# Title

Sample preparation for the analysis of key metabolites from cannabinoids biosynthesis in phytoplankton using GC-MS

# Running title

sample preparation for cannabinoids analysis

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# Abstract

Cannabinoids biosynthesis in phytoplankton has attracted much attention due to the rapid development of genetic tools and the optimization of genetic transformation methods in microalgae. To monitor the biosynthesis process, proper sample preparation and practical instrumental methods are needed to measure the various precursors, intermediates, cannabinoids, and their degradation products. The objective of this study was to develop a sample preparation procedure for the quantification of olivetolic acid (OA), cannabigerolic acid (CBGA), cannabidiolic acid (CBDA), tetrahydrocannabinolic acid (THCA), olivetol (OL), cannabidiol (CBD), and tetrahydrocannabinol (THC) using single-quadrupole gas chromatography-mass spectrometry (GC-MS). *Isochrysis galbana* was used as the model matrix. After methanol extraction, samples were purified using solid phase extraction (SPE), silylated with N-methyl-N-(trimethylsilyl)trifluoroacetamide, and analyzed using GC-MS in electron ionization mode. A strong anion-exchange SPE efficiently recovered OA, CBGA, CBDA, and THCA. A graphitized carbon black SPE was necessary to purify OL, CBD, and THC. Both columns removed amino acids, sugars, polyols, and pigments from the algae extract and prepared samples that are suitable for silylation and GC-MS analysis. The total protocol, including solvent extraction, SPE, silylation, and GC-MS analysis, was validated in accordance with the ICH guidelines. Performance characteristics of our method are superior to existing protocols with similar complexity in the literature.

# Key words

phytoplankton, cannabinoids biosynthesis, olivetolic acid, olivetol, SPE, method validation

# Introduction

Cannabis has been known for centuries for its undeniable medicinal properties by the general public. Cannabinoids, the hallmark constituents of cannabis, have been intensively investigated for their potential medical applications (Whiting et al., 2015). Substantial evidence exists that cannabinoids are effective for treating pain, chemotherapy-induced nausea and vomiting, and spasticity associated with multiple sclerosis (Abrams, 2018). Moderate evidence for secondary sleep disturbances and limited evidence for appetite improvement, Tourette syndrome, anxiety, posttraumatic stress disorder, cancer, irritable bowel syndrome, epilepsy, and a variety of neurodegenerative disorders were comprehensively reviewed by the National Academies of Sciences, Engineering and Medicine (Abrams, 2018). However, due to the low *in planta* abundances of most cannabinoids and their compositional complexity, pharmacokinetic studies and new drug development has been hampered. Compounding this, the structural and stereochemical complexity of these metabolites hinders most attempts to produce these compounds in large amounts using traditional chemical synthesis or metabolic engineering in plants (Pyne, Narcross, & Martin, 2019). Recent advances in synthetic biology and synthetic biochemistry overcome many of these obstacles, thus providing an alternative avenue for cannabinoids research and development (Tauraa, Tanayaa, & Sirikantaramasb, 2019).

The most well-known cannabinoids biosynthesis pathway is composed of three major stages: 1) olivetolic acid (OA) formation from hexanoyl-CoA and malonyl-CoA, 2) prenylation of OA to produce cannabigerolic acid (CBGA), and 3) oxidative cyclization of CBGA to form Δ⁹-tetrahydrocannabinolic acid A (THCA) and cannabidiolic acid (CBDA) (Tauraa et al., 2019). Pioneering work by Luo *et al.* (2019) reported the complete biosynthesis of the cannabinoids following this pathway in *Saccharomyces cerevisiae*. Currently, at least 18 companies are racing to produce cannabinoids in similar ways in various microbial hosts (Dolgin, 2019). Among them, phytoplankton has attracted much attention (Castro, 2019) due to the rapid development of genetic tools and the optimization of genetic transformation methods in microalgae (Fabris et al., 2020). To monitor the experimental progress of cannabinoids biosynthesis in phytoplankton, proper sample preparation and reliable instrumental methods are needed to measure the various precursors, intermediates, cannabinoids, and their degradation products. Following the abovementioned biosynthesis pathway, we identified OA, CBGA, THCA, and CBDA as the most important metabolites to track. Olivetol (OL), Δ⁹-tetrahydrocannabinol (THC), and cannabidiol (CBD), as suspected byproducts generated through decarboxylation of the biosynthesized acids (Hazekamp, Fischedick, Díez, Lubbe, & Ruhaak, 2010), were also included in the method development. The complex matrix of phytoplankton contains large amounts of interferences for chromatographic analysis. If not removed, these compounds may co-elute with target analytes, leading to ion suppression or enhancement and reducing peak resolution, or contaminate analytical instruments, shortening column life and causing unnecessary maintenance downtime. Reduction of interferences also lowers the requirements on the measurement instrument, making it possible to use a more affordable instrument, such as a single quadrupole gas chromatography-mass spectrometry (GC-MS), instead of two-dimensional GC or tandem MS (Gasse, Pfeiffer, Köhler, & Schürenkamp, 2016).

Although many sample preparation procedures exist in the literature for cannabinoids analysis in plant matrices (Citti, Braghiroli, Vandelli, & Cannazza, 2018), biological samples (Battista et al., 2014), and cannabis edibles (Leghissa, Hildenbrand, & Schug, 2019), none of them meet our needs because of the dramatic differences in sample matrices and target analytes. Plant inflorescence is generally analyzed for chemotyping or quality control, with a focus on the active principles, THC and CBD. The dry matrix and high cannabinoids content afford a simple procedure, which includes solvent extraction and decarboxylation, followed by chromatographic analysis (Citti et al., 2018). Because of the high concentrations of cannabinoids in the plant matrix, matrix load on the analytical instrument can be largely reduced by proper dilution before injection. Biological samples, such as urine, blood, and hair, are mainly analyzed to provide evidence of drug abuse or for pharmacokinetics studies. The complex matrices and low levels of cannabinoids require extra sample preparation steps, such as deproteination, digestion, saponification, and various forms of solid phase extraction (SPE) (Battista et al., 2014). Although published methods for complex biological samples may seem to be appropriate references for phytoplankton matrices, most of these procedures focused solely on the recovery of THC and its metabolites in human, such as 11-hydroxy-Δ⁹-THC (THC-OH), 11-nor-9-carboxy-Δ⁹-THC (THC-COOH), THC glucuronide (THC-gluc), and THC-COOH glucuronide (THC-COOH-gluc), and were not designed to remove large amounts of hydrophobic and hydrophilic pigments such as chlorophylls. Sample preparation procedures for cannabis edibles differ case by case, due to large variations between food matrices. QuEChERS (quick, easy, cheap, effective, rugged, and safe), which combines liquid-liquid extraction and dispersive solid-phase extraction (dSPE), has been applied for cannabinoids recovery in gummy bears, brownies (Shimelis, Stenerson, & Wesley, 2019) and chocolate (Yousefi‐Taemeh & Ifa, 2019) and shows great potential for phytoplankton sample preparation.

In this study, we aimed at developing a sample preparation procedure for the analysis of OA, CBDA, THCA, CBGA, OL, CBD, and THC during their biosynthesis in phytoplankton using single quadruple GC-MS. *Isochrysis galbana* was used as a sample matrix for method development and validation. A number of common organic solvents were compared for the initial extraction of cannabinoids from the phytoplankton matrix. Multiple SPE procedures were tested to remove instrument contaminants and reduce matrix peaks in the chromatographs. Since none of them was able to recover all the seven compounds simultaneously while eliminating the matrix peaks, a two-step cleanup was developed: 1) recovery of OA, CBDA, THCA, and CBGA using a reverse phase strong anion-exchange SPE and 2) recovery of OL, CBD, and THC using a graphitized carbon black SPE. The total protocol, including solvent extraction, SPE cleanup, silylation, and GC-MS analysis, was validated for specificity, linearity, range, accuracy, repeatability, and intermediate precision in accordance with the ICH Harmonised Tripartite Guideline (ICH Expert Working Group, 2005).

# Materials and Methods

## Reagents

Methanol and acetonitrile of HPLC grade, 2-propanol, hexanes, and chloroform of ACS grade, and anhydrous ethanol were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid, ammonium acetate, anhydrous pyridine, n-methyl-n-trimethylsilyltrifluoroacetamide (MSTFA), and chlorotrimethylsilane (TMCS) were purchased from Sigma Aldrich Canada Co.

## Standards

OL with >95% purity, 1.0 mg/mL THCA in acetonitrile, 1.0 mg/mL CBDA in acetonitrile, 1.0 mg/mL CBGA in acetonitrile, THC Cannabinoids Mixture-3 solution containing 1.0 mg/mL each of CBD, THC, and cannabinol (CBN) in methanol, and tribenzylamine (TBA) with >99% purity were purchased from Sigma Aldrich Canada Co. OA with purity >90% was supplied by Cayman Chemical Company (Ann Arbor, MI, USA). One hundred microliters of 10 ppm TBA in acetonitrile was used as internal standard (I.S.) throughout this study. Standard stock solutions containing a combination of OL, OA, CBD, THC, CBN, CBDA, THCA, and CBGA at 20 ppm, 5 ppm, and 1 ppm in acetonitrile were prepared for future use. Although CBN was not in our experimental plan, it was included in some parts of this study as it was present in the THC Cannabinoids Mixture-3 solution from Sigma Aldrich and it did not interfere with our target analytes.

## Algae samples

*Isochrysis galbana* culture was donated by the Aquatron Laboratory at Dalhousie University. The algae culture was centrifuged in weighed falcon tubes at 2000 RPM (~200 g) to concentrate the biomass. After careful removal of supernatant, the falcon tubes were weighed to evaluate the amount of wet algae collected. More culture was added to the same tubes and centrifuged to remove the media. This step was repeated until the desired amount of algae biomass was obtained in each tube. Three aliquots of algae samples were weighed before and after drying in a 40°C oven for 2 days to determine the dry biomass content (~8%) in order to estimate the calibration ranges on dry weight basis.

### Preparation for solvent extraction comparison

To prepare one algae sample for solvent extraction, about 50 mg wet algae (concentrated from 50.0 mL algae culture as described above) was spiked with 500 ng of each standard (100 µL of the 5 ppm standard stock solution). To minimize the impact of residual water on solvent polarity and extraction efficiency, the spiked sample was dried under a nitrogen stream at 30 °C for > 60 min, before extraction with 1.00 mL organic solvent. The final concentration of each analyte was expected to be 0.5 ppm in extraction solvent (1.00 mL), which is equivalent to 10 ppb in the algae culture (50.0 mL), about 10 ppm in concentrated wet algae (~50 mg), or about 125 ppm in dry algae (~4 mg).

### Preparation for validation study

For the recovery test of the total protocol, each ~50 mg wet algae sample (collected from 50.0 mL algae culture) was spiked with 100 ng, 300 ng, 500 ng, or 5000 ng of each standard in acetonitrile and dried with nitrogen at 30 °C for 60 min before solvent extraction and SPE cleanup. This was equivalent to 2 ppb, 6 ppb, 10 ppb, or 100 ppb of each analyte in the algae culture (50.0 mL). Assuming analytes were fully extracted, 0.1 ppm, 0.3 ppm, 0.5 ppm, or 5 ppm was expected in the methanol (1.00 mL).

## Solvent extraction comparison

Solvent extractions using methanol, ethanol, acetonitrile, and a mixture of methanol and chloroform (9:1) and a QuEChERS extraction procedure were performed in triplicate. Spiked and dried algae samples were mixed with 1.00 mL organic solvent and sonicated for 15 min before centrifuging at ~200 g. Four hundred microliters of the supernatant were then collected and spiked with I.S. Solvent was removed under a nitrogen stream, and samples were then silylated (see below) and analyzed using GC-MS. For the QuEChERS extraction, each spiked algae sample was rehydrated with 10.0 mL DI water for 30 min before mixing with 10.0 mL acetonitrile, 1.0 g sodium chloride, and 4.0 g anhydrous magnesium sulfate. Immediately after salt addition, the mixture was vortexed for 1 min to avoid conglomerates. After centrifugation at ~200 g for 15 min, the top acetonitrile layer was carefully transferred to a new tube and dried over sodium sulfate. Four milliliters of the acetonitrile extract were collected, spiked with I.S., dried under a nitrogen stream, silylated, and analyzed using GC-MS.

## SPE cleanup of methanol extract

After the solvent extraction comparison, the methanol extract was selected for further cleanup and analysis. Graphitized carbon black SPE columns (StrataTM GCB, 8B-S528-FCH) and reverse phase strong anion-exchange SPE columns (Strata™-X-A, 8B-S123-UBJ) were supplied by Phenomenex (Torrance, CA, USA). They were conditioned with 2 mL methanol and 2 mL Milli-Q water right before use. Four hundred microliters of the methanol extract was mixed with 1 mL 25 mM ammonium acetate and loaded on a Strata™-X-A (XA) column to recover acids (OA, CBDA, THCA, and CBGA). After sample-loading, the XA column was washed twice with 1.5 mL 25 mM ammonium acetate and twice with 1.5 mL methanol. To collect the acids, the column was eluted three times with 1.5 mL methanol containing 5% formic acid. Another 400 μL of the methanol extract was loaded on a StrataTM GCB (GCB) column to recover OL, CBD, and THC. After sample-loading, the GCB column was washed twice with 1.5 mL Milli-Q water containing 5% methanol. To collect the neutral species, OL, CBD, and THC, the column was rinsed three times with 1.5 mL 2-propanol containing 5% formic acid.

## Silylation

The collected SPE fractions containing target compounds were spiked with I.S. and solvent was evaporated under a nitrogen stream at 30 °C. After evaporation, 75 μL pyridine and 150 μL MSTFA with 2% TMCS were added. The mixture was vortexed for 10 seconds and heated at 70 °C for 45 min. After silylation, samples were either directly injected for GC-MS analysis or evaporated to dryness under nitrogen and dissolved in hexane before injection.

## GC-MS conditions

Analysis was performed on a Thermo Trace 1310 GC coupled with a Thermo ISQ 7000 single quadrupole mass spectrometer. One microliter of the silylated sample was injected in split mode with a split ratio of 100:1 at 280 °C. Separation of compounds was achieved on a Zebron ZB-5 capillary column (5% phenyl, 95% dimethylpolysiloxane; 5 m Guardian; 30 m length, 0.25 mm I.D., 0.25μm film thickness). The oven temperature was first held at 50 °C for 2 min, then increased to 90 °C at a rate of 5 °C/min, and then increased again to 300 °C at a rate of 10 °C/min, which was held for 7 min. For compound identification, the MS was run in TIC mode, with a scanning range from 60 to 600 amu and dwell time of 0.2 sec. The fragmentation pattern and ion ratio consistency of each compound were investigated by exploring the mass spectra of target analytes. Quantification was performed in SIM mode. The selected quantification and confirmation ions are listed in Table S1. In most cases, the most abundant ion was selected as the quantification ion. However, when the base peak had a low or common m/z (e.g. 73 m/z, which represents the TMS group and is a common fragment in many peaks), the second or third most abundant ion was chosen instead to minimize interference. With similar ion intensity, preference was given to the ions with higher m/z. The consistency of ratios between quantification ion, molecular ion (M.+), M-15 fragment, and M-73 fragment for each target analyte across different sample matrices and on different days were assessed to ensure the chosen quantification ions were reliable. Chromatographs of the non-silylated standard mixture (OL, OA, CBD, THC, CBN, CBDA, THCA, and CBGA) contained only 4 analyte peaks (OL, CBD, THC, and CBN). These 4 peaks were included in the data processing method to verify the complete silylation of the 8 compounds in various matrices.

## Calibration curves

Following the internal standard method, calibration curves were built by plotting the theoretical amount ratio between each analytical standard and I.S. in the derivatization mixture against their response area ratio. Linearity of OL, CBD, THC, CBN, OA, CBDA, THCA, and CBGA was evaluated in two ranges, 20 – 200 ng and 500 – 3000 ng of each compound in the derivatization mixture (~225 μL). These two ranges were chosen based on the practical needs for cannabinoids analysis in our lab at the time of this study. Assuming the total protocol fully recovered each analyte, these ranges corresponded to 1 – 10 ppb and 25 – 150 ppb in the algae culture (50.0 mL), or about 1 – 10 ppm and 25 – 150 ppm in wet algae (~50 mg), taking into account that 400 µL out of the 1.00 mL methanol extract was transferred to each SPE tube. Within each range, six levels were tested in triplicate (3 separate preparations) in a random order. For 20 – 200 ng: 20, 40, 80, 120, 160, and 200 ng; for 500 – 3000 ng: 500, 1000, 1500, 2000, 2500, and 3000 ng.

## Method validation

Selectivity was investigated by analyzing the chromatographs of standards, spiked samples, and blank samples. Linearity was evaluated by visual inspection and regression analysis of calibration curves in the two ranges: 20 – 200 ng and 500 – 3000 ng of each standard in the derivatization mixture (~225 μL). To evaluate the matrix effect, another set of standards were prepared in the corresponding SPE fractions from a methanol extract of non-spiked algae. Matrix effect was calculated as the percent ratio between the slopes of the standard curves prepared in SPE fractions and in pure solvent.

Accuracy was reported in the form of percent recovery by the assay of spiked samples. Two sets of spiked samples at 4 spiking levels in triplicates were prepared. In the pre-extraction spiked samples, 100, 300, 500, and 5000 ng of each analyte was spiked in wet algae (~50 mg) collected from 50.0 mL culture before solvent extraction. Considering 400 µL out of 1.00 mL methanol extract was transferred to each SPE, these corresponded to 40, 120, 200, and 2000 ng in the derivatization mixture as targeted levels. In the post-SPE spiked samples, 40, 120, 200, and 2000 ng of each analyte was spiked in SPE fractions from a methanol extract of non-spiked algae matrix. Recovery rate (%) was calculated using the response area ratio between analytes and I.S. in the pre-extraction spiked samples and dividing by the area ratio in the post-SPE spiked samples and multiplying by 100.

Precision was expressed as relative standard deviation (RSD). Repeatability was evaluated by analyzing three individual samples at each of the 4 spiking levels on the same day and calculating the intraday precision of the analysis. To estimate the intermediate precision, replications at each of the 4 spiking levels were performed on different days using new preparations of reagents, different batches of algae, various sample storage time, new calibration curves, different batches of SPE columns, and varied silylation time (30 – 60 min) at random. RSDs of these replicates represented the degree of agreement when operating conditions, except for analyst and instrument, were as different as possible. We referred to these RSDs as “interday precision” to differentiate it from the strict term of “intermediate precision”, which often involves multiple instruments and multiple analysts.

Stability of silylated analytes were evaluated using twelve samples, each containing 3000 ng of OL, OA, CBD, THC, CBN, CBDA, THCA, and CBGA. To assess analytes’ stability at room temperature, 3 samples in the derivatization mixture (without evaporation and reconstitution in hexane) and 3 samples in hexane were analyzed every day from Day 1 to Day 5. For stability at -20°C, another set of 3 samples in the derivatization mixture and 3 samples in hexane were analyzed on Day 4, Day 6, and Day 8. Sample vials were re-capped after each analysis.

## Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics. Curve estimation including various models was carried out on the calibration standards. Multiple R, R squared, adjusted R squared, standard error, and mean squared error of those models were compared. Residuals from simple linear regression were examined for heteroscedasticity. General linear model multivariate tests were performed to investigate: 1) effect of extraction solvents on compounds’ recovery, 2) effect of concentration on compounds’ recovery by SPE columns, and 3) stability of analytes over time. Post Hoc Ryan-Einot-Gabriel-Welsch and Quiot (REGWQ) test was used at 95% confidence level to separate means, as REGWQ test is great at controlling type I error while retaining a statistical power higher than that of Tukey’s test (Omer, 2013).

# Results and Discussion

## GC-MS method development

A GC-MS method in the Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products by United Nations Office on Drugs and Crime (2009) was adapted. The GC oven starting temperature was lowered and the temperature program was elongated to resolve the peaks of silylated short-chain carboxylic acids, dicarboxylic acids, tricarboxylic acids, and ketone acids of metabolic importance (data not shown). Silylation with 100 µL MSTFA in 1.5 mL chloroform at 70 °C for 30 min, as suggested in the UN manual for THCA analysis, did not apply to all the compounds of our interest; derivatization was not complete for THC and CBD, as demonstrated by the presence of chromatographic peaks for non-silylated forms of these compounds (data not shown). Derivatization using 75 µL pyridine and 150 µL MSTFA (with 2% TMCS) at 70 °C for 45 min achieved complete silylation of OL, OA, CBD, THC, CBDA, THCA, and CBGA and was chosen as the ultimate method. In the literature, silylated samples were either directly injected for GC-MS analysis (Fodor, Boldizsár, & Molnár-Perl, 2018; Tan, Clomburg, & Gonzalez, 2018) or evaporated and reconstitued in hexane (Cardenia, Gallina Toschi, Scappini, Rubino, & Rodriguez-Estrada, 2018). In this study, we found that reconstitution in hexane significantly changed the assay results for OL, THC, CBDA, THCA, and CBGA (Fig. 1). Only half of the OL was detected after this extra step. For all the other compounds, the detected amounts were significantly increased after reconstitution in hexane. Loss of OL during evaporation under the nitrogen stream or compound discrimiation in the injection port might have occurred. It is also possible that the TMS derivative of OL is more sensitive to humidity in the air, leading to a much faster hydrolysis compared with other analytes during drying and reconstitution in hexane. This finding indicates we can not simply add this extra reconstitution step during sample preparation and still use the same calibration curves made from standards in pyridine and MSTFA. If hexane is desired as the final sample solvent, the protocol needs to be adjusted and revalidated.

Except for THC, in both silylated and non-silylated forms, the intensity of M.+ was low (<15% of base peak) for all the compounds. However, the relative intensity was always > 1%. M-15 fragment corresponds to the loss of a methyl group and was chosen as the most appropriate quantification ion for the non-silylated THC and CBN and silylated OA, THC, CBN, THCA, and CBGA (Table S1). This information may help when searching for specific compounds in exploratory studies. For example, by extracting 371 m/z (M-15 of silylated THC) and 487 m/z (M-15 of silylated THCA) from a TIC chromotograph, we tentatively identified positional isomers of THC and THCA in authentic biosynthesized samples, which had similar MS fragmentation patterns but eluted at different times. For both non-silylated and silylated forms of OL, the M-56 fragment, which corresponds to the loss of C4H8 on the side chain, had the highest intensity. Silylated CBD had M-68 as its base peak, which corresponds to the loss of C5H8 fragment in the side ring. Both CBD and silylated CBDA had M-83 as their quantification ions, which represent the loss of side chain, C6H11. Although 73 m/z and M-73, which represent the loss of trimethylsilyl group, have been used in the literature as confirmation ions (Cardenia et al., 2018), we found the relative intensity of 73 m/z was not consistent in sample matrices from different extraction solvents. These solvents might have extracted different types or quantities of interferences, leading to different amounts of additional 73 m/z fragment. For each target analyte, the ratios between M.+, M-15, and the selected quantification ion were consistent across different sample matrices and on different days of analysis.

## Solvent extraction comparison

Methanol, ethanol, methanol: chloroform (9:1), and acetonitrile effectively extracted cannabinoids in previous studies (Citti et al., 2018). Here, we found methanol was the best choice (Fig. 2). Although the methanol: chloroform (9:1) solvent system performed as well as methanol alone, the introduction of a highly toxic solvent without additional benefits made it unfavorable. Ethanol was as effective as methanol in extracting OL, CBD, THC, OA, CBDA, and THCA. However, its extraction efficiency for CBGA was significantly lower than methanol. Acetonitrile did not extract OA, THCA, and CBGA as well as methanol, although it largely reduced matrix load based on TIC chromatographs. One should note that we did not consider matrix effects in this part of the study. Different matrix fractions extracted by these organic solvents may have different ion suppression or enhancement effects, which may have contributed to the difference that we observed.

QuEChERS protocols for cannabinoids extraction in foods usually omit the dSPE step, because high concentrations of cannabinoids are collected in the organic layer (acetonitrile) during the liquid-liquid extraction step (Christinat, Savoy, & Mottier, 2020; Wang, Mackowsky, Searfoss, & Telepchak, 2017; Yousefi‐Taemeh & Ifa, 2019). In the study of gummy bears and brownies (Shimelis et al., 2019), QuEChERS extraction resulted in high recoveries (≥80%) of all the cannabinoids under investigation. However, in our study, recovery rates of all the target compounds were significantly lower than methanol, especially for the acids, OA, CBDA, THCA, and CBGA (Fig. 2). This is probably due to their higher polarity compared to the neutral compounds, which caused their partitioning into the aqueous phase. In addition, the upper acetonitrile phase collected all the undesirable pigments from the algae matrix, making it necessary to apply further treatment, such as SPE or dSPE. In this case, QuEChERS extraction could not provide any advantages over simple solvent extraction.

## SPE method development

Five types of SPE sorbents, C18, silica, strong anion exchange, weak anion exchange, and graphitized carbon, with previously published elution procedures for cannabinoids recovery from biological samples were compared in our preliminary experiments. Among these methods, a two-step strong anion exchange SPE for the recovery of THC, THC-OH, and THC-COOH in plasma and serum (Gasse et al., 2016) showed great potential and thus was adapted and optimized for the recovery of OL, CBD, THC, OA, CBDA, THCA, and CBGA in algae matrix. Instead of using acetone and acidic acetone, better recovery of neutral (OL, CBD, and THC) and acidic compounds (OA, CBDA, THCA, CBGA) was achieved using methanol and acidic methanol, respectively. Yellow and orange pigments were rinsed off before the concentration steps, while some dark-green pigments were retained on the column even after the elution with acidified methanol. However, challenges remained with recovery of the neutral components as all solvents investigated that recovered the targeted neutral components also recovered certain bright-green pigments. These green pigments in the collected neutral fraction are not volatile even after silylation and may form active reaction sites in the injection port. To solve this issue, additional cleanup was needed. Initially, the neutral fraction collected from the XA column was subject to solvent reduction and fractionation on the GCB column. Later, to better estimate the true recovery of the GCB column alone and to have the highest recovery possible, the methanol extract of the algae was divided into two steams, with one separated with the XA column to recover acidic components and the other directly loaded on a GCB column to recover neutral compounds. The GCB column firmly retained all the pigments, acidic analytes, and CBN in the sorbent during all kinds of solvent wash, while OL, CBD, and THC could be collected using acidic isopropanol. As a downstream degradation product of THC (Hazekamp et al., 2010), CBN was out of the focus of this study. It was included solely because it came in a more affordable standard solution, and because its data in other parts of this study may be interesting to some readers.

Methanol extraction without SPE cleanup yielded sediments during silylation, which required a further filtration step before GC-MS analysis. Silylated SPE fractions from the XA and GCB columns remained clear, even when left in the autosampler tray or stored at -20°C for several weeks. GC-MS chromatographs of silylated SPE fractions were much cleaner compared to the silylated methanol extract (Fig. S1. a & S1. b). Most peaks representing silylated amino acids, sugars, and polyols were removed by SPE cleanup. These included alanine, valine, leucine, glycerol, proline, serine, threonine, glutamic acid, phenylalanine, 1,2,4,5-cyclohexanetetrol, asparagine, ribonic acid, xylopyranose, galactopyranose, mannopyranose, glucopyranose, mannitol, glucopyranose, glycerol-glycosides, mannobiose, and lactose. Fatty acids, including myristic acid, palmitelaidic acid, palmitic acid, eicosapentaenoic acid, linoleic acid, octadecenoic acid, linolenic acid, and octadecenoic acid, were not removed by SPE cleanup. They were present in the collected fractions from both GCB and XA columns. GCB cleanup fully resolved silylated OL, CBD, and THC from interfering peaks (Fig. S1. c), making peak identification using selected confirmation ions much easier and more reliable. Although XA cleanup did not fully resolve OA and CBDA from the interferences occurred around their retention times, the resolution was sufficient for compound confirmation (Fig. S1. d).

## Method validation

### Selectivity, linearity, and range

No interferences from endogenous substances that would impact the accuracy of the method were observed (Fig. S2). Resolution between any close peaks was larger than 2. Linearity of all the target compounds was generally satisfactory (0.983 ≤ R2 ≤ 0.997) in the two testing ranges (Table S2). Visual inspection and residual analysis revealed a slightly curved trend in the regression lines for most compounds in both ranges. This was also reflected in the increased slope of regression lines in the higher concentration range. Quadratic models showed improved fitting (Table S3). However, as the improvement was minimum, linear models were chosen for standard calibration in this study. If only one calibration curve is desired for a wider concentration range, a quadratic model would likely be necessary. The two validated ranges correspond to 50 – 500 ng and 1250 – 7500 ng of each standard in 50.0 mL algae culture. These are equivalent to 1 – 10 ppb and 25 – 150 ppb in the algae culture (50.0 mL) or 0.05 – 0.5 ppm and 1.25 – 7.5 ppm in methanol extract (1.00 mL). The low concentration levels make the method suitable for sensitive detection and quantification of very low amounts of cannabinoids during their biosynthesis in novel aqueous matrices.

### Accuracy

Matrix effects ranged from 61.9% to 185.4% and from 66.8% to 120.2% in the low and the high concentration ranges, respectively (Table 1). As the matrix effect was non-negligible, especially in the lower range, it was necessary to consider this effect by including the post-SPE spiked samples in the recovery study.

At relatively lower spiking levels (100 – 500 ng), the recovery rate was not significantly different across different concentrations for all the seven target analytes (Table 2). However, there was a significant difference in the recovery of CBD and CBDA when their spiking amount was increased to 5000 ng. Mean recoveries for the seven target analytes ranged from 67.91% to 91.36%. For organic chemicals in food and environmental samples, recommended limits of mean recovery by the Codex Alimentarius Commission (CAC) are between 50 – 120% and 70 – 110% for concentration levels between “<1 ppb” and “>1 ppm” (Ellis, 2008). According to the AOAC guideline for dietary supplements and botanicals, recoveries less than 60 – 70% should be subject to investigations and improvement (Horwitz, 2002). Based on these criteria, the recoveries of all the seven compounds in this study were generally satisfactory.

OA and OL, as important metabolites, have been monitored in cannabinoids’ biosynthesis studies. They are usually quantified using simple HPLC (Fellermeier & Zenk, 1998) or GC-FID (Tan et al., 2018) with authentic standards. To our knowledge, there is no validated method for simultaneous quantification of OA and OL, together with cannabinoids that are important in the biosynthesis pathway. This is probably because OA and OL have a very short life and have never been isolated from the cannabis plants (Hazekamp et al., 2010). During innovative biosynthesis research, their quantity is important to monitor and thus was included in this method development. However, there is no available data on their recovery rate in the literature with which to compare.

Although validated methods for cleanup and analysis of most known cannabinoids exist in the literature, they are scattered over a large amount of scientific papers. Recovery rate of cannabinoids varies dramatically across these studies. Biological samples are usually saponified and cleaned up using SPE or QuEChERS, before subject to LC-MS analysis. Recoveries ranging from 21% to 98 % and from 12% to 118% have been reported for THC and CBD in these validated methods (Ramnarine, Poklis, & Wolf, 2019; Wei, McGuffey, Blount, & Wang, 2016). In a study where samples were not saponified before SPE, recovery rate ranged from 60.2% to 67.2% and from 79.3% to 87.4% for THC and CBD, respectively (Sobolesky et al., 2019). Our protocol yielded higher average recovery rates for THC and CBD (Table 2). Recovery data on THCA, CBDA, and CBGA have been reported in validation studies on food samples, where QuEChERS extraction was used without the dSPE step. Recoveries within the tolerated range of 70 – 120% were reported (Brighenti et al., 2019; Christinat et al., 2020). Our recoveries of CBDA and CBGA were comparable to the reported values (Table 2). The recovery rate of THCA here, however, was lower than the reported value and may be due to the additional SPE cleanup protocol. In general, the acidic compounds (THCA, CBDA, and CBGA) had lower recovery rates compared to the neutral compounds (OL, THC, and CBD) (Table 2). This is probably due to the stronger binding of those acids on the XA column than the neutral compounds’ binding on the GCB column.

### Precision

According to ICH guidelines (ICH Expert Working Group, 2005) for assay validation, as a minimum, precision should be evaluated at two levels: repeatability and intermediate precision. Repeatability, also termed intra-assay precision, expresses the precision under the same operating conditions over a short interval of time (ICH Expert Working Group, 2005). As recommended by the CAC, repeatability limits of analysis (including sample processing) are 14%, 18%, 22%, 32%, and 36% for levels “>1 ppm”, “100 ppb – 1 ppm”, “10 – 100 ppb”, “1 – 10 ppb”, and “<1 ppb”, respectively. Repeatability limits set by AOAC are between 8% and 15% for levels between 10 ppb and 1 ppm (Horwitz, 2002). All the intraday precisions in this study were within these two sets of limits except for OL at 5 ppm with RSD ~28% (Table 3). Considering the recovery rate of OL was quite high (Table 2), it is possible that ion enhancement by artifacts or algae matrix components had happened in the GC-MS and increased the uncertainty of the analytical method. If higher precision is needed, one may need to use stable isotope-labeled internal standards to compensate for matrix effects.

Intermediate precision, also called intra-lab reproducibility, may vary greatly depending on the conditions that are considered and the extents that are varied. Thus, no specific criteria were given for intermediate precision (Ellis, 2008; Horwitz, 2002). As a minimum, intermediate precision should be lower than reproducibility limits. Reproducibility is the precision between laboratories (ICH Expert Working Group, 2005). Reproducibility limits of analysis (including sample processing) by CAC are 19%, 25%, 34%, 46%, and 54% for levels “>1 ppm”, “100 ppb – 1 ppm”, “10 – 100 ppb”, “1 – 10 ppb”, and “<1 ppb”, respectively (Tadeo, 2019). Reproducibility limits set by AOAC are between 16% and 32% for levels between 1 ppm and 10 ppb (Horwitz, 2002). All the interday precisions of our method were well below these limits (Table 3). Thus, we conclude that repeatability and intermediate precision of the tested protocol are generally satisfactory based on CAC and AOAC standards. It is worth noting that interday precisions of some analytes are similar to or even lower than their intraday precisions in our study (e.g., OL at 0.1 ppm and 5 ppm), possibly due to the limited extent of variation in analytical conditions between days. To have achieved a better evaluation of intermediate precision, we would have needed to use multiple instruments and multiple analysts.

Due to the various combinations of sample matrix, solvent extraction, sample cleanup, and chromatography techniques, reported precision of analytical protocols for cannabinoids in complex matrices varies dramatically in the literature. For cannabis plant materials, protocols often only include solvent extraction and chromatographic analysis due to the high levels of cannabinoids (Brown et al., 2019; Cardenia et al., 2018; Elkins, Deseo, Rochfort, Ezernieks, & Spangenberg, 2019; Leiman, Colomo, Armenta, de la Guardia, & Esteve-Turrillas, 2018). When GC-MS was used, analytical precision of CBD, THC, CBDA, THCA, and CBGA ranged from 6.11% to 8.86% and from 0.80% to 8.63% for intraday and interday, respectively (Brown et al., 2019; Cardenia et al., 2018; Leiman et al., 2018). For biological samples, most analytical protocols include a SPE cleanup step and the use of LC-MS (Escrivá, Andrés-Costa, Andreu, & Picó, 2017; Sobolesky et al., 2019; Sørensen & Hasselstrøm, 2017; Wei et al., 2016). Precision data of these protocols in the literature are limited to THC, CBD, THCA, and metabolites of THC. For THC, CBD, and THCA, both intraday and interday precisions ranged from 2% to 16% in most studies (Sobolesky et al., 2019; Sørensen & Hasselstrøm, 2017; Wei et al., 2016). Precision of THC analysis ranging from 5% to 24% was reported by Escrivá et al. (2017). Studies involving the complete QuEChERS protocol, including solvent extraction and dSPE, reported even higher RSDs, ranging from 3% to 38% for THC and from 3% to 16% for CBD (Ramnarine et al., 2019; Yousefi‐Taemeh & Ifa, 2019). The additional SPE or dSPE step in these protocols introduced more uncertainties which might have led to the higher RSDs. Precision is largely dependent on the sample matrix and the complexity of sample preparation procedures. Intraday and interday precisions of our current method (Table 3) are superior or comparable to those existing protocols, even with the additional silylation step, which is a prerequisite for GC-MS analysis.

### Stability

To our knowledge, the stabilities of TMS derivatives of OL, CBD, THC, OA, CBDA, THCA, and CBGA have not been reported in literature. In order to guide sample handling practice after TMS drivatization, we added a preminary stability study. Analytes in the original silylation mixture (150 μL MSTFA with 2% TMCS and 75 μL pyridine) and in hexane, either at room temeprature or at -20°C, were evaluated. At room temperature, silylated THC, CBDA, THCA, and CBGA in the MSTFA and pyridine mixture were stable during the 5-day stability study (Fig. 3a). Silylated CBD and OA started to decrease from Day 4. To investigate if freezing improved their stability, samples stored frozen were analyzed from Day 4 (Fig. 3b). The stabilities of silylated CBD and OA in the MSTFA and pyridine mixture were not significantly improved by frozen storage; they showed a significant decrease from Day 4 to Day 6. Based on these results, such samples should be analyzed as soon as possible after the preparation, ideally within 24 hours.

When analytes were reconstituted in hexane, only silylated OL was stable over the 5-day study at room temperature (Fig. 3c). Silylated CBD and THC started to decrease from Day 2, while other analytes started from Day 3. Samples stored in a freezer demonstrated improved stability of silylated THC, OA, CBDA, THCA, and CBGA in hexane at -20°C (Fig. 3d). However, most compounds still began to degrade on Day 6 or Day 8. Silylated analytes seem to be more stable in the MSTFA and pyridine mixture (Fig. 3a & 3b) than in hexane (Fig. 3c & 3d). Reconstitution in hexane should be used with caution; a stability study on the specific analytes should be carried out before using this extra reconstitution step.

As a preliminary investigation on analytes stability, our experiment design had some drawbacks. The same sets of vials were used repeatedly during the study. A later literature review revealed that multiple injections of one sample of TMS derivatives could lead to a higher variability than multiple analysis of one sample distributed as aliquots in multiple independent vials. In one study, the coefficients of variation for TMS derivatives of amino acids ranged from 18% to 100% when using multiple injections of one sample (Quéro et al., 2014). The authors suspected the degradation of TMS derivatives might be due to the accumulation of trace water issuing from the syringe needle exposed to air and methanol (used for rinsing the syringe) between injections. In our study, re-capping the vials after injections and water condensation from the thawing-freezing cycles may have introduced additional moisture that led to the hydrolysis of TMS derivatives. To confirm the stability of TMS derivatives of OL, CBD, THC, OA, CBDA, THCA, and CBGA without the influence of such factors, a more systematic experiment using “one vial-one injection” analysis should be performed. If more accurate estimation of stability is needed, perhaps a higher sampling frequency (e.g., every 6 hours) should be employed.

## Limitations and future work

The validated protocol is limited to the recovery of OA, OL, and biosynthesized cannabinoids that are within phytoplankton cells or attached to the cell membranes. In practice, these compounds may exist in the culture media, depending on the cellular location of biosynthesis and the life stages of the cells. The phytoplankton types and harvesting time may dictate whether it is necessary to recover metabolites from the culture media. Since the method was validated in only one species of phytoplankton and the matrix effect was not negligible, it will be necessary to use calibration standards prepared in authentic sample matrix. The validation study was performed in two separate ranges. Recovery rates and precisions were evaluated at 3 concentrations across the lower range, and only at one concentration in the higher range. Strictly speaking, only the lower range was validated. However, it is interesting to see if the performance characteristics significantly change when the concentration dramatically increases, even at only one concentration level. Thus, the limited validation data in the higher range are included in this manuscript. The GC oven program is long, as we were interested in analyzing some smaller metabolites in the matrix, which elute within the first 20 min of the run. If only OA, OL, and cannabinoids need to be analyzed, the GC oven program can be shortened. In addition, the use of two SPE columns makes this method less economical but the value of cannabinoids biosynthesis research will likely offset the cost of such an analytical procedure. As our knowledge of cannabinoids metabolic pathways grows, more precursors and metabolites may need to be included in such a method.

# Conclusion

In this study, we developed a sample preparation procedure, which includes solvent extraction, SPE purification, and silylation, for the analysis of OA, CBGA, THCA, CBDA, OL, THC, and CBD in bioengineered phytoplankton using single quadruple GC-MS. Methanol was found to be the most efficient solvent in recovering all 7 compounds of interest. Although acetonitrile and QuEChERS extraction largely reduced the matrix load, their recovery rates of OA, CBGA, THCA, and CBDA were significantly lower than the other solvents tested. The proposed SPE procedure effectively removed most amino acids, sugars, polyols, and pigments from the methanol extract, decreased the number and intensity of matrix interferences in the chromatographs, and reduced matrix contamination in the GC-MS system. The complete analytical method, including solvent extraction, SPE, silylation, and GC-MS analysis, was validated based on ICH guidelines. Recovery rate (67.91 – 91.36%), repeatability (7.16 – 17.36%), and intermediate precision (7.59 – 20.31%) of the method were comparable or superior to existing protocols with similar complexity in the literature. Reconstitution in hexane reduced the stability of the silylated analytes; thus, it is not recommended. Silylated samples should be analyzed within 3 days, if stored at room temperature or in a freezer. The validated method can be used in exploratory studies on cannabinoids biosynthesis in phytoplankton or similar matrices.

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# Tables

Table 1. Matrix effect (%) of SPE fractions from methanol extract of *Isochrysis galbana.* Matrix effect was calculated as the percent ratio between the slopes of the standard curves prepared in SPE fractions and in pure solvent.

|  |  |  |
| --- | --- | --- |
| Compound | Matrix effect (%) | |
| 20-200 ng | 500-3000 ng |
| OL | 64.4 | 66.8 |
| CBD | 185.4 | 107.3 |
| THC | 147.4 | 102.6 |
| OA | 98.8 | 106.2 |
| CBDA | 111 | 120.2 |
| THCA | 104.1 | 115.3 |
| CBGA | 61.9 | 70.4 |

Table 2. Recovery rate (%) at four concentrations. Recover rate was calculated using the response area ratio between analyte and internal standard in the pre-extraction spiked samples and dividing by the area ratio in the post-SPE spiked samples and multiplying by 100. Numbers reported are the mean values from three separate preparations (n=3). Post Hoc REGWQ test was used at 95% confidence level to separate means. Under each analyte, means that do not share a same letter are significantly different.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Spiking amount (ng) | Expected concentration | | OL | CBD | THC | OA | CBDA | THCA | CBGA |
| In culture (ppb) | In methanol (ppm) |
| 100 | 2 | 0.1 | 78.07±10.12a | 77.92±6.27a | 79.90±8.35a | 77.59±3.31a | 75.68±0.26a | 66.37±1.93a | 99.36±6.88a |
| 300 | 6 | 0.3 | 91.59± 7.55a | 82.41±3.26ab | 86.98±4.09a | 70.44±4.30a | 79.22±1.08ab | 71.42±2.34a | 86.09±10.53a |
| 500 | 10 | 0.5 | 99.13±8.72a | 80.93±2.69ab | 86.69±0.93a | 65.91±7.57a | 77.60±2.22a | 71.56±2.46a | 79.02±6.17a |
| 5000 | 100 | 5 | 96.64±27.41a | 91.66±5.88b | 93.22±3.82a | 72.18±4.92a | 87.55±4.22b | 62.28±2.95a | 84.73±8.20a |

Table 3. Intraday and interday precisions at 4 concentrations. Precisions are expressed as relative standard deviations (%). Numbers reported here are the mean values from three separate preparations (n=3).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Intraday precision** | | | | | | | | | |
| Spiking amount (ng) | Expected concentration | | OL | CBD | THC | OA | CBDA | THCA | CBGA |
| In culture (ppb) | In methanol (ppm) |
| 100 | 2 | 0.1 | 12.97 | 8.05 | 10.45 | 9.45 | 7.67 | 9.99 | 4.00 |
| 300 | 6 | 0.3 | 8.24 | 3.95 | 4.71 | 6.10 | 1.37 | 3.28 | 12.23 |
| 500 | 10 | 0.5 | 8.80 | 3.32 | 1.07 | 11.48 | 2.86 | 3.44 | 7.81 |
| 5000 | 100 | 5 | 28.36 | 6.41 | 4.10 | 6.81 | 4.82 | 4.74 | 9.68 |
| **Interday precision** | | | | | | | | | |
| Spiking amount (ng) | Expected concentration | | OL | CBD | THC | OA | CBDA | THCA | CBGA |
| In culture (ppb) | In methanol (ppm) |
| 100 | 2 | 0.1 | 4.35 | 13.39 | 10.34 | 5.07 | 6.60 | 5.68 | 10.00 |
| 300 | 6 | 0.3 | 9.68 | 11.58 | 13.16 | 6.02 | 2.63 | 4.50 | 4.92 |
| 500 | 10 | 0.5 | 14.74 | 11.77 | 9.78 | 7.09 | 1.64 | 2.27 | 5.49 |
| 5000 | 100 | 5 | 9.03 | 12.13 | 9.99 | 10.20 | 4.79 | 8.75 | 7.49 |

# Figure legends

Fig. 1. Recovery rates of TMS derivatives after reconstitution in hexane. Three micrograms of each analyte were derivatized and reconstituted in hexane. The experiment was done in triplicate. Error bars represent 95% confidence intervals. Asterisk (\*) indicates a significant (p < 0.05) difference between before and after reconstitution in hexane based on t-test.

Fig. 2. Extraction efficiency (%) of solvents. Column heights represent mean values (n=3). Error bars represent standard deviations. For each analyte, asterisk (\*) indicates the solvent had a significantly (p < 0.05) lower extraction efficiency than methanol in *Post Hoc* REGWQ test.

**Fig. 3.** Stability of TMS derivatives: (a) in pyridine and MSTFA at room temperature, (b) in pyridine and MSTFA at -20°C, (c) in hexane at room temperature, and (d) in hexane at -20°C. Column heights represent mean values (n=3). Error bars represent standard deviations. For each analyte, asterisk (\*) indicates a significant difference (p < 0.05) from Day 1 in *Post Hoc* REGWQ test.