap HRMS). Qualitative identification was performed through the quasi-molecular ion accurate masses of target compounds. Confirmation was carried out by secondary ion segments. It is the first time that all of 27 controlled fentanyl substances was tested. The proposed method was simple, rapid and accurate, applicable for the rapid screening and confirmation of 27 controlled fentanyl substances without standards.

Keywords: Fentanyl, UPLC/Orbitrap HRMS, Screening

1. Introduction

Since 2000, the global reporting of the abuse of new psychoactive substances (NPS) has increased dramatically [1]. Between 2008 and 2015, 102 countries reported 644 NPS to the United Nations Office on Drugs and Crime. NPS is a single substance or mixture that is not controlled by the UN international conventions but is harmful to public health. It is a drug analogue that the criminals have obtained to chemically modify the controlled drugs in order to escape the blow of law enforcement agencies. NPS has the similar or stronger effect of controlled drugs and has become the third-generation drug that is globally popular after traditional drugs and synthetic drugs, generally including synthetic cannabinoids, stimulants (such as phenethylamine, cathinone, piperazine, etc.), magic agents or isolated hallucinogens (ketamine and phencyclidine), tranquilizers, tryptamine, aminoguanidine and fentanyl

synthetic opioids [2]. The Belgian pharmacologist and pharmaceutical chemist Paul Janssen developed fentanyl in 1960. It was at the time a revolutionary compound with a potency much greater than that of the natural opioid morphine, and a significantly greater safety margin than other pharmaceutical synthetic opioids such as dextromoramide, meperidine and phenoperidine. Under Janssen's leadership, a deeper understanding of the structure/activity relationships led to the development of the very potent analgesics sufentanil, alfentanil, lofentanil and carfentanil, which could also be used for anaesthesia. These drugs have been successfully used clinically for over fifty years, and have made possible complex surgeries and the successful management of chronic pain for millions of patients worldwide. Fentanyl, together with codeine, methadone and morphine are four opioid analgesics on the World Health Organization's (WHO) List of Essential Medicines [3]. In California, United States in 1979, a series of deaths occurred

in people who injected drugs that were subsequently attributed to an analogue of fentanyl, α-methylfentanyl. Initially discovered and patented by Janssen, α-methylfentanyl had neither been evaluated in humans for safety or efficacy, nor approved as a pharmaceutical drug. This was the first recorded instance of a completely novel clandestinely synthesized opioid, and earned the sobriquet "designer drug". Through the early 1980s, α-methylfentanyl was quickly joined by a series of other fentanyl analogues including *para*- fluorofentanyl, 3-methylfentanyl, β-hydroxyl fentanyl, and a number of others that were identified in deaths in the west coast of the United States. Subsequently, the use of fentanyl itself became widespread due to its availability worldwide in injectable, sublingual and transdermal forms, and it also became subject to diversion and abuse. At the beginning of 2014, the availability of fentanyl in the United States had started to increase exponentially [4], with crime laboratories reporting more than 4,500 fentanyl cases in 2014 and over 14,400 in 2015. Around the same time, a group of fentanyl new psychoactive substances (NPS) began to appear in the street drug market, beginning with acetylfentanyl, and butyrylfentanyl. In view of the abuse and addiction of fentanyl substances, international organizations have restricted their use and the number of restricted species increase gradually. Fentanyl was first controlled under the 1961 Single Convention on Narcotic Drugs in 1964. Sufentanyl and alfentanyl were then controlled in 1980 and 1984, respectively. Acetyl-α-methylfentyl, α-methylfentanyl and 3-methylfentanyl were controlled in 1988. Thiofentanyl, α-methyl-thiofentanyl, β-hydroxy fentanyl, 3-methylthiofentanyl, β-hydroxy-3- methylfentanyl, para-fluorofentanyl were controlled in 1990. Remifentanil was controlled in 1999, while acetylfentanyl and ocfentanyl were controlled in 2013. Butyrfentanyl and furanfentanyl were controlled in 2015 while acryfentanyl and para-fluoroisobutyrfentanyl were controlled in 2016. By 2017, 15 fentanyl analogues and two precursor chemicals had been placed under international control and more than 20 individual substances had been detected in deaths. They have been routinely detected in drug markets in Europe and North America, and several more have been encountered in smaller, more isolated incidents, along with other non-morphine or fentanyl-related opioid receptor agonists. Up to now, two precursors and 25 fentanyl substances are regulated by China. In recent years, the number of people who have been addicted to fentanyl in the United States has risen sharply. Trump thus announced in October 2018 that the United States entered a "public health emergency." At the summit meeting of Chinese and American leaders held in December 2018, the leaders of two countries reached consensus on the control of fentanyl substances and this became a new bright spot in Sino-US cooperation.

Numerous analysis methods for fentanyl and its analogues have been published in the scientific literatures. Analysis technique such as liquid chromatography (HPLC) [5-9], gas chromatography/mass spectrometry (GC/MS) [10-18], liquid chromatography/high resolution mass spectrometry

(LC-HRMS) [19-20], liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) [21-30] has been reported to apply in the precision determination and quantification of fentanyl and its analogues. GC/MS analysis method has been considered a gold standard for decades due to its combination of good selectivity and high specificity. HPLC-MS/MS analysis method provides excellent selectivity, as well as specificity and limits of detection that are usually better than those obtainable on GC/MS systems. On the other hand, surface enhanced Raman spectrometry has been applied for the rapid screening of fentanyl and its analogues [31-33]. However, they are all come with one critical limitation: they are "known unknown" method in which instrumental parameters must be specified for each compounds of interest, that is to say, standard substances of fentanyl and its analogues must be provided for the analysis. As a class of controlled substances, standard substances of fentanyl and its analogues are rather difficult to purchase. Therefore, not many types of fentanyl and its analogues have been preferred in all the existing literatures. It is mainly focused on fentanyl, alfentanil, remifentanil, sufentanil, carfentanil and orfentanil. As to other fentanyl substances, they are rarely mentioned because of the difficulty to find standard or reference. An LC-MS/MS method has been established by Strayer et al. [30] to determine the contents of fentanyl and its analogues in blood, in which a total of 24 fentanyl and its analogues were detected. It is the most widely reported fentanyl species in the literature. However, it only covers 13 kinds of fentanyl substances controlled by China. At present, there are no reports in the literature on the determination of 27 fentanyl substances regulated in China.

In view of the difficulty in obtaining all controlled fentanyl standards, it is necessary to develop a screening method that does not require standards. Methods based upon full scan mass spectral data avoid the targeting limitations of the analytical technique mentioned above, but selectivity can suffer due to increased background, and specificity is hampered by the fact that most compounds of interest yield only a pseudomolecular ion under typical ESI and APCI conditions. Acquiring MS data with high spectral resolution and high mass accuracy can counter both these problems: high resolution can restore selectivity by permitting a given analyte signal to be extracted from a near-isobaric background signal, while mass accuracy improves specificity by restricting the number of possible chemical formulae that might give signals indistinguishable from the analyte of interest. Some chromatographic separation must still be retained in order to distinguishing true isobaric compounds. The increased availability of robust and easy to use high resolution mass spectrometers over the last few years has finally allowed people to begin realizing the potential of full scan MS methods in practice. This can be seen in the increasing number of broad-spectrum drug screening methods employing either time-of-flight or Orbitrap technology that have appeared within the last few years [34-40]. In this paper we present an ultra-high performance liquid chromatography/Orbitrap high resolution mass spectrometry (UPLC/Orbitrap HRMS) technique to simultaneously screen

for the presence of 27 compounds, including two precursors and 25 fentanyl substances.

2. Experimental

Formic acid (purity>98.0%) and ammonium formate (purity>97.0%) were purchased from CNW Technologist Co, Germany. Acetonitrile and methanol (both HPLC grade) were purchased from Fisher Scientific, Pittsburgh, Pennsylvania, USA. Fentanyl standard (purity 98.5%) was purchased from Alta Scientific Co. Ltd, USA. De-ionized water (18+ mega-ohm grade) was obtained from an inhouse Millipore purification system. An UPLC analytical column (Accucore Phenylhexyl C_{18}, 100mm× 2.1mm, 1.6μm) was purchased from ThermoFisher Scientific, Bremen, Germany. Information of 27 fentanyl substances studied in this paper was listed in Table 1.

Table 1. Information of 27 fentanyl substances.

No.	Name	IUPAC Name	Formula	CAS No
1#	acetyl-alpha-methylfentanyl	*N*-Phenyl-*N*-[1-(1-phenylpropan-2-yl) piperidin-4-yl]acetamide	$C_{22}H_{28}N_2O$	101860-00-8
2#	Fentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl)piperidin -4-yl]propanamide	$C_{22}H_{28}N_2O$	437-38-7
3#	Alfentanil	*N*-{1-[2-(4-Ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl]-4-(methoxymethyl)-4-piperidinyl}-*N*-phenylpropanamide	$C_{21}H_{32}N_6O_3$	71195-58-9
4#	alpha-methyl fentanyl	*N*-Phenyl-*N*-[1-(1-phenyl-2-propanyl) -4-piperidinyl]propanamide	$C_{23}H_{30}N_2O$	79704-88-4
5#	3-Methylfentanyl	*N*-[3-Methyl-1-(2-phenylethyl)-4-piperidinyl] -*N*-phenylpropanamide	$C_{23}H_{30}N_2O$	42045-86-3
6#	Butyryl fentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl) -4-piperidinyl]butanamide	$C_{23}H_{30}N_2O$	1169-70-6
7#	Isobutyryl fentanyl	2-Methyl-*N*-phenyl-*N*-[1-(2-phenylethyl) -4-piperidinyl]propanamide	$C_{23}H_{30}N_2O$	119618-70-1
8#	alpha-Methyl thiofentanyl	*N*-Phenyl-*N*-{1-[1-(2-thienyl)-2-propanyl] -4-piperidinyl}propanamide	$C_{21}H_{28}N_2OS$	103963-66-2
9#	3-Methyl thiofentanyl	*cis*-*N*-[3-Methyl-1-(2-thienyl)ethyl]-4- piperidinyl]-*N*-phenyl-propanamide	$C_{21}H_{28}N_2OS$	86052-04-2
10#	Beta-Hydroxy fentanyl	*N*-[1-(2-Hydroxy-2-phenylethyl)-4- piperidinyl]-*N*-phenylpropanamide	$C_{22}H_{28}N_2O_2$	78995-10-5
11#	Beta-hydroxy-3-methylfentanyl	*N*-[1-(2-Hydroxy-2-phenylethyl)-3-methylpiperidin -4-yl]-*N*-phenylpropanamide	$C_{23}H_{30}N_2O_2$	78995-14-9
12#	para-Fluorofentanyl	*N*-(4-Fluorophenyl)-*N*-[1-(2-phenylethyl)-4- piperidinyl]propanamide	$C_{22}H_{27}FN_2O$	90736-23-5
13#	Remifentanil	Methyl 1-(3-methoxy-3-oxopropyl)-4-(*N*-propanoylanilino)piperidine-4-carboxylate	$C_{20}H_{28}N_2O_5$	132875-61-7
14#	Sufentanil	*N*-{4-(Methoxymethyl)-1-[2-(2-thienyl)ethyl]-4-piperidinyl}-*N*-phenylpropanamide	$C_{22}H_{30}N_2O_2S$	56030-54-7
15#	Thiofentanyl	*N*-Phenyl-*N*-{1-[2-(2-thienyl)ethyl] -4-piperidinyl}propanamide	$C_{20}H_{26}N_2OS$	1165-22-6
16#	Acetyl fentanyl	N-Phenyl-N-[1-(2-phenylethyl) -4-piperidinyl]acetamide	$C_{21}H_{26}N_2O$	3258-84-2
17#	beta-Hydroxythiofentanyl	*N*-{1-[2-Hydroxy-2-(2-thienyl)ethyl] -4-piperidinyl}-*N*-phenylpropanamide	$C_{20}H_{26}N_2O_2S$	1474-34-6
18#	para-Fluorobutyryl fentanyl	*N*-(4-Fluorophenyl)-*N*-[1-(2-phenylethyl) -4-piperidinyl]butanamide	$C_{23}H_{29}FN_2O$	244195-31-1
19#	para-Fluoro-isobutyrylfentanyl	*N*-(4-Fluorophenyl)-2-methyl-*N*-[1- (2-phenylethyl)piperidin-4-yl]propanamide	$C_{23}H_{29}FN_2O$	244195-32-2
20#	Ocfentanil	*N*-(2-Fluorophenyl)-2-methoxy-*N*-[1- (2-phenylethyl)-4-piperidinyl]acetamide	$C_{22}H_{27}FN_2O_2$	101343-69-5
21#	Carfentanil	Methyl 1-(2-phenylethyl)-4-[phenyl (propionyl) amino]-4-piperidinecarboxylate	$C_{24}H_{30}N_2O_3$	59708-52-0
22#	Furanyl fentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl)-4- piperidinyl]-2-furamide	$C_{24}H_{26}N_2O_2$	101345-66-8
23#	Acryl fentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl)-4- piperidinyl]acrylamide	$C_{22}H_{26}N_2O$	82003-75-6
24#	Valeryl fentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl)- 4-piperidinyl]pentanamide	$C_{24}H_{32}N_2O$	122882-90-0
25#	tetrahydrofuranyl fentanyl	N-(1-phenethylpiperidin-4-yl)-N- phenyltetrahydrofuran-2-carboxamide	$C_{24}H_{30}N_2O_2$	2142571-01-3
26#	NPP	N-phenethyl-4-piperidone	$C_{13}H_{17}NO$	39742-60-4
27#	4-ANPP	*N*-Phenyl-1-(2-phenylethyl) piperidin-4-amine	$C_{19}H_{24}N_2$	21409-26-7

Accurately weigh 10mg of the sample, dissolve it with 10mL of acetonitrile, then dilute to the appropriate concentration, followed by UPLC/Orbitrap HRMS analysis.

Analysis was carried out with a Dionex Ultimate 3000-Q Exactive ultra-high performance liquid chromatography/ Orbitrap high resolution mass spectrometry supplied by ThermoFisher Scientific, Bremen, Germany.

Chromatographic separation was performed on an Accucore Phenylhexyl column (100mm× 2.1mm×2.6μm) supplied by ThermoFisher Scientific, Bremen, Germany. A binary gradient separation was used with mobile phase of aqueous 2mmol/l ammonium formate with 0.1% formic acid (mobile phase A), and 49.5% methanol/49.5%acetonitrile /1.0%water, containing 0.1% formic acid and 2mmol/l ammonium formate (mobile phase B). The initial gradient start at 99% mobile phase A and 1% mobile phase B, and maintained for 1.0 minute, followed by a gradual ramp to 1% mobile phase A and 99% mobile phase B at 10.0 minute. This mobile phase maintained to 11.5 minute, and then re-equilibrated back to the initial conditions for the next injection with a total runtime of 15.5 minutes. The autosampler needle and loop, and injection valve were washed with 800μl of 45/40/10/5 methanol/acetonitrile/water /isopropanol (strong wash), followed by 1000μl of 90/10 water/acetonitrile (weak wash). An injection volume of 1μl, a flow rate of 0.5μl/min, an autosampler compartment temperature of 7°C and a column temperature of 40°C were used for the entire study.

The mass spectrometry analysis was performed using electrospray ionization in the positive mode with a HESI II probe at an inlet capillary temperature of 320°C and spray voltage of 3200V. Sheath and auxiliary gas flows of 50 and 10 arbitrary unites respectively were used. The temperature of auxiliary gas was 400°C. Data acquisition on the Q-Exactive

was performed with Xcalibur version 2.2, SP1.48, and tune page version 2.2, SP1. Data were collected with a resolution of 35000 in the full mass scan mode and 17500 in ddMS^2 mode over a scan range from m/z 50 to m/z 750. Data were collected in profile mode with maximum IT time of 50ms and automatic gain control setting of 1e6. Identification of the target compounds was performed using the retention time and the accurate mass of the quasi-molecular ion. Confirmation was carried out by secondary ion segments with a mass tolerance of 5ppm.

3 Results and Discussion

3.1. Establishment of the Screening List

Only one peak existed in the full scan mass spectra of 27 fentanyl substances in ESI$^+$ mode, and this peak was the quasi-molecular ion of 27 fentanyl substances. It was broken under a certain collision voltage to produce secondary ion segments. The fracture mechanism of 27 fentanyl substance was illustrated taking fentanyl as an example. The C-N bond of the N-phenylamide moiety in quansi-molecular ion [M+H]$^+$ of fentanyl is cleaved, and a propionaldehyde group is removed to form ion segment A while the C-N bond and C-C bond in the piperidine ring are broken to generate ion segment F and ion segment G. Ion segment A can be further broken to form ion segment B and ion segment C. Quansi-molecular ion [M+H]$^+$ can also be broken to form ion segment D. Piperidine

ring and N-phenylamide substituent in quansi-molecular ion can also be cleaved to form ion segment E. Figure 1 shows the possible cleavage pathway for fentanyl. The fracture mechanism of other fentanyl species is similar to that of fentanyl except for precursor NPP (26#) and 4-ANPP (27#). No ion segment A appears in secondary ion segments of 4-ANPP. Similarly, no ion segment A, C and G appear in secondary ion segments of NPP. On the other hand, the piperidine ring in NPP is broken and a carbonyl group is lost, ring closure occurs to form ion segment E. Information of the quansi-molecular ion and secondary ion fragments of the 27 target analytes are shown in Table 2. The serial number of each target analyte in Table 2 is the same as Table 1.
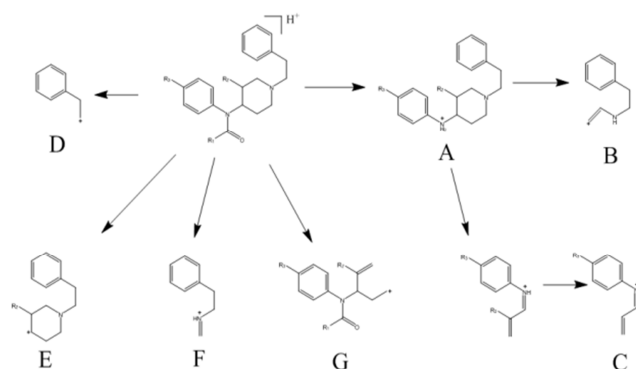

Figure 1. *Proposed fragmentation pathways of product ion for fentabyl in ESI$^+$ mode.*

Table 2. *Quasi-molecular ion and secondary ion fragment of 27 fentanyl substances/m/z.*

No.		[M+H]	Segment A	Segment B	Segment C	Segment D	Segment E	Segment F	Segment G
1#	formula	$C_{22}H_{29}N_2O$	$C_{20}H_{27}N_2$	$C_{11}H_{14}N$	$C_9H_{10}N$	C_9H_{11}	$C_{14}H_{20}N$	$C_{10}H_{14}N$	$C_{13}H_{16}NO$
	accurate mass	337.22744	295.21168	160.11208	132.08078	119.08553	202.15903	148.11208	202.12264
2#	formula	$C_{22}H_{29}N_2O$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{14}H_{18}NO$
	accurate mass	337.22744	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	216.13829
3#	formula	$C_{21}H_{33}N_6O_3$	$C_{18}H_{29}N_6O_2$	$C_7H_{12}N_5O$	$C_{11}H_{14}NO$	$C_5H_9N_4O$	$C_{12}H_{22}N_5O_2$	$C_6H_{12}N_5O$	$C_{16}H_{22}NO_2$
	accurate mass	417.26087	361.23465	182.10364	176.10699	141.07709	268.17680	170.10364	260.16451
4#	formula	$C_{23}H_{31}N_2O$	$C_{20}H_{27}N_2$	$C_{11}H_{14}N$	$C_9H_{10}N$	C_9H_{11}	$C_{14}H_{20}N$	$C_{10}H_{14}N$	$C_{14}H_{18}NO$
	accurate mass	351.24309	295.21688	160.11208	132.08078	119.08553	202.15903	148.11208	216.13829
5#	formula	$C_{23}H_{31}N_2O$	$C_{20}H_{27}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{14}H_{20}N$	$C_9H_{12}N$	$C_{15}H_{20}NO$
	accurate mass	351.24309	295.21168	146.09643	132.08078	105.06988	202.15903	134.09643	230.15394
6#	formula	$C_{23}H_{31}N_2O$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{15}H_{20}NO$
	accurate mass	351.24309	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	230.15394
7#	formula	$C_{23}H_{31}N_2O$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{15}H_{20}NO$
	accurate mass	351.24309	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	230.15394
8#	formula	$C_{21}H_{29}N_2OS$	$C_{18}H_{25}N_2S$	$C_9H_{12}NS$	$C_9H_{10}N$	C_7H_9S	$C_{12}H_{18}NS$	$C_8H_{12}NS$	$C_{14}H_{18}NO$
	accurate mass	357.19951	301.17330	166.06850	132.08078	125.04195	208.11545	154.06850	216.13829
9#	formula	$C_{21}H_{29}N_2OS$	$C_{18}H_{25}N_2S$	$C_8H_{10}NS$	$C_9H_{10}N$	C_6H_7S	$C_{12}H_{18}NS$	$C_7H_{10}NS$	$C_{15}H_{20}NO$
	accurate mass	357.19951	301.17330	152.05285	132.08078	111.02630	208.11545	140.05285	230.15394
10#	formula	$C_{22}H_{29}N_2O_2$	$C_{19}H_{25}N_2O$	$C_{10}H_{12}NO$	$C_9H_{10}N$	C_8H_9O	$C_{13}H_{18}NO$	$C_9H_{12}NO$	$C_{14}H_{18}NO$
	accurate mass	353.22235	297.19614	162.09134	132.08078	121.06479	204.13829	150.09134	216.13829
11#	formula	$C_{23}H_{31}N_2O_2$	$C_{20}H_{27}N_2O$	$C_{10}H_{12}NO$	$C_9H_{10}N$	C_8H_9O	$C_{14}H_{20}NO$	$C_9H_{12}NO$	$C_{15}H_{20}NO$
	accurate mass	367.23800	311.21179	162.09134	132.08078	121.06479	218.15394	150.09134	230.15394
12#	formula	$C_{22}H_{28}N_2OF$	$C_{19}H_{24}N_2F$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{14}H_{17}NOF$
	accurate mass	355.21802	299.19180	146.09643	132.08078	105.06988	188.14338	134.09643	234.12887
13#	formula	$C_{20}H_{29}N_2O_5$	$C_{17}H_{25}N_2O_4$	$C_6H_{10}NO_2$	$C_{11}H_{12}NO_2$	$C_4H_7O_2$	$C_{11}H_{18}NO_4$	$C_5H_{10}NO_2$	$C_{16}H_{20}NO_3$
	accurate mass	377.20710	321.18088	128.07061	190.08626	87.04406	228.12303	116.07061	274.14377
14#	formula	$C_{22}H_{31}N_2O_2S$	$C_{19}H_{27}N_2OS$	$C_8H_{10}NS$	$C_{11}H_{14}NO$	C_6H_7S	$C_{13}H_{20}NSO$	$C_7H_{10}NS$	$C_{16}H_{22}NO_2$
	accurate mass	387.21008	331.18386	152.05285	176.10699	111.02630	238.12601	140.05285	260.16451
15#	formula	$C_{20}H_{27}N_2OS$	$C_{17}H_{23}N_2S$	$C_8H_{10}NS$	$C_9H_{10}N$	C_6H_7S	$C_{11}H_{16}NS$	$C_7H_{10}NS$	$C_{14}H_{18}NO$
	accurate mass	343.18386	287.15765	152.05285	132.08078	111.02630	194.09980	140.05285	216.13829
16#	formula	$C_{21}H_{27}N_2O$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{13}H_{16}NO$
	accurate mass	323.21179	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	202.12264

No.		[M+H]	Segment A	Segment B	Segment C	Segment D	Segment E	Segment F	Segment G
17#	formula	$C_{20}H_{27}N_2O_2S$	$C_{17}H_{23}N_2SO$	$C_8H_{10}NSO$	$C_9H_{10}NO$	C_6H_7SO	$C_{11}H_{16}NSO$	$C_7H_{10}NSO$	$C_{14}H_{18}NO$
	accurate mass	359.17878	303.15256	182.05084	148.07569	127.02121	210.09471	156.04776	216.13829
18#	formula	$C_{23}H_{30}N_2OF$	$C_{19}H_{24}N_2F$	$C_{10}H_{12}N$	C_9H_9NF	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{15}H_{19}NOF$
	accurate mass	369.23367	299.19180	146.09643	150.07135	105.06988	188.14338	134.09643	248.14452
19#	formula	$C_{23}H_{30}N_2OF$	$C_{19}H_{24}N_2F$	$C_{10}H_{12}N$	C_9H_9NF	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{15}H_{19}NOF$
	accurate mass	369.23367	299.19180	146.09643	150.07135	105.06988	188.14338	134.09643	248.14452
20#	formula	$C_{22}H_{28}N_2O_2F$	$C_{19}H_{24}N_2F$	$C_{10}H_{12}N$	C_9H_9NF	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{14}H_{17}NO_2F$
	accurate mass	371.21293	299.19180	146.09643	150.07135	105.06988	188.14338	134.09643	250.12378
21#	formula	$C_{24}H_{31}N_2O_3$	$C_{21}H_{27}N_2O_2$	$C_{10}H_{12}N$	$C_{11}H_{12}NO_2$	C_8H_9	$C_{15}H_{20}NO_2$	$C_9H_{12}N$	$C_{16}H_{20}NO_3$
	accurate mass	395.23292	339.20670	146.09643	190.08626	105.06988	246.14886	134.09643	274.14377
22#	formula	$C_{24}H_{27}N_2O_2$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{16}H_{16}NO_2$
	accurate mass	375.20670	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	254.11756
23#	formula	$C_{22}H_{27}N_2O$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{14}H_{16}NO$
	accurate mass	335.21179	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	214.12264
24#	formula	$C_{24}H_{33}N_2O$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{16}H_{22}NO$
	accurate mass	365.25874	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	244.16959
25#	formula	$C_{24}H_{31}N_2O_2$	$C_{19}H_{25}N_2$	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{16}H_{20}NO_2$
	accurate mass	379.23800	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	258.14886
26#	formula	$C_{13}H_{18}NO$	-	$C_{10}H_{12}N$	-	C_8H_9	$C_{12}H_{18}N$	$C_9H_{12}N$	-
	accurate mass	204.13829	-	146.09643	-	105.06988	176.14338	134.09643	-
27#	formula	$C_{19}H_{25}N_2$	-	$C_{10}H_{12}N$	$C_9H_{10}N$	C_8H_9	$C_{13}H_{18}N$	$C_9H_{12}N$	$C_{11}H_{14}N$
	accurate mass	281.20123	-	146.09643	132.08078	105.06988	188.14338	134.09643	160.11208

-: segment did not appear

A database was created using the accurate mass and formula of quasi-molecular ion and secondary ion segments listed in Table 2, then this database was used to establish a screening list utilizing TraceFinder software, in which rapid screening of the analytes was performed using the accurate mass of the quansi-molecular ion and confirmation was performed utilizing the accurate mass and formula of secondary ion segments while the accurate mass deviation less than 5×10^{-6}. At least 4.0 identification points are required to confirm an analyte using secondary mass spectrometry according to EU Directive 2002/657/EC while a primary ion provided 2.0 identification points and a secondary ion segment provided 2.5 identification points. In order to ensure the reliability of the confirmation results, at least 4 secondary ion segments were provided for each target analyte, and the identification point were all larger than 12.0, far exceeding the identification requirements of 2002/657/EC.

3.2. Rapid Screening and Conformation of Real Samples

The proposed method was used for the rapid screening of the target compounds in nine white powder samples transferred from the customs anti-smuggling department and the results showed that both fentanyl and precursor 4-ANPP existed simultaneously in sample 1# and sample 2# while fentanyl, acetylfentanyl and precursor 4-ANPP were detected simultaneously in other seven samples. Figure 2(a) and Figure 2(b) were UPLC/Orbitrap HRMS extracted ion chromatograms of samples 1# and 3#, respectively. The peaks appearing at 5.20min and 5.42min in Figure 2(a) were 4-ANPP and fentanyl, respectively. The peaks appearing at 4.93min, 5.20min and 5.42 min in Figure 2(b) were acetylfentanyl, 4-ANPP and fentanyl, respectively.
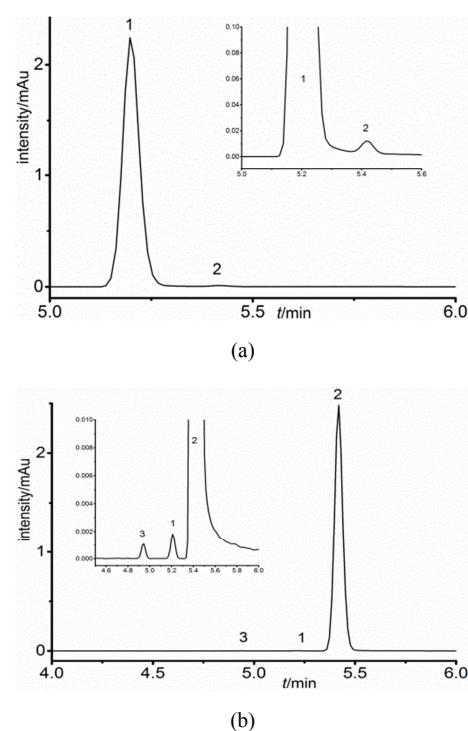

(a)

(b)

Figure 2. *Extracted ion chromatograms of real samples, (a) sample 1#; (b) sample 3#, 1:4-ANPP; 2: fentanyl; 3: acetylfentanyl.*

In ESI$^+$ mode, the formula of the quasi-molecular ion $[M+H]^+$ of 4-ANPP, fentanyl and acetylfentanyl are $C_{19}H_{25}N_2$, $C_{22}H_{29}N_2O$, $C_{21}H_{27}N_2O$, respectively. The information of secondary ion segments that may be generated after fracture was shown in Table 2 but some of the secondary ion segments may disappear completely. Secondary mass spectrometry analysis was performed for sample 3# in which 4-ANPP, fentanyl and acetylfentanyl were detected simultaneously as shown in Figure 2(b). The results were shown in Figure 3, all

other secondary ion segments were detected except for ion segment A and G in precursor 4-ANPP and ion segment A in acetylfentanyl. The theorectical and measured values of accurate mass of quasi-molecular ion and secondary ion segments of 4-ANPP, fentanyl and acetylfentanyl detected in the 3# sample were listed in Table 3. The deviation from the corresponding theoretical values was calculated and also shown in Table 3. The deviation changed in the range from -4.13×10^{-6} to $+0.48\times10^{-6}$, and all less than $\pm5\times10^{-6}$, meeting the requirements of the high-resolution mass spectrometric identification of the European Union Regulation SANCO/12571/2013. For comparison, fentanyl standard was also analyzed and its secondary ion segments were also listed in Table 3. The accurate mass of ion segment C and D is the same in sample and fentanyl standard. The deviation of accurate mass of quansi-molecular ion and ion segment A, B, E, F and G in sample and fentanyl standard was all less than $\pm5\times10^{-6}$. That is to say, fentanyl was really detected in the 3# sample. European Directive 2002/657/EC stipulates that at least 4.0 identification points are required for confirmation using secondary mass spectrometry. In this paper, one primary parent ion and five to seven secondary ion segments were used for confirmation, and the identification point was high as 14.5 to 19.5. Therefore, 4-ANPP, fentanyl and acetylfentanyl were confirmed to exist in 3# sample.
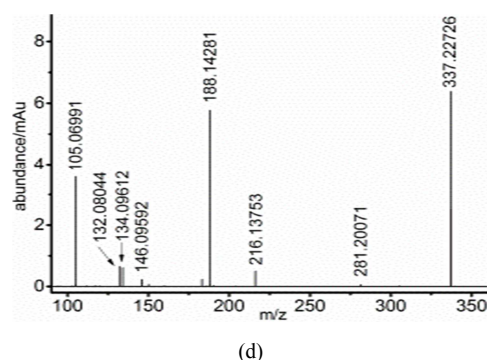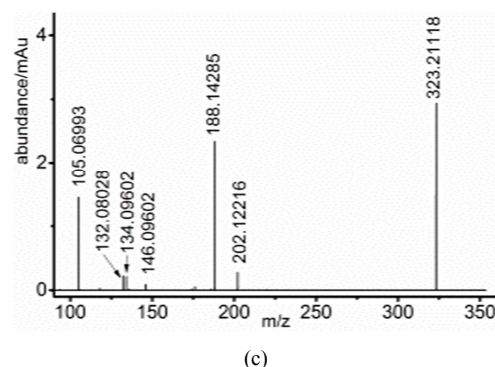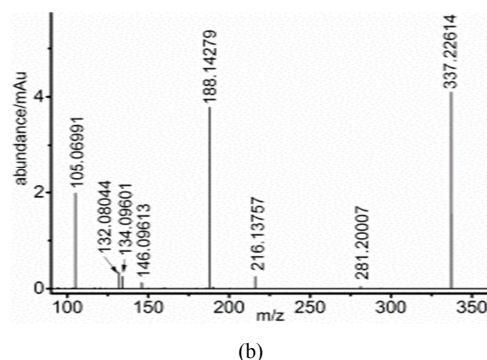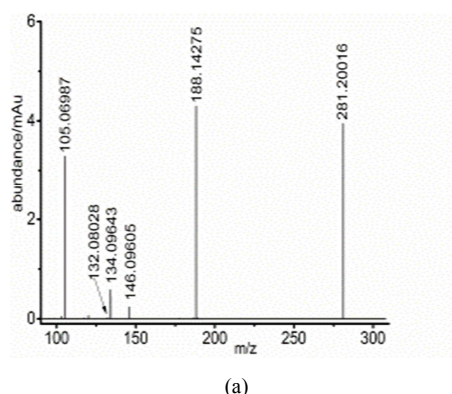

(a)

(b)

(c)

(d)

Figure 3. Secondary ion segments of three target compounds in a positive sample and fentanyl standard (a): 4-ANPP;(b): fentanyl; (c): acetylfentanyl; (d): fentanyl standard.

Table 3. Quasi-molecular ion and secondary ion fragments of three fentanyl substances detected in a positive sample /m/z.

Peak No	Compound		[M+H]	Segment A	Segment B	Segment C	Segment D	Segment E	Segment F	Segment G
1#	4-ANPP	Theoretical mass	281.20123		146.09643	132.08078	105.06988	188.14338	134.09643	160.11208
		measured mass	281.20016	-	146.09605	132.08028	105.06987	188.14275	134.09643	-
		deviation/×10⁻⁶	-3.81	-	-2.60	-3.79	-0.10	-3.35	0.00	-
2#	fentanyl	Theoretical mass	337.22744	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	216.13829
		measured mass	337.22614	281.20007	146.09613	132.08044	105.06991	188.14279	134.09601	216.13757
		deviation/×10⁻⁶	-3.85	-4.13	-2.05	-2.57	+0.29	-3.14	-3.13	-3.33
3#	acetylfentanyl	Theoretical mass	323.21179	281.20123	146.09643	132.08078	105.06988	188.14338	134.09643	202.12264
		measured mass	323.21118	-	146.09602	132.08028	105.06993	188.14285	134.09602	202.12216
		deviation/×10⁻⁶	-1.89	-	-2.81	-3.79	+0.48	-2.82	-3.06	-2.38
standard	fentanyl	measured mass	337.22726	281.20071	146.09592	132.08044	105.06991	188.14281	134.09612	216.13753
		deviation/×10⁻⁶	-0.53	-1.85	-3.49	-2.57	+0.29	-3.03	-2.31	-3.52

-: segment did not appear

The purity of drugs is of great significance for the sentencing of drug trafficking cases. Therefore, for positive samples, standards are required for purity analysis. Different levels of fentanyl were detected in all 9 samples. Fentanyl standard (purity of 98.5%) purchased form Alta Scientific Co. Ltd. was analyzed and used to estimate the purity of fentanyl in nine positive samples. The purity of fentanyl was 0.84% and 1.02%, respectively, for 1# sample and 2# sample, in

which precursor 4-ANPP was the main component. The purity of fentanyl was 85.25%, 74.93%, 86.42%, 79.38%, 66.18%, 69.84% and 59.58%, respectively, for 3# sample to 9# sample. Acetylfentanyl and 4-ANPP at trace levels were also detected in these seven positive samples.

4. Conclusion

Fentanyl substances are newly regulated psychoactive substances. They are strictly controlled and it is quite difficult to obtain their standards or reference materials. Therefore, it is very difficult to establish an analytical method that can simultaneously detect all 27 controlled fentanyl substances. In this paper, a rapid screening and confirmation method of all 27 controlled fentanyl substances without standards was established based on Orbitrap technology. Rapid screening of the analytes was performed using the accurate mass of the quansi-molecular ion and confirmation was performed utilizing the accurate mass and formula of secondary ion segments. The fentanyl standard was analyzed and the analysis results were compared with those of fentanyl in a positive sample. It was found that the accurate mass deviation of the quasi-molecular ion and the second ion segment between fentanyl standard and fentanyl in a positive sample were all less than 5ppm. Therefore, fentanyl was really detected in a positive sample. The experimental results fully demonstrated the feasibility of the proposed method. The proposed method was simple, rapid, sensitive and accurate, and could be applied to the rapid screening and confirmation of fentanyl-like new psychoactive substances without standard.

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