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Salvia divinorum increases alcohol intake and tonic immobility whilst decreasing food intake in Wistar rats

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The kappa-opioid system (KOP) is the key in drug abuse. Of all the compounds isolated from *Salvia divinorum* (*S. divinorum*), salvinorin-A (Sal-A) is predominant. Further, Sal-A is the only compound within *S. divinorum* which is reported to have psychoactive properties as a powerful kappa-opioid receptor (KOPr) agonist. Based on the key role of the KOP system in the consumption of drugs, *S. divinorum* extract (SDE) and Sal-A may modify the alcohol intake in Wistar rats. Assessing voluntary alcohol intake as a drug consummatory behavior, food intake as natural reward behavior and tonic immobility as indicative of anxiety-like behavior, the present study sought to identify the role of both SDE and Sal-A in the Wistar rat model. Forty-eight adult male rats were randomly divided into six groups: control, alcohol naive and vehicle, alcohol-naive and SDE, alcohol-naive and Sal-A, alcohol-consumption and vehicle, alcohol-consumption and SDE, and alcohol-consumption and Sal-A. Alcohol and food intake were assessed for two weeks. In the middle of these two weeks, vehicle, SDE (containing ~1 mg/kg of Sal-A) or Sal-A was injected intraperitoneally once a day for a week. Tonic immobility testing was performed once. The administration of SDE produced a significant increase in voluntary alcohol intake especially in rats with a history of forced alcohol consumption from a juvenile age, Sal-A elicited an increase in alcohol intake in animals with or without previous alcohol exposure, SDE and Sal-A prolonged the tonic immobility duration and decreased food intake. In conclusion, *S. divinorum* or Sal-A stimulated alcohol consumption in rats with a history of alcohol intake and independent of previous exposure respectively, also SDE or Sal-A elicited an anorexigenic effect, and increased tonic immobility as indicative of anxious-like behavior.

Key words: *Salvia divinorum*, salvinorin A, alcohol, food intake, tonic immobility

INTRODUCTION

Salvia divinorum (*S. divinorum*) (Lamiaceae family; formerly Labiatae) contains diterpenes such as salvinorin A and B, as well as additional diterpenoids, divinatorines (A-F), salvidivines (A-D), salvinicins (A and B) and nine additional salvinorins (C-J) (Keasling and Zjawiony, 2016). Salvinorin A (Sal-A) is the only known psychoactive/hallucinogenic constituent of *S. divinorum*. Structurally, Sal-A is a unique furanolactone neoclerodane diterpene which acts as a potent non-nitrogenous selective kappa-opioid receptor (KOPr) agonist (Roth et al., 2002; Willmore-Fordham et al., 2007). In contrast to LSD, psilocybin, dimethyltryptamine, and mescaline, Sal-A has no known activity at the classical hallucinogen drug-receptor

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serotonergic system (5-HT_{2A}) (Roth et al., 2002; Prisinzano, 2005; Johnson et al., 2011). Due to its powerful hallucinogenic effects, *S. divinorum* is often co-consumed (smoking) with alcohol. Sal-A isolated from *S. divinorum* leaves produced a unique profile of subjective effects in humans, similar to other classic hallucinogens (Johnson et al., 2011). When smoked, a single dose of Sal-A (200-500 μg) results in strong, yet brief (5-10 min), hallucinogenic effects and a change in perception (Siebert, 1994).

The toxicity of *S. divinorum* or Sal-A is relatively low. For instance, this observation is highlighted by the doses at which humans are commonly exposed to during healing/ritual practices by the Mazateca ethnic group in Oaxaca, Mexico (Mowry et al., 2003). Further, it is well known that *S. divinorum* is traditionally used for divination practices, spiritual rituals, and training of medical practitioners (Valdés et al., 1983). More recently *S. divinorum* leaves are smoked on recreational/hallucinogenic use worldwide (Casselman and Heinrich, 2011). Internet vendors and "head shops" sell *S. divinorum* plants, dried leaves, leaf extracts with increased Sal-A concentrations for smoking, or as tinctures for oral administration (Johnson et al., 2011).

Clinically, there is increasing evidence that links the action of the endogenous opioid neuropeptide, dynorphin, in modulating mood and increasing the reward effects of abused drugs (Bruchas et al., 2010). It has been hypothesized that the kappa-opioid system (KOP) largely modulates drug consumption, in order of the number of publications: cocaine, alcohol, opioids and nicotine and cannabinoids to a lesser extent (Wee and Koob, 2010). Previous studies have provided evidence of an anti-addictive effect resulting from KOPr stimulation (Prisinzano et al., 2005), while others have found that KOPr antagonists may reverse motivational aspects of dependence (Wee and Koob, 2010). Furthermore, in animal models of drug reinstatement, KOPr stimulation can induce a relapse *via* a stress-like mechanism (Wee and Koob, 2010). Previous animal studies suggest that manipulations which reduce the activity of the KOP system may be effective in the treatment of depression and drug addiction (Shippenberg, 2009).

Additionally, the activity of kappa agonists, including the highly selective KOPr agonist U50, 488H, stimulates food consumption in rats (Cooper et al., 1985). Further, food ingestion was increased after treating animals with different peptide chains of dynorphin (1-17, 1-10, 1-11, 1-13 and 3-13) the endogenous KOPr ligand. However, in rhesus monkeys, the intramuscular administration of bremazocine (a μ and KOPr agonist at 0.00032-, 0.001-, and 0.0025-mg/kg i.m.) under a fixed ratio schedule reduced the response to cocaine, ethanol, phencyclidine, saccharin, and food in a dose-dependent manner (Crosgrove and Carroll, 2002).

The activation of the dynorphin/KOP system has also been shown to be necessary for stress-induced behavioral responses in animal models of anxiety, depression, and drug-seeking behaviors (Bruchas et al., 2010). The cataleptic immobility, or catalepsy, can be defined as a failure to correct externally imposed "awkward postures", whereas tonic immobility (TI) is a reversible state of immobility which can be caused by a wide range of external stimuli. In the laboratory, TI involves the physical restraint and release of the animal, generally in a supine position. Additionally, TI is used as a proxy for innate passive defensive terminal behavior in several species and is characterized by a profound, yet temporary, state of motor inhibition which is often assessed as the latency time immediately before of the righting reflex in rats (Miranda-Páez et al., 2016). TI can be considered as an index of an anxiety-like state (Vázquez-León et al, 2017). In an exploratory environment where cues were introduced to elicit a variety of spontaneous reactive behaviors (e.g. righting reflex, response to pencil-pokes, and reaction to food stimuli), 0.1 and 0.3 mg/kg doses of Sal-A produced no-effects in female rats. However, higher doses (1.0 mg/kg, 3.0 mg/kg and 5.6 mg/kg) produced motor incoordination during the last 15 min of observation as well as dose-dependent catalepsy at times proximal to i.p. drug administration (Willmore-Fordham et al., 2007). Moreover, KOPr activation is associated with depression-like behaviors, characterized by increased immobility in the forced swim test (FST) in rats (Carlezon et al., 2006), while KOPr antagonists have been shown to improve this immobility through anxiolytic activity (Knoll et al., 2007). However, some reports suggest that Sal-A produces both anxiolytic and antidepressant-like effects in rodents (Braida et al., 2009).

Due to the heterogeneity of the findings regarding the behavioral profile of *S. divinorum* and Sal-A administration, the present study aimed to provide clear insights into the effects of *S. divinorum* extract (SDE) containing Sal-A as the main pharmacological active constituent, and Sal-A itself, on alcohol and food intake as well as tonic immobility. To our knowledge, this is the first evaluation of the influence exerted by SDE or Sal-A injection on voluntary alcohol consumption, food intake, and TI in rats.

METHODS

Animals

Male Wistar rats (21 days old) (N=48) were obtained from the vivarium of the Escuela Nacional de

Ciencias Biológicas of the Instituto Politécnico Nacional (Mexico). Animals were randomly divided into six groups (n=8) as follows: control, with standard lab chow diet and tap water, and injected vehicle (AN-VEH), alcohol-naive, with standard lab chow diet and tap water, plus *S. divinorum* extract injection (ANSDE), alcohol-naive, with standard lab chow diet and tap water, plus Sal-A injection (ANSALA), forced alcohol intake plus vehicle injection (ACVEH), forced alcohol intake and SDE injection (ACSDE), and forced alcohol intake and Sal-A injection (ACSALA). All injections were administered i.p. The rats were housed four per cage (32.5 × 20 × 40 cm), kept on a 12/12-h light/dark cycle (lights on at 7:00 a.m.) in a room with controlled temperature (20-22°C) and humidity (45-55%), and, in the case of the three alcohol-naive groups, provided *ad libitum* access to food and tap water. The alcohol consumption groups (ACVEH, ACSDE and ACSALA) were provided with an ethanol solution (6% v/v) instead of tap water from the post-natal day (PND) 21 to 67 (first forced alcohol intake period), followed by a one week with tap water as the only liquid in the diet (first imposed alcohol withdrawal period). A second forced alcohol intake period (10% ethanol solution v/v) was carried out at PND 76–106, followed by a second imposed alcohol withdrawal period at PND 107–114 (as previous treatment or condition). The experimental design was based on Mendoza-Ruiz et al. (2018) (Fig. 1).

Body weights were measured twice a week from PND 21 to 67 and from PND 76 to 106, and daily during the voluntary alcohol consumption evaluation period. The experimental protocol of the study was carried out in accordance with procedures established by the NIH in the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and the Mexican Guidelines for Animal Care (NOM-062-ZOO-1999).

Drugs

The Salvia divinorum extract

The leaves of *S. divinorum* were purchased from Salvia Mx Smartshop (México City – voucher no. 3158). The plant used was identified by Dafné Córdova-Maqueda (Palynology Laboratory, Botany Department, Escuela Nacional de Ciencias Biológicas, México City). SDE was obtained by means of a simple maceration using methanol as solvent. 40 g of the dry leaf were weighed, the sample was pulverized with a mortar for about 10 to 15 min and it was transferred to a 100 mL flask, 80 mL of methanol was added and left to stand for a 3-day period with an occasional stirring such was eliminated as a first decanted high in chlorophyll. 80 mL of methanol was added once again and left in maceration for a week. The same procedure was repeated from the methanol addition; the product was collected from the 2 washes before it was then filtered. Following filtration, the solvent was removed by means of distillation under reduced pressure using a rotary evaporator (Prendo™, Model 1750, Puebla, Mexico) to obtain 0.528 g of the crude extract. 250 mg of the crude extract was dissolved. Two independent chemical analyses of the SDE were conducted. First, a qualitative analysis of the components of the SDE was carried out by Miss Maricarmen Morales and Dr. Jorge Mendoza, both from Escuela Nacional de Ciencias Biológicas at Nanosciences Center of the IPN, México City, using nuclear magnetic resonance proton spectroscopy (NMR^{1}H) using autoflexTOF/TOF and D:\Methods\flexControlMethods\RP_700-3500_Da_MBGP.par. Second, SDE was evaluated by mass spectrometry coupled to high-performance liquid chromatography (MS-HPLC), salvinorin A ($C_{23}H_{28}O_8$; Molecular Weight: 432.5 g/mol) constituted 4% of the methanolic extract (Method: MassLynx 4.1 SCN810; Instrument: XEVO-TQMS#VBA605) with ref-

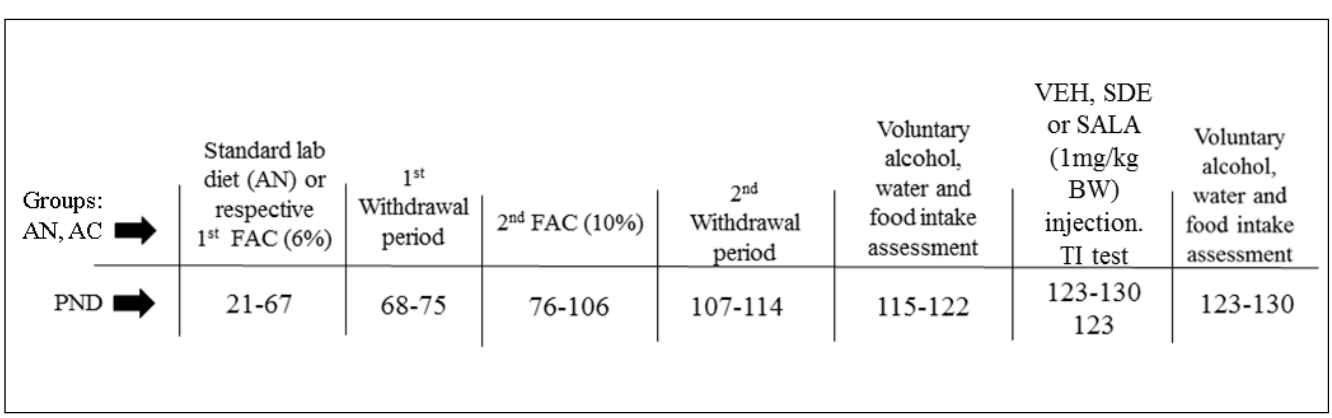

Fig. 1. Experimental design. AN, alcohol naive; BW, body weight; FAC, forced alcohol consumption; PND, postnatal day; SALA, Salvinorin A; SDE, *Salvia divinorum extract*; TI, tonic immobility; VEH, vehicle.

erence to salvinorin A (Cayman chemical®, Ann Arbor, Michigan, USA) [See supplemental materials]. Using these data, the approximate concentration of Sal-A from the SDE to be administered to the rats within this study was calculated to be 1 mg/kg body weight (BW). For the SDE, the vehicle (VEH) was DMSO (10%) and Tween 80 (10%) in sterile saline isotonic solution (Sufka et al., 2014). The SDE, or its respective vehicle, was injected *via* i.p. at a dose of 1 mL/kg, according to Sufka et al. (2014).

Salvinorin A

Salvinorin A. IUPAC Name: methyl (2S,4aR,6aR, 7R,9S,10aS,10bR)-9-acetyloxy-2-(furan-3-yl)-6a,10b-dimethyl-4,10-dioxo-2,4a,5,6,7,8,9,10a-octahydro-1H-benzo[f]isochromene7-carboxylate. (Cayman chemical®, Ann Arbor, Michigan, USA), was dissolved in 10% DMSO and 10% Tween 80 in a sterile saline isotonic solution (Sufka et al., 2014). 1 mg/kg BW in a volume of 1 mL/kg was injected *via* i.p daily from days 7 to 14 of food and fluids consumption assessment.

Alcohol intake assessment

Voluntary ethanol consumption was measured for the rats kept in individual cages. Each rat had access to three standard glass tubes (70 mL, 25 × 200 mm) equipped with a glass mouthpiece containing a terminal hole (diameter=1 mm) to allow for fluid intake by licking with a minimum of spillage. The glass tubes were mounted on the front of the cage and each was previously filled with a different solution: tap water, 10% v/v ethanol-water, and 20% v/v ethanol-water. Ethanol solutions were freshly prepared from 96% ethyl alcohol (C_2H_5OH, Merck® Darmstadt, Germany). Both alcohol concentrations were chosen based on previous reports (Spanagel and Hölter, 1999; Mendoza-Ruiz et al., 2018). The positions of the three tubes were randomly rearranged daily to avoid position preference. Each assay tube was weighed to quantify the amount of alcohol consumed per solution. Alcohol ingestion was calculated in grams of absolute alcohol per kg of body weight (g/kg) daily at two periods: one week before and one week during vehicle, SDE or Sal-A injection (i.p.) once a day.

Food intake assessment

Approximately 50 g of standard lab-chow food pellets (Propecua™) were available in a stainless-steel container at the front of the individual cage. The lab-chow pellets were replaced daily and located next to the three glass tubes. The food container was briefly removed and the food was weighed daily (during the two weeks of examination of voluntary alcohol consumption) to determine the quantity (g) eaten. The food intake measurement for each rat was rectified for unavoidable spillage.

Tonic immobility test

On day-123 (15 min after the vehicle, SDE or Sal-A corresponding i.p. injection), animals were subjected once to the tonic immobility test by using two 5-cm rubber-tipped alligator clips, one clamped to the dorsal and the other to the ventral part of the neck. Each clip exerted 1300 g/cm^2 of force on approximately 1 cm^2 of neck skin. The animal was then inverted to a supine-lateral position and gently maintained in this posture until it stopped struggling (if applicable) and remained immobile. The duration of TI was measured from the time the experimenter's hand was removed until the animal recovered the prone position (latency of righting reflex) for a maximum of 180 s (Miranda-Páez et al., 2016; Vázquez-León et al., 2017). Consumption of fluids and food measurement began at 19:00, while TI assessment between 17:30 and 19:00.

Statistical analyses

All data in the text and figures are expressed as the mean ± SEM. Alcohol and food intake were examined with a two-way analysis of variance for repeated measures (two-way RM-ANOVA), considering alcohol initiation procedure (alcohol-naive or alcohol forced groups), and injected drug (VEH, SDE or Sal-A), followed by a multiple comparison procedure (Student-Newman-Keuls). The duration of tonic immobility was scrutinized with a non-parametric one-way ANOVA (Kruskal-Wallis test) followed by a multiple comparison procedure (Student-Newman-Keuls), therefore, the values represent the median as the measure of central tendency, and the lower (25%) and the upper (75%) corresponding quartile as the variation. Statistically significant differences for all tests were set at $P<0.05$. All statistical analyses were carried out using Sigma Plot® 11.0 (Systat Software Inc. San Jose, CA, USA).

RESULTS

No significant differences were found between all experimental groups in regard to body weight or water intake across all experimental phases (data not shown).

Table I shows that, during the first week of alcohol intake assessment, there was a significant difference between the forced alcohol consumption groups (ACVEH, ACSDE, and ACSALA) and alcohol-naive groups (ANVEH, ANSDE, and ANSALA). However, there was no significant difference between days and treatment/days interaction during the first week.

We performed a statistical analysis of the 14-day alcohol intake. Regarding consumption of the 10% ethanol at respective injection days, a significant difference was found between groups [$F_{(5,546)}$=13.06; P<0.001], and between days [$F_{(13,546)}$=4.211; P<0.001]. We found that ACVEH, ACSDE, and ACSALA differ significantly from ANVEH, ANSDE, and ANSALA from day-1 to day-14. Also, groups ACVEH, ACSDE, ANSALA and ACSALA showed a greater ethanol intake at days 8 and 9 relative to the ANVEH and ANSDE groups. However, there was no significant interaction between treatment/injected drug/day [$F_{(65,546)}$=1.055; P=0.367] (Fig. 2A).

Table I. Statistical analysis of the first week of alcohol intake assessment.

	Treatment	Days	Treatment/ Days
Alcohol 10%	$F_{(5,252)}$=8.773; P<0.001[§]	$F_{(6,252)}$=0.263; P=0.953	$F_{(30,252)}$=0.120; P=1.00
Alcohol 20%	$F_{(5,252)}$=4.942; P<0.001[§]	$F_{(6,252)}$=0.474; P=0.827	$F_{(30,252)}$=0.164; P=1.00
Total Alcohol	$F_{(5,252)}$=8.808; P<0.001[§]	$F_{(6,252)}$=0.351; P=0.909	$F_{(30,252)}$=0.153; P=1.00

Results of two-way RM-ANOVA from days 1 to 7. [§]P<0.001 difference between treatments (ACVEH, ACSDE, and ACSALA *vs.* ANVEH, ANSDE, and ANSALA). (n=8 per group).

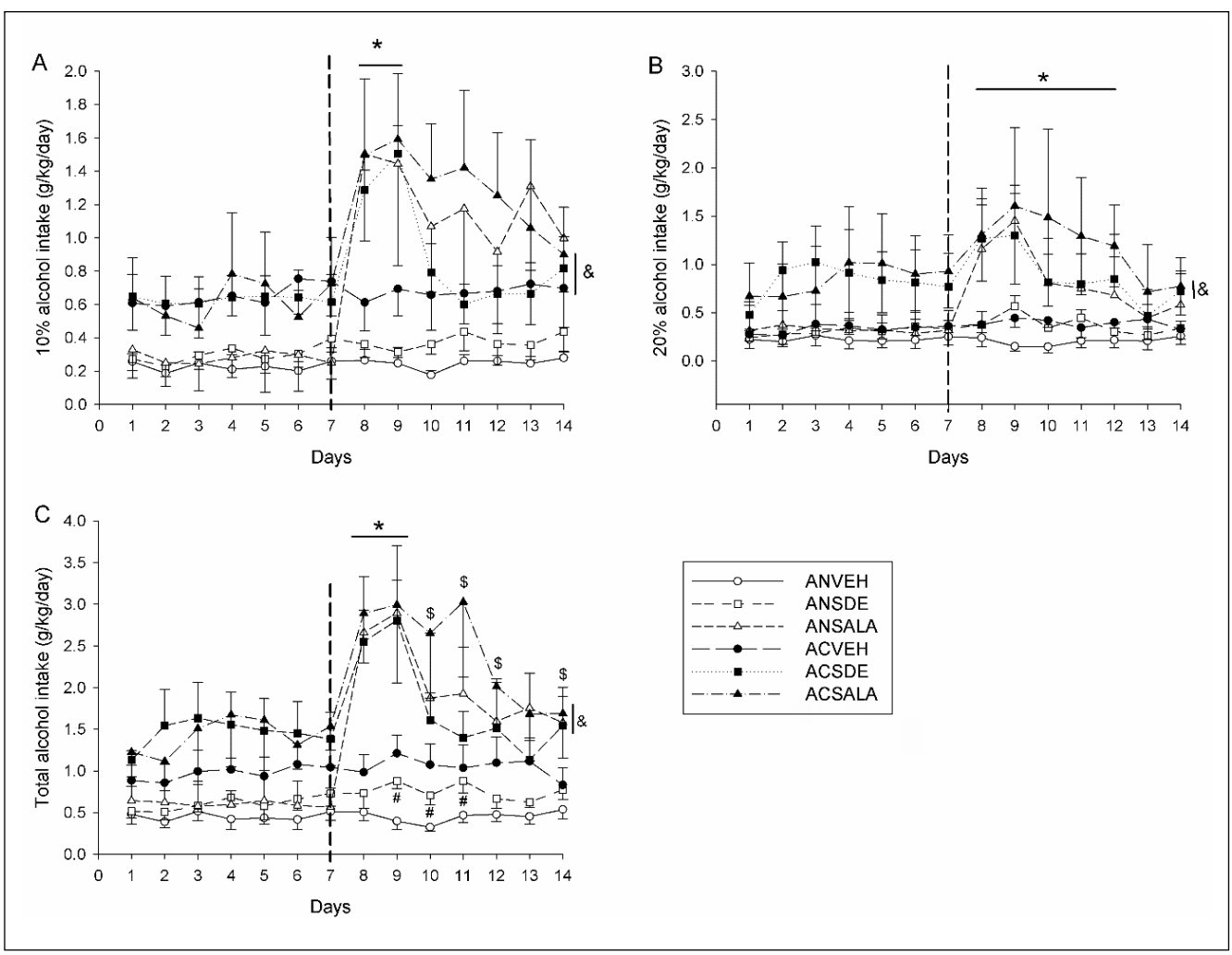

Fig. 2. Alcohol intake (g/kg/day). (A) 10% alcohol intake, (B) 20% alcohol intake, and (C) total alcohol intake. Data are expressed as the mean ± SEM. *P<0.05 days 8 and 9 or 8, 9, 10, 11, and 12 *vs.* days 1, 2, 3, 4, 5, 6, 7, 13, and 14; [&]P<0.05 ACVEH, ACSDE and ACSALA *vs.* ANVEH, ANSDE, ANSALA; [#]P<0.05 ANSDE *vs.* ANVEH and ANSALA; [$]$P$<0.05 ACSALA *vs.* ACVEH, ACSDE. Dotted line represents the daily vehicle or drug injection starting at day-7 of assessment.

The intake of 20% ethanol was significantly different between the ACSDE and ACSALA groups relative to the other treatment groups [$F_{(5,546)}$=12.119; P<0.001]. ANSALA, ACSDE, and ACSALA exhibited an increased alcohol intake relative to ANVEH, ANSDE, and ACVEH from days-8 to day-12 of assessment [$F_{(13,546)}$=1.780; P=0.043]. There was no interaction between treatment/injected drug/day [$F_{(5,546)}$=0.442; P=1.00] (Fig. 2B).

The total alcohol intake (10% and 20%) was significant different between the ACSDE and ACSALA groups relative to the alcohol-naive groups from day-1 to day-14 [$F_{(5,546)}$=24.986; P<0.001]. Namely, the groups with forced alcohol consumption during their juvenile age increased their alcohol intake from day-7, i.e., day-1 of respective injection. Interestingly, between the alcohol-naive groups, the ANSALA group significantly increased its alcohol consumption relative to the ANVEH and ANSDE groups. However, ANSDE group also increased their alcohol intake relative to ANVEH. In addition, we found a significant difference between days 8 and 9, the greatest effect after respective vehicle or drug injection [$F_{(13,546)}$=4.742; P=0.01]. There was no interaction between treatment/injected drug/day [$F_{(5,546)}$=0.075; P=0.242] (Fig. 2C).

Food consumption between groups before vehicle or drug injections was similar. However, there were statistically significant differences in the food intake between the injected SDE and Sal-A groups relative to the injected VEH groups [$F_{(5,546)}$=10.774; P<0.001]. The food intake in the ANSDE and ACSDE groups was significantly reduced at days 8 and 9 of measurement (days 2 and 3 of injection) [$F_{(13,546)}$=60.761; P<0.001]. A two-way RM-ANOVA also revealed a significant interaction between groups and days [$F_{(65,546)}$=9.898; P<0.001]. On days 10, 11, and 12, food intake was significantly decreased in the ANSALA and ACSALA groups as compared to the other groups (ANVEH, ANSDE, ACVEH, and ACSDE). Particularly, on day-11, the ANSALA and ACSALA groups differed significantly and food intake was further decreased in the ANSALA group (Fig. 3).

A significant increase was shown on the duration of tonic immobility in the injected SDE and Sal-A groups (ANSDE, ANSALA, ACSDE and ACSALA) relative to the vehicle groups (ANVEH and ACVEH) [$H_{(3)}$=21.323; P<0.001] (Fig. 4).

DISCUSSION

In the present study, we report that the forced treatment of cyclic alcohol intake and withdrawal at two periods during juvenile age is sufficient to enhance the voluntary consumption of alcohol in the experimental rats relative to alcohol-naive rats. This data agrees with

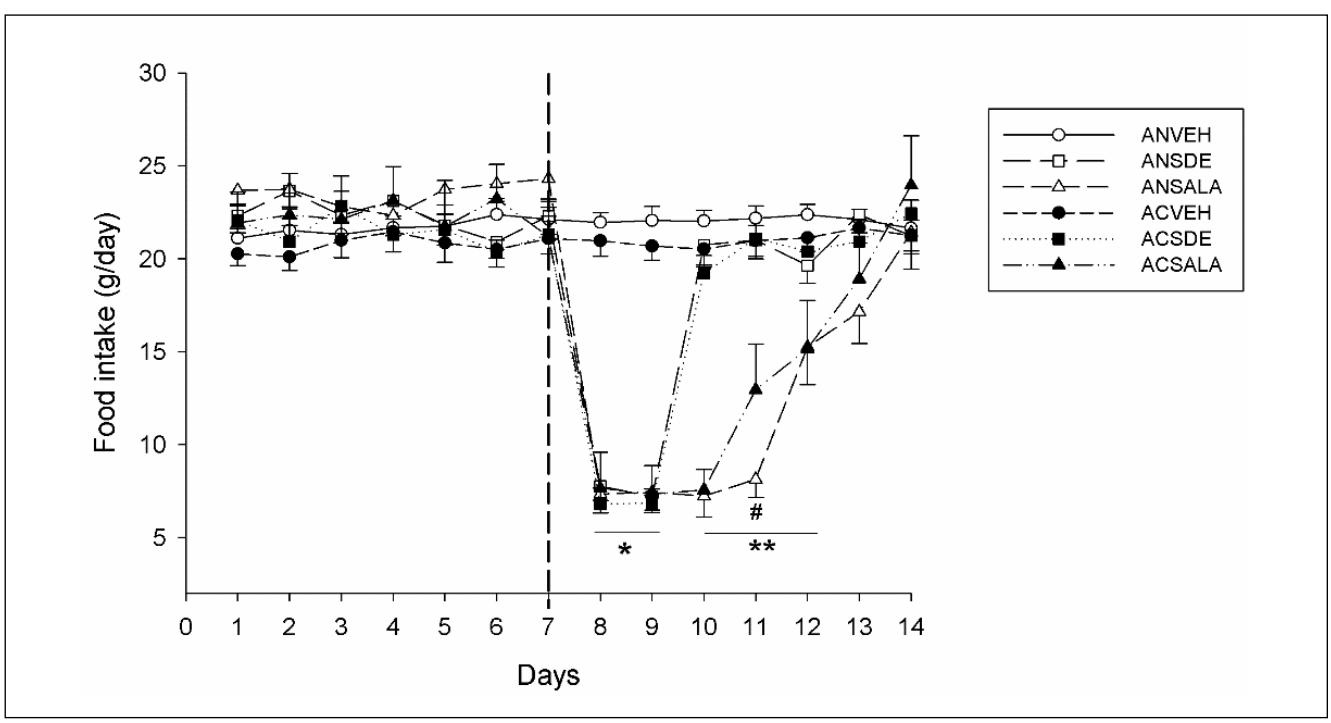

Fig. 3. Food intake (g/day). Data are expressed as the mean ± SEM. *P<0.05 ANSDE, ANSALA, ACSDE and ACSALA *vs.* ANVEH and ACVEH. **P<0.05 ANSALA and ACSALA *vs.* ANVEH, ANSDE, ACVEH, and ACSDE; #P<0.05 ANSALA *vs.* ACSALA. Dotted line represents the daily vehicle or drug injection starting at day-7 of assessment.

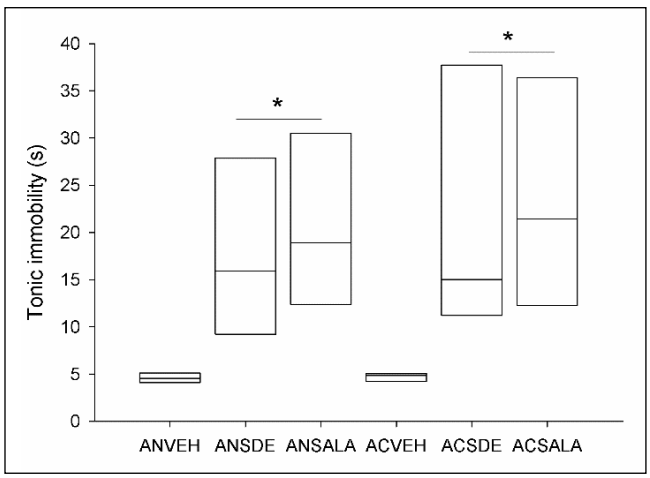

Fig. 4. Tonic immobility (s). Data are expressed as the median and the interquartile range 25 and 75. *P<0.05 ANSDE, ANSALA, ACSDE and ACSALA *vs.* ANVEH and ACVEH.

the findings shown by Mendoza-Ruiz et al. (2018) who provided evidence of same phenomenon. Moreover, it has been reported that prolonged exposure to ethanol intake can induce a dependence-like state (Walker and Koob, 2008). As has been previously hypothesized, such a dependence-like state might be modulated by the KOP system. Here, we show that exposure to SDE increased the alcohol intake in alcohol-naive rats but mainly in rats with a history of alcohol consumption. Further, our data indicate that Sal-A enhanced alcohol intake in rats with, or without, previous alcohol exposure. To our knowledge, this is the first report which links the administration of *S. divinorum* extract to increased alcohol intake. Further, this observation was subsequently confirmed in our study through the injection of Sal-A into experimental rats.

In rats previously subjected to forced alcohol consumption during juvenility, the injection of the SDE or Sal-A, significantly increased the voluntary alcohol intake: 10% ethanol for the first two days after injection, and both 20% ethanol and total ethanol for the first five days of administration. Further, SDE also increased alcohol intake in alcohol-naive rats (ANSDE) at the level of the group previously subjected to forced alcohol consumption and injected vehicle (ACVEH) but solely at 20% and total for the third day and both the third and fourth days of administration, respectively. At the first day of injection, ACSDE rats drank significantly more 20% alcohol than ANVEH group, but not more than the ACVEH and ANSDE groups. Furthermore, after the daily injection of Sal-A, the compound was found to increase alcohol intake: 10%, 20% and total for the first days: 1-2, 1-5 and 1-7 days (except the sixth day) in rats with, or without, previously forced alcohol consumption.

The present findings are in accordance with data reported by Hölter et al. (2000) which demonstrated that the stimulation of KOPr with the highly selective agonist, CI-977 (enadoline), can increase ethanol intake, at least in long-term ethanol-experienced Wistar rats. Since KOPr agonists have aversive motivational consequences, increased ethanol drinking might be an attempt to counteract the aversive effects of this treatment (Hölter et al., 2000). The level of stress, as well as a history of brain alcohol exposure, may be important determinants of the consequences of KOPr activation/blockade for alcohol reward and self-administration (Hölter et al., 2000). Our data support the hypothesis that behaviors associated with withdrawal from ethanol are regulated by the KOP system.

In another study, male Wistar rats with a history of ethanol dependence showed a significant decrease in open-arm exploration, which can be interpreted as immobility, on the elevated plus maze (EPM) after exposure to restraint, indicating an anxiety-like state, compared to similarly treated controls, an effect that was blocked by a KOPr antagonist nor-BNI (0-20 mg/kg, i.p.) (Gillett et al., 2013). In the same study, the highest dose of a KOPr agonist U50, 488H decreased open-arm exploration and the total number of arm entries in ethanol-exposed and control rats; such is in line with the present findings.

Interestingly, the administration of *S. divinorum* at a similar level to its main compound Sal-A produced a robust conditioned place aversion (CPA) in Sprague-Dawley rats (Sufka et al., 2014) similar to other KOPr agonists such as U50, 488H (Tzschentke, 2007; Sufka et al., 2014). CPA might represent a decrease in dopamine levels by 30% to 70% at a dose of 1 to 3.2 mg/kg respectively of Sal-A *via* i.p. in the caudate putamen, but not at nucleus accumbens (NAc) in mice (Zhang et al., 2005) which is fully according to a decreased in locomotor activity at both concentrations. In other studies, Sal-A which was isolated and purified from *S. divinorum* plant leaves, dose-dependently increased immobility in the FST, an opposite effect to that of standard antidepressant drugs. The doses of Sal-A which produced these effects in the FST did not affect locomotor activity in an open field test, which infers a depressive-like effect (Carlezon et al., 2006) and not any ataxic or sedative effects. Moreover, Sal-A dose dependently elevated intracranial self-stimulation (ICSS) thresholds, an effect similar to that produced by treatments that cause depressive symptoms in humans. Further, Sal-A produced anxiogenic effects in rats decreasing time spent in open arms on the EPM (Ewald et al., 2017). According to the present results, a significantly longer period of immobility occurs after the administration of SDE or Sal-A in rats. To the

best of our knowledge, the present findings are the first to show a significant increase in tonic immobility following the i.p. injection of SDE or Sal-A in rats. As a passive defensive behavior, tonic immobility may serve to evaluate an anxiety-like state (Vázquez-León et al., 2017), and this result fits with the pharmacologic profile accepted for the *S. divinorum,* or its main compound Sal-A, as an agonist of dynorphin/KOPr system and as an anxiogenic drug.

KOPr agonists such as dynorphin and U50, 488H have been shown enhanced food intake in rats (Morley and Levine, 1983). In contrast to our data, we report a reduction in food consumption following the administration of SDE – whose effect is attributable to its main active principle, Sal-A. The administration of KOPr agonists in the context of a free-access drinking paradigm may involve more general appetitive actions (Wee and Koob, 2010). Additionally, it is suggested that the KOP system could affect ingestive behaviors mediating palatability, especially in regard to a sweet taste (Woolley et al., 2007). Sal-A and its analogues can also evoke different responses in the modulation of natural reward (Ewald et al., 2017). However, in previous studies, Sal-A (0.3, 1.0 mg/kg i.p.) was shown to have no significant effects on the intake of 10% sucrose solution (Morani et al., 2009). Within this study, SDE administration produced a significant but transient anorexigenic effect with both pre-treatment conditions: in the presence and absence of previously forced alcohol consumption. The period of the decreased food intake only lasted two days, probably due to the activation of homeostatic mechanisms involved in metabolic/energetic balance which would lead to the reinstatement of the previous level of food intake. In the present study, the concentration of SDE was the same every day during the injection period. Perhaps, if the concentration of Sal-A from SDE had been gradually increased, the anorexigenic effect would have been sustained. The occurrence of significant anorexia was observed with the administration of Sal-A during the first five days of injection. Interestingly, food consumption returned to the previous range without "rebound" after the 2-days for SDE group, and 5-days for Sal-A group, respectively. An alternative, but a less likely, explanation for the anorexigenic effect of SDE and Sal-A is that the boost in ethanol consumption, which entailed a lightly greater caloric intake, coincided with the decrease in food consumption, at least during the first 2-day of injection. However, the anorexigenic effect was observed in the SDE injected group, independently of previous alcohol exposure and was highlighted as well as prolonged by the Sal-A injection. Likely, the caloric value provided by alcohol in all experimental groups does not reach significant difference. Such calculation is beyond the goals of the present study. However, the top level of alcohol intake was 2.8 ± 0.7 g/kg/day in the ANSALA, ACSDE and ACSALA groups at days 2 and 3 of i.p. injection.

In a two-week treatment study, minimal histological differences were observed after high doses of Sal-A when administered by i.p. (Mowry et al., 2003; Grundmann et al., 2007). In the present study, injection of the SDE or Sal-A was carried out daily for a week. This approach shortens the likelihood of toxicity, even at the dose used. Nevertheless, to the present, no study has exhaustively investigated chronic or acute toxicity of the leaf extract of *S. divinorum* (Grundmann et al., 2007). In the present study, we suggest an anorexigenic effect for the SDE and its main active component, Sal-A. The anorexigenic, dipsogenic for alcohol, and anxiogenic effects of SDE or Sal-A, could support, at least in part, the thought as to why *S. divinorum* is considered a non-addictive drug. Moreover, current data is suggestive minimal abuse potential for *S. divinorum*. Previous studies have illustrated this idea by showing that Sal-A was able to elevate the thresholds for intracranial stimulation and decreased extracellular dopamine concentrations in the NAc in rats (Carlezon et al., 2006). Furthermore, reports from human case studies have not mentioned any toxic side effects linked to the use of *S. divinorum* as a recreational drug (Grundmann et al., 2007).

Future research is needed to elucidate the role of *S. divinorum* and its main active constituent, Sal-A, in the KOP system as well as to better understand the dipsogenic, orexigenic, depressive-like and anxiety-like behaviors in animals and humans during drug intake.

CONCLUSION

Injection of *S. divinorum* extract fostered greater alcohol intake in Wistar rats, especially in the animals with a history of forced ethanol consumption (that began at a juvenile age). Injection of Sal-A increased alcohol consumption independently of previous alcohol exposure. Additionally, there was an anorexigenic effect with the administration of SDE or salvinorin A, as well as an increase in tonic immobility, suggesting an anxiety-like effect.

ACKNOWLEDGEMENTS

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y Tecnologia (824142). PVL, ERSJ and AMP are fellows of the SNI (CONACYT). We thank Dr. Jorge Mendoza and Miss Maricarmen Morales-Ramírez (both of ENCB-IPN) and Dr. Leticia Contreras Zavala of Pharmometrica® for the chemical/quantitative analysis of the SDE (RMN^{1}H and Mass spectrometry coupled to HPLC), and Professors Bruce Allan Larsen and Mirza Rojas for proofreading the English language version of the manuscript.

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SUPPLEMENTAL MATERIALS

Anexo I

Waters Xevo TQ MS Detector Tune Parameters - MassLynx 4.1 SCN810 Page 1 of 2

File: C:\MassLynx\salvinorin A.PRO\ACQUDB\salvinorin A_18FEB19.ipr

Instrument: XEVO-TQMS#VBA605

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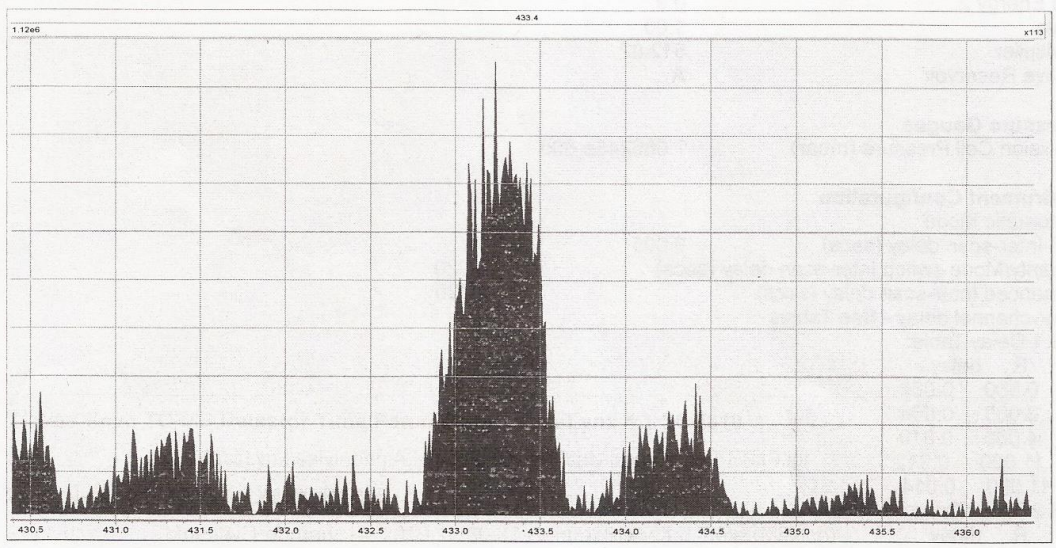


Type	Start Mass	End Mass	Set Mass
MS1 Scan	430.39	436.39	

Source (ES+)	Settings	Readbacks
Capillary (kV)	3.00	3.01
Cone (V)	10.00	14.72
Extractor (V)	3.00	4.59
Source Temperature (°C)	150	151
Desolvation Temperature (°C)	500	500
Cone Gas Flow (L/Hr)	150	150
Desolvation Gas Flow (L/Hr)	1000	1002
Collision Gas Flow (mL/Min)	0.15	0.00

Analyser	Settings	Readbacks
LM 1 Resolution	3.0	
HM 1 Resolution	15.0	
Ion Energy 1	0.5	
MS Mode Collision Energy	2.00	
MSMS Mode Collision Energy	20.00	
MS Mode Entrance	0.50	
MS Mode Exit	0.50	
Gas On MS Mode Entrance	0.50	
Gas On MS Mode Exit	0.50	
Gas On MSMS Mode Entrance	0.50	
Gas On MSMS Mode Exit	0.50	
Gas Off MS Mode Entrance	30.00	
Gas Off MS Mode Exit	30.00	
Gas Off MSMS Mode Entrance	0.50	
Gas Off MSMS Mode Exit	0.50	
ScanWave MS Mode Entrance	0.50	
ScanWave MS Mode Exit	0.50	
ScanWave MSMS Mode Entrance	0.50	
ScanWave MSMS Mode Exit	0.50	
LM 2 Resolution	2.8	
HM 2 Resolution	14.9	

Waters Xevo TQ MS Detector Tune Parameters - MassLynx 4.1 SCN810 Page 2 of 2

File: C:\MassLynx\salvinorin A.PRO\ACQUDB\salvinorin A_18FEB19.ipr

Instrument: XEVO-TQMS#VBA605

Printed: Monday, February 18, 2019 12:30:37 Central Standard Time (Mexico)

Ion Energy 2	0.9
Gain	1.00
Multiplier	512.82
Active Reservoir	A

Pressure Gauges

Collision Cell Pressure (mbar)	7.686246e-005

Instrument Configuration

Automatic Mode

MS Inter-scan delay (secs)	0.005	
Polarity/Mode switch Inter-scan delay (secs)		0.020
Enhanced Inter-scan delay (secs)		0.020

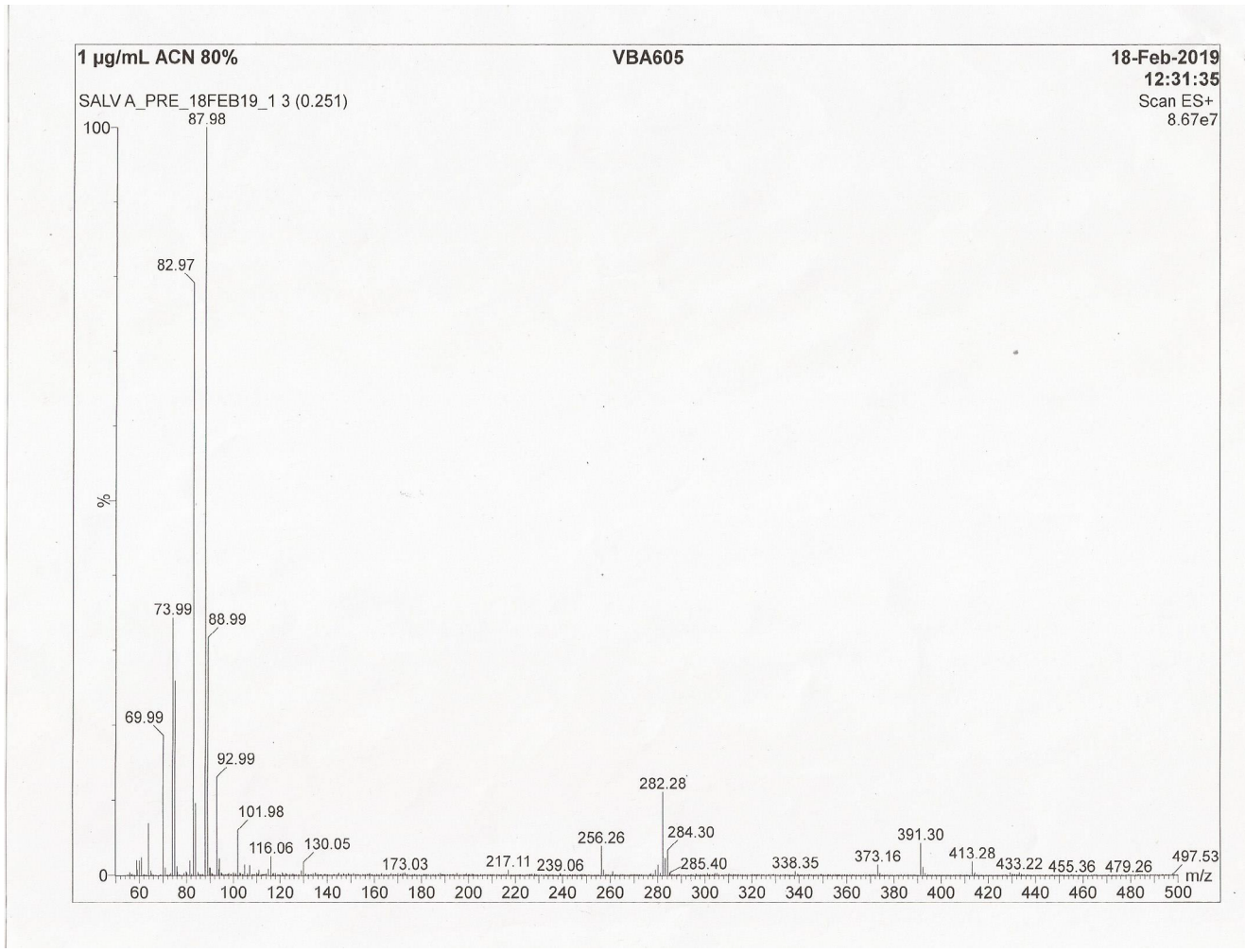
Inter-channel delay - See Tables

MS 1 Delay Table:

R	delay
<= 0.500	0.005
<= 2.000	0.008
<= 4.000	0.010
<= 11.000	0.012
> 11.000	0.014

MS 2 Delay Table:

R	delay
<= 8.000	0.005
<= 25.000	0.005
> 25.000	0.007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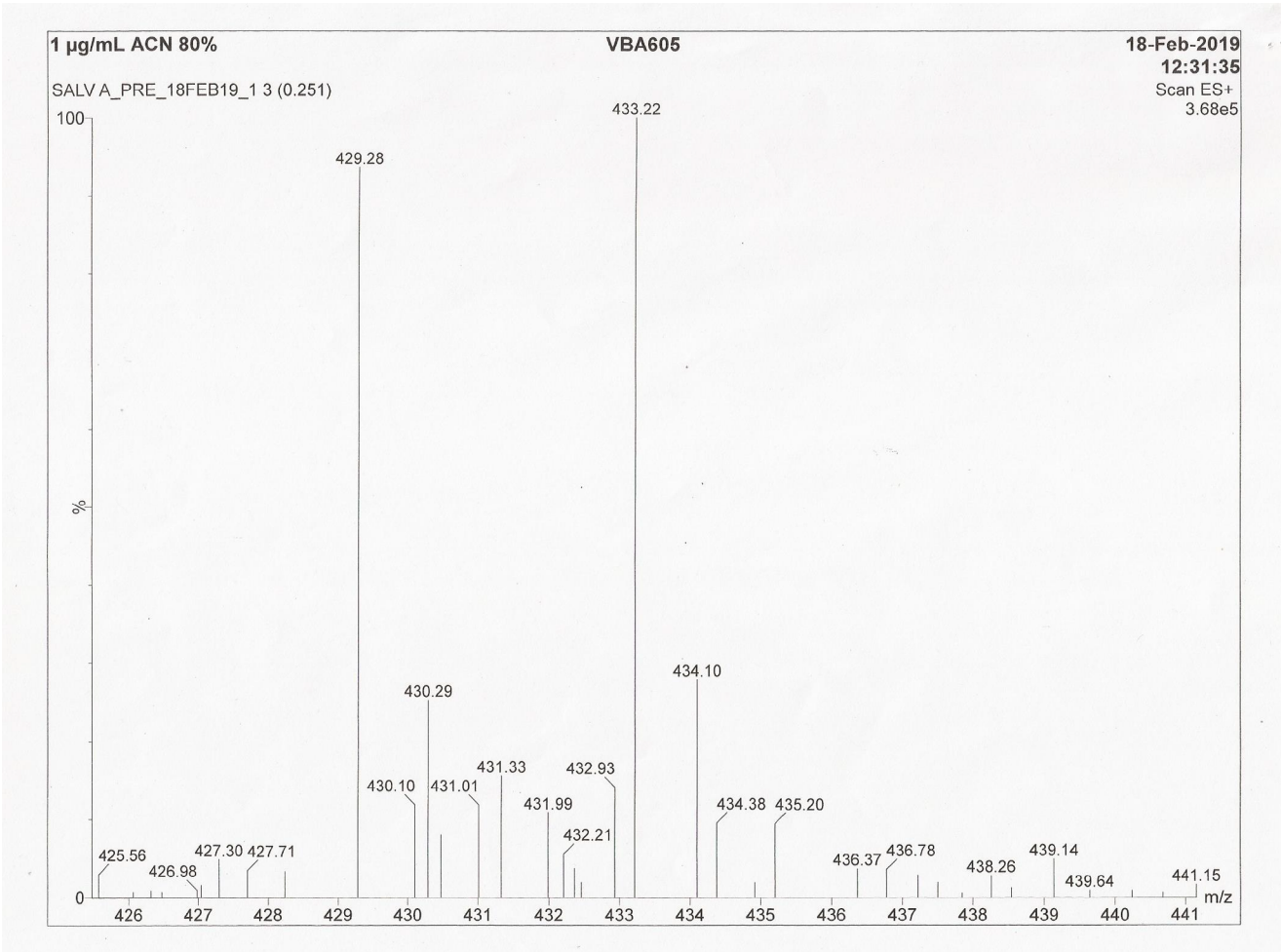
SALV A_PRE_18FEB19_1 3 (0.251)
Scan ES+
8.67e7
100
%
0
87.98
82.97
73.99
88.99
69.99
92.99
101.98
116.06
130.05
173.03
217.11
239.06
256.26
282.28
284.30
285.40
338.35
373.16
391.30
413.28
433.22
455.36
479.26
497.53
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500
m/z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VBA605
18-Feb-2019
12:31:35
SALV A_PRE_18FEB19_1 3 (0.251)
Scan ES+
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100
%
0
433.22
429.28
434.10
430.29
431.33
432.93
430.10
431.01
431.99
434.38
435.20
432.21
425.56
427.30
427.71
426.98
436.37
436.78
438.26
439.14
439.64
441.15
m/z
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441

Waters Xevo TQ MS Detector Tune Parameters - MassLynx 4.1 SCN810 Page 1 of 2

File: C:\MassLynx\salvinorin A.PRO\ACQUDB\salvinorin A_18FEB19.ipr

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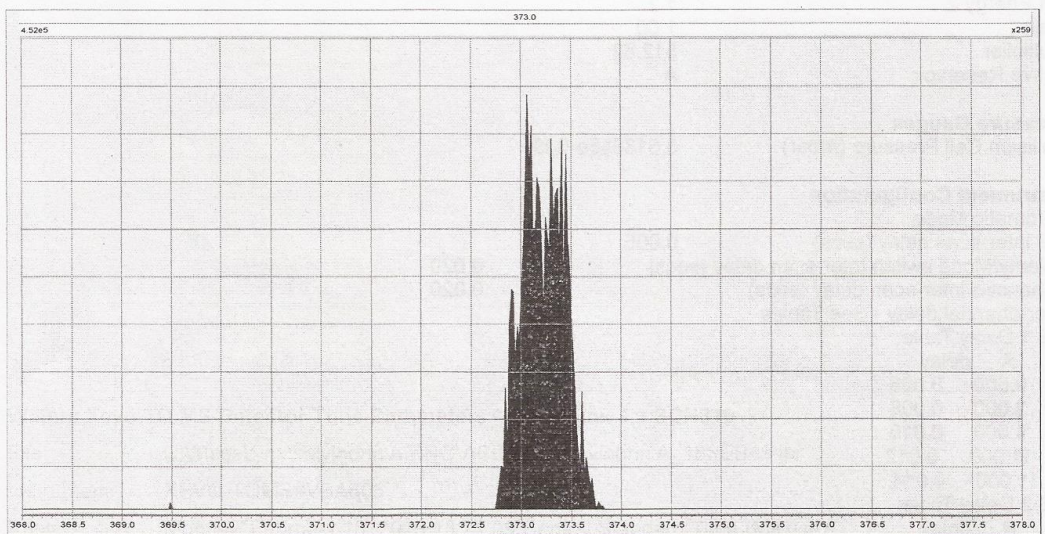


Type	**Start Mass**	**End Mass**	**Set Mass**
Daughter Scan	368.00	378.00	433.22

Source (ES+)	**Settings**	**Readbacks**
Capillary (kV)	3.00	3.03
Cone (V)	10.00	22.32
Extractor (V)	3.00	12.51
Source Temperature (°C)	150	150
Desolvation Temperature (°C)	500	500
Cone Gas Flow (L/Hr)	150	149
Desolvation Gas Flow (L/Hr)	1000	990
Collision Gas Flow (mL/Min)	0.13	0.13

Analyser	**Settings**	**Readbacks**
LM 1 Resolution	3.0	
HM 1 Resolution	15.0	
Ion Energy 1	0.5	
MS Mode Collision Energy	2.00	
MSMS Mode Collision Energy	10.00	
MS Mode Entrance	0.50	
MS Mode Exit	0.50	
Gas On MS Mode Entrance	0.50	
Gas On MS Mode Exit	0.50	
Gas On MSMS Mode Entrance	0.50	
Gas On MSMS Mode Exit	0.50	
Gas Off MS Mode Entrance	30.00	
Gas Off MS Mode Exit	30.00	
Gas Off MSMS Mode Entrance	0.50	
Gas Off MSMS Mode Exit	0.50	
ScanWave MS Mode Entrance	0.50	
ScanWave MS Mode Exit	0.50	
ScanWave MSMS Mode Entrance	0.50	
ScanWave MSMS Mode Exit	0.50	
LM 2 Resolution	3.0	
HM 2 Resolution	15.0	

Waters Xevo TQ MS Detector Tune Parameters - MassLynx 4.1 SCN810 Page 2 of 2

File: C:\MassLynx\salvinorin A.PRO\ACQUDB\salvinorin A_18FEB19.ipr

Instrument: XEVO-TQMS#VBA605

Printed: Monday, February 18, 2019 13:21:09 Central Standard Time (Mexico)

Ion Energy 2	1.2
Gain	1.00
Multiplier	512.82
Active Reservoir	A

Pressure Gauges

Collision Cell Pressure (mbar)	3.513885e-003

Instrument Configuration

Automatic Mode

MS Inter-scan delay (secs)	0.005	
Polarity/Mode switch Inter-scan delay (secs)		0.020
Enhanced Inter-scan delay (secs)		0.020

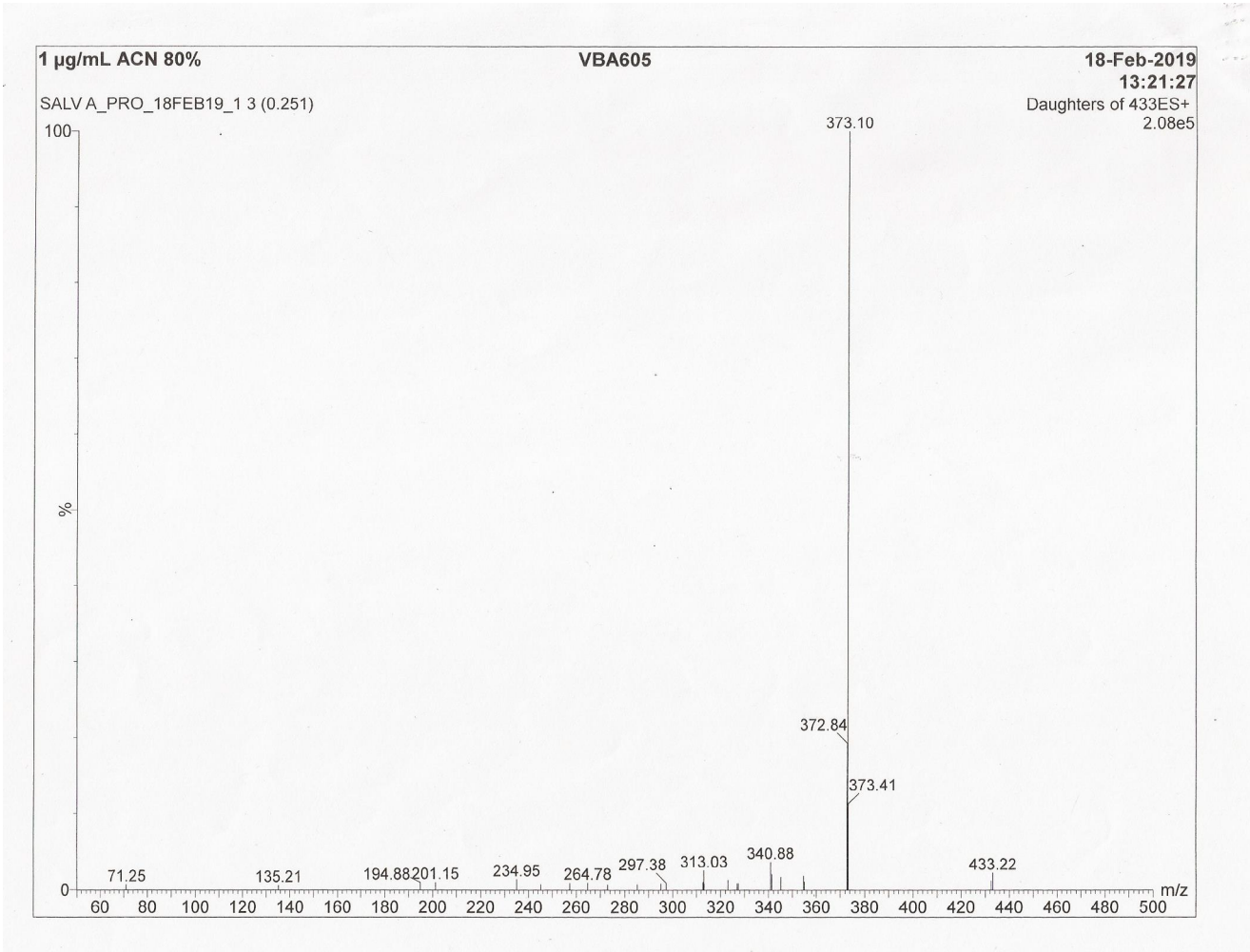
Inter-channel delay - See Tables

MS 1 Delay Table:

R	delay
<= 0.500	0.005
<= 2.000	0.008
<= 4.000	0.010
<= 11.000	0.012
> 11.000	0.014

MS 2 Delay Table:

R	delay
<= 8.000	0.005
<= 25.000	0.005
> 25.000	0.007


1 µg/mL ACN 80%
VBA605
18-Feb-2019
13:21:27
SALV A_PRO_18FEB19_1 3 (0.251)
Daughters of 433ES+
2.08e5
100
%
0
373.10
372.84
373.41
71.25
135.21
194.88
201.15
234.95
264.78
297.38
313.03
340.88
433.22
m/z
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500

Method Development Report

MS1 Resolution	0.75
MS2 Resolution	0.75
Calibration Method	C:\MassLynx\IntelliStart\Results\Unit Mass Resolution\Calibration_20180731_1.cal
Tune Method	C:\MassLynx\salvinorin A.PRO\Acqudb\IntelliStart_18feb19.ipr
Cone Voltage Range	2 - 100
Collision Energy Range	2 - 80
Lowest Fragment Mass	40.00
Excluded Losses	18.00, 44.00

Date: Generated on Mon 18 Feb 2019 at 12:24

Results

IntelliStart generated the following experiments:

MRM Experiment	C:\MassLynx\salvinorin A.PRO\Acqudb\Intelli-MRM-18feb19.exp

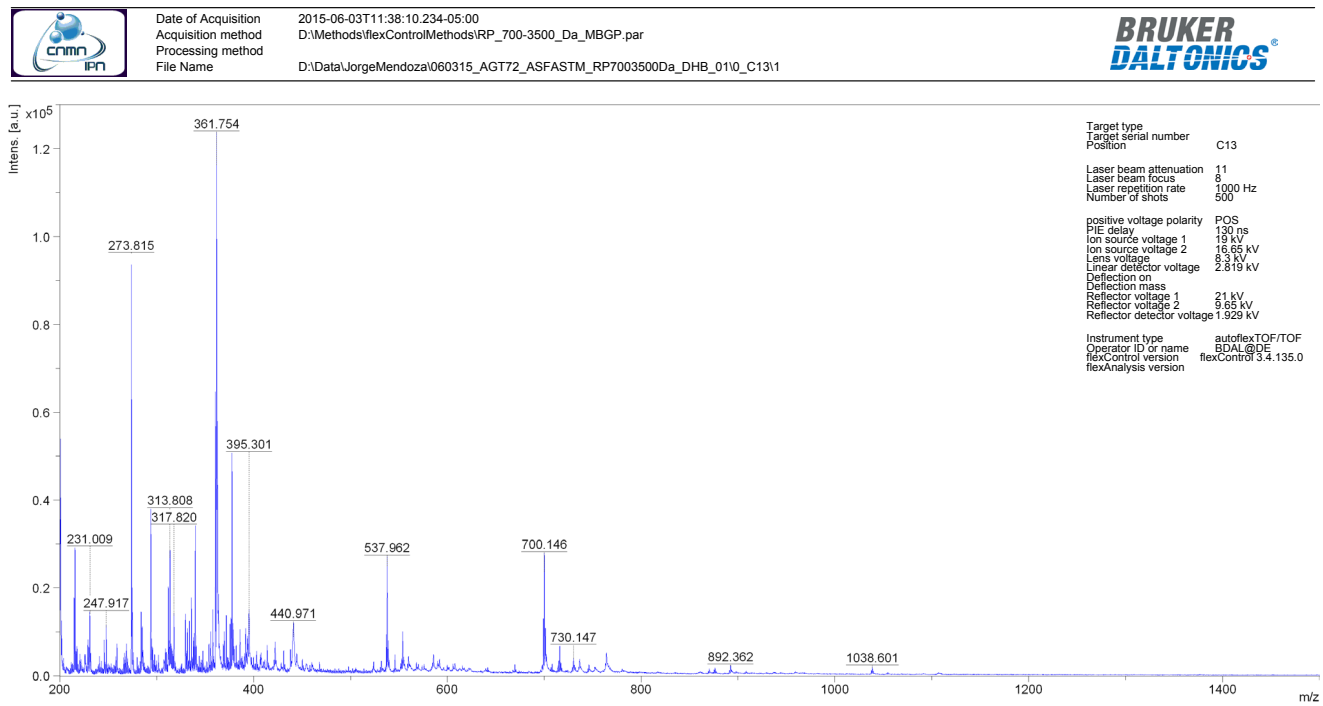
IntelliStart found the following compounds:

Compound	Formula/Mass		Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode
salvinorin	432.5	1	433.21	12	373.14	8	ES+

Compound

salvinorin (432.5)

Transition 1: ES+, m/z 433.21 -> 373.14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Processing method
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273.815
395.301
313.808
317.820
231.009
247.917
537.962
700.146
440.971
730.147
892.362
1038.601
m/z
Target type
Target serial number
Position C13
Laser beam attenuation 11
Laser beam focus 8
Laser repetition rate 1000 Hz
Number of shots 500
positive voltage polarity POS
PIE delay 130 ns
Ion source voltage 1 19 kV
Ion source voltage 2 16.65 kV
Lens voltage 8.3 kV
Linear detector voltage 2.819 kV
Deflection on
Deflection mass
Reflector voltage 1 21 kV
Reflector voltage 2 9.65 kV
Reflector detector voltage 1.929 kV
Instrument type autoflexTOF/TOF
Operator ID or name BDAL@DE
flexControl version flexControl 3.4.135.0
flexAnalysis version