UNIWERSYTET MARII CURIE-SKŁODOWSKIEJ
W LUBLINIE
Szkoła Doktorska Nauk Ścisłych i Przyrodniczych

Dziedzina: **Nauki ścisłe i przyrodnicze**
Dyscyplina: **Nauki chemiczne**

Michał Rombel
nr albumu: 261758

Problemy w ilościowej ocenie kannabidiolu, kannabigerolu i kannabinolu w matrycach biologicznych

(Issues related to the quantitative assessment of cannabidiol, cannabigerol, and cannabinol in biological matrices)

Rozprawa doktorska przygotowywana pod kierunkiem naukowym
prof. dr. hab. Andrzeja L. Dawidowicza

w Instytucie Nauk Chemicznych

LUBLIN, 2023

Spis treści

Wykaz stosowanych skrótów

Δ8-THC	(−)-trans-Δ^8-tetrahydrokannabinol
Δ9-THC	(−)-trans-Δ^9-tetrahydrokannabinol
8-OH-iso-HHC	8-hydroksy-izo-heksahydrokannabinol
9α-OH-HHC	9α-hydroksyheksahydrokannabinol
CBD	kannabidiol
CBG	kannabigerol
CBN	kannabinol
GC	chromatografia gazowa
GC-MS	chromatografia gazowa sprzężona ze spektrometrią mas
LC	chromatografia cieczowa
LLE	ekstrakcja w układzie ciecz-ciecz
MS	spektrometria mas
NMR	spektroskopia magnetycznego rezonansu jądrowego
SPE	ekstrakcja do fazy stałej
TCA	kwas trichlorooctowy
TFA	kwas trifluorooctowy
QuEChERS	metoda ekstrakcji z ang. *Quick, Easy, Cheap, Effective, Rugged, and Safe*

Wprowadzenie

Konopie uznaje się za jedną z najbardziej kontrowersyjnych roślin w naszym społeczeństwie. Z jednej strony jest źródłem marihuany oraz haszyszu – popularnie przyjmowanych przez człowieka przetworów wywołujących efekt narkotyczny [1], z drugiej jednak strony konopie posiadają niezaprzeczalne właściwości lecznicze, przez co są w grupie najstarszych roślin uprawnych wykorzystywanych przez człowieka [2,3]. Podstawowymi substancjami czynnymi odpowiedzialnymi za efekt działania konopi na ludzki organizm jest grupa związków organicznych określanych mianem kannabinoidów. Najbardziej znanymi przedstawicielami tej grupy są: (−)-trans-Δ9-tetrahydrokannabinol (Δ9-THC) oraz kannabidiol (CBD). Należy wspomnieć również o dwóch zyskujących coraz większą popularność kannabinoidach: kannabigerolu (CBG) oraz kannabinolu (CBN).

Zainteresowanie chemią oraz farmakologią konopi rozpoczęło się w latach 40-tych XX wieku, kiedy po raz pierwszy Adams i współpracownicy wyizolowali z roślin konopnych blisko 90 kannabinoidów [4,5]. Kolejnym punktem przełomowym w historii konopi było opisanie struktury i stereochemii CBD oraz Δ9-THC przez Raphaela Mechoulama, odpowiednio w roku 1963 i 1964 [6]. Na przełomie lat 80-tych i 90-tych XX wieku dokonano kolejnego istotnego odkrycia opisując istnienie układu endokannabinoidowego z receptorami kannabinoidowymi CB1 i CB2 w ludzkim organizmie [7]. Wykazano, że nasz organizm jest zdolny do wytwarzania kannabinoidów – określanych mianem endokannabinoidów – których działanie opiera się na interakcjach ze wspomnianymi receptorami. Efekt terapeutyczny bądź narkotyczny przyjmowania kannabinoidów pochodzenia egzogennego również oparty jest na ich oddziaływaniu z układem kannabinoidowym [8,9]. Stąd też w najczęściej spotykanej obecnie definicji kannabinoidów określane są one jako organiczne związki, naturalne lub syntetyczne, które oddziałują z receptorami kannabinoidowymi.

Ogólna charakterystyka kannabidiolu, kannabigerolu, kannabinolu oraz Δ9-tetrahydrokannabinolu

Najlepiej przebadanym i opisanym w literaturze ze wszystkich kannabinoidów jest (−)-trans-Δ9-tetrahydrokannabinol (nazwa systematyczna: 6aR,10aR)-6a,7,8,10a-tetrahydro-6,6,9-trimetylo-3-pentylo-6H-dibenzo[b,d]piran-1-ol). Związek ten jest w największym stopniu odpowiedzialny za psychoaktywny efekt działania środków narkotycznych pozyskiwanych z konopi [10]. Warto zauważyć, że największa zawartość Δ9-THC występuje w roślinach z gatunku *Cannabis indica* [11]. Według raportu opublikowanego w 2021 roku narkotyki pochodzenia konopnego są najczęściej przyjmowanymi środkami psychoaktywnymi na świecie. W raporcie tym szacuje się również, że w samym 2019 roku przynajmniej jednego kontaktu z wytworami z konopi indyjskich doświadczyło 4% globalnej populacji [12]. Należy jednak podkreślić iż, w literaturze coraz częściej eksponowane są właściwości lecznicze Δ9-THC [13]. Obecność tego związku w preparatach medycznych może wywoływać efekt terapeutyczny lub wzmacniać ten wywołany przez inne kannabinoidy np. CBD. Jednak właściwości narkotyczne Δ9-THC powodują, że dystrybucja produktów leczniczych zawierających ten związek jest mocno ograniczona, bądź nielegalna w wielu krajach świata [14,15]. Natomiast w państwach, w których preparaty tego typu są dopuszczalne, zawierają one głównie mieszaninę Δ9-THC i CBD (np. popularny Sativex), ze względu na podobieństwo efektu terapeutycznego obu kannabinoidów [16].

Obecnie drugim równie popularnym kannabinoidem jest wspomniany kannabidiol (nazwa systematyczna: 2-[(1R,6R)-3-metylo-6-(prop-1-en-2-ylo)cykloheks-2-enylo]-5-pentylobenzeno-1,3-diol). Związek ten jest pozbawionym właściwości psychoaktywnych izomerem Δ9-THC. Spośród wszystkich kannabinoidów znajduje on na ten moment największą ilość zastosowań w medycynie. Uznaje się, że CBD ma szczególny potencjał w leczeniu chorób neurologicznych np. epilepsji [17]. Zwraca się również uwagę na możliwe wykorzystanie go w terapii cukrzycy [18], uzależnień [19], schizofrenii [20] oraz nowotworów [21]. Przedkliniczne badania wykazują również działanie przeciwwymiotne i przeciwbólowe [22]. Głównym naturalnym źródłem CBD są konopie z gatunku *Cannabis sativa*, gdyż w przeciwieństwie do *Cannabis indica* zawierają znacznie mniej psychoaktywnego Δ9-THC [11,23].

Kolejnym wartym uwagi związkiem wyizolowanym z konopi jest kannabigerol (nazwa systematyczna: 2-[(2E)-3,7-dimetylookta-2,6-dienylo]-5-pentylobenzeno-1,3-

diol). Kannabinoid ten, podobnie jak CBD, nie wykazuje właściwości psychoaktywnych po przyjęciu przez człowieka. CBG jest wymieniane jako składnik preparatów stosowanych przede wszystkim w uśmierzaniu przewlekłego bólu i leczeniu stanów zapalnych [24]. Wskazuje się, że może obniżać ciśnienie tętnicze oraz polecany jest w terapii depresji i zaburzeń nastroju [25]. W roślinach konopi CBG występuje w bardzo małych ilościach, bowiem związek ten jest prekursorem pozostałych kannabinoidów, przekształcając się w Δ9-THC oraz CBD w trakcie biosyntezy w roślinie [6,26].

Czwartym z wymienionych w tytule podrozdziału kannabinoidów jest kannabinol (nazwa systematyczna: 6,6,9-trimetylo-3-pentylobenzo[c]chromen-1-ol). Kończy on szlak przemian wymienionych wyżej kannabinoidów i powstaje na drodze dehydrogenacji Δ9-THC [27]. Fakt ten powoduje, że CBN jest uznawany przez wielu jako marker starzenia się konopi [28]. Ze względu na to, że tworzy się z Δ9-THC i ma podobną do niego budowę, to wykazuje niewielką psychoaktywność. Nie jest ona jednak tak silna i odurzająca, jak w przypadku Δ9-THC [29]. CBN znalazł zastosowanie szczególnie w leczeniu bezsenności, jako związek ułatwiający zaśnięcie i poprawiający jakość snu [30]. Ma działanie przeciwzapalne, przeciwdrgawkowe i antybiotyczne [31].

Cel pracy

Wszystkie prozdrowotne właściwości kannabinoidów, a w szczególności CBD, zwiększają nie tylko liczbę pojawiających się na rynku preparatów medycznych zawierających je w swoim składzie, ale również kosmetyków, suplementów diety oraz produktów spożywczych. Zarówno stosowanie przez człowieka coraz większej ilości dostępnych legalnie produktów, których kannabinoidy są głównymi składnikami, jak również nielegalnych środków psychoaktywnych pochodzenia konopnego, wzmaga potrzebę dokładnego oznaczania tych związków w samych preparatach, materiale roślinnym oraz ludzkich i zwierzęcych płynach ustrojowych. Zagadnienie to jest tak samo istotne z punktu widzenia medycyny, weterynarii, jak i kryminalistyki.

Procedura analizy kannabinoidów z matryc biologicznych, takich jak ludzkie i zwierzęce płyny ustrojowe lub materiał roślinny, jest procesem złożonym, w trakcie którego pojawiają się problemy utrudniające rzetelne oznaczanie analizowanych związków. Dotyczą one zarówno etapu przygotowania próbki do analizy, jak i etapu samej analizy oraz wiążą się:

- ze stopniem odzysku analitów w pierwszym etapie procedury analitycznej,
- z możliwą ich transformacją w pierwszym i drugim etapie procedury oraz
- z czułością analizy, która jest pochodną optymalizacji pierwszego etapu procedury, jak i właściwego doboru aparatury wykorzystywanej w drugim etapie przy jednoczesnej wiedzy o możliwościach i ograniczeniach samej aparatury chromatograficznej.

Dlatego też celem niniejszej pracy było poznanie problemów, z którymi może spotkać się analityk zajmujący się oznaczeniem kannabinoidów w złożonych matrycach (głównie płynach ustrojowych), oraz zaproponowanie sposobów rozwiązania niektórych z nich.

Lista artykułów wchodzących w skład rozprawy doktorskiej

Poniżej przedstawiono listę artykułów opublikowanych w renomowanych czasopismach, które stanowią podstawę niniejszej dysertacji doktorskiej:

[D1] M.P. Dybowski, A.L. Dawidowicz, R. Typek, **M. Rombel**, Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – Troubles with reliable CBD quantitation when acidic precipitation agents are applied, Talanta. 220 (2020) 121390. https://doi.org/10.1016/j.talanta.2020.121390. (IF 2-letni: 6,1; MEiN: 100 pkt)

[D2] A.L. Dawidowicz, M.P. Dybowski, **M. Rombel**, R. Typek, Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: Oleamide-induced transient matrix effect, J. Pharm. Biomed. Anal. 204 (2021) 114265. https://doi.org/10.1016/J.JPBA.2021.114265. (IF 2-letni: 3,4; MEiN: 100 pkt)

[D3] A.L. Dawidowicz, M.P. Dybowski, **M. Rombel**, R. Typek, Oleamide as analyte protectant in GC analysis of THC and its metabolites in blood, J. Pharm. Biomed. Anal. 215 (2022) 114800. https://doi.org/10.1016/J.JPBA.2022.114800. (IF 2-letni: 3,4; MEiN: 100 pkt)

[D4] P. Holowinski, R. Typek, A.L. Dawidowicz, **M. Rombel**, M.P. Dybowski, Formation of trifluoroacetic artefacts in gas chromatograph injector during Cannabidiol analysis, J. Chromatogr. A. 1671 (2022) 463020. https://doi.org/10.1016/J.CHROMA.2022.463020. (IF 2-letni: 4,1; MEiN: 100 pkt)

[D5] R. Typek, P. Holowinski, A.L. Dawidowicz, M.P. Dybowski, **M. Rombel**, Chromatographic analysis of CBD and THC after their acylation with blockade of compound transformation, Talanta. 251 (2023) 123777. https://doi.org/10.1016/J.TALANTA.2022.123777. (IF 2-letni: 6,1; MEiN: 100 pkt)

[D6] A.L. Dawidowicz, M.P. Dybowski, R. Typek, **M. Rombel**, P. Holowinski, Unexpected formation of dichloroacetic and trichloroacetic artefacts in gas chromatograph injector during Cannabidiol analysis, J. Pharm. Biomed. Anal. 230 (2023) 115388. https://doi.org/10.1016/J.JPBA.2023.115388. (IF 2-letni: 3,4; MEiN: 100 pkt)

[D7] A.L. Dawidowicz, R. Typek, M.P. Dybowski, P. Holowinski, **M. Rombel**, Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry, Forensic Toxicol. (in press). (IF 2-letni: 2,2; MEiN: 100 pkt)

[D8] M.P. Dybowski, A.L. Dawidowicz, **M. Rombel**, R. Typek, GC vs. HPLC in quantitation of CBD, CBG, Δ9-THC and CBN in plasma using different sample preparation methods, J. Pharm. Biomed. Anal. 234 (2023) 115563. https://doi.org/10.1016/J.JPBA.2023.115563. (IF 2-letni: 3,4; MEiN: 100 pkt)

Sumaryczny *impact factor*: 32,1

Sumaryczna liczba punktów MEiN: 800

Skrótowe omówienie problemów badawczych podjętych w pracach stanowiących podstawę doktoratu

Procedura analizy ksenobiotyków w matrycach biologicznych to zazwyczaj proces złożony, w którym należy wyróżnić etap przygotowania próbki do analizy oraz samą analizę końcową. Problemy utrudniające rzetelną ocenę ilościową analizowanych związków mogą pojawiać się zarówno na pierwszym, jak i drugim etapie stosowanej procedury. Bardzo często wynikają one z przemian, jakim ulegają anality w trakcie etapu przygotowania próbki do analizy, jak i podczas samej analizy, szczególnie jeśli warunki panujące na tych etapach (fizykochemiczne parametry danego procesu/etapu) można zaliczyć do ekstremalnych w odniesieniu do oznaczanych związków.

Jednym z najpopularniejszych obecnie kannabinoidów, który nie wykazuje psychoaktywnego działania, jest CBD. Z literatury [32] wiadomo, że związek ten *in vivo* w soku żołądkowym przekształca się do 9α-hydroksyheksahydrokannabinol (9α-OH-HHC), 8-hydroksy-izo-heksahydrokannabinol (8-OH-iso-HHC), CBN i Δ9-THC. Badania przeprowadzone w ramach prezentowanej pracy doktorskiej wykazały, że związki te tworzą się również w trakcie analizy CBD w próbkach osocza, jeśli analizę tę wykonuje się techniką chromatografii gazowej wykorzystując jako metodę przygotowania próbki precypitację białek osocza odczynnikiem strącającym białka o charakterze kwasowym. Oprócz wyżej wymienionych związków w trakcie analizy CBD, wykorzystującej strącanie białka jako procedurę przygotowania próbki osocza do analizy, powstaje także Δ8-THC, co dodatkowo utrudnia możliwość dokładnego ilościowego oznaczenia kannabidiolu w próbkach osocza. Wyniki tych badań zostały opublikowane w pracy [D1].

Spośród szeregu związków o charakterze kwasowym, które wykorzystuje się do wytrącania białek osocza przed analizą zawartych w nim ksenobiotyków warto wyróżnić kwas trifluorooctowy (TFA) i trichlorooctowy (TCA). W wyniku badań przeprowadzonych z wykorzystaniem TFA jako czynnika wytrącającego białka osocza z jego próbek zawierających CBD wykazano, że w trakcie analizy tego kannabinoidu, poza wyżej wymienionymi produktami jego transformacji (9α-OH-HHC, 8-OH-iso-HHC, CBN, Δ9-THC i Δ8-THC), tworzą się dodatkowo trifluoroacylowe pochodne Δ9-THC i Δ8-THC. Ich ilość zależy od temperatury panującej w dozowniku GC. Wprawdzie wymienione pochodne Δ9-THC i Δ8-THC są znane z literatury i otrzymano je wcześniej na drodze derywatyzacji CBD i Δ9-THC za pomocą bezwodnika kwasu trifluorooctowego, ale dopiero w trakcie badań prowadzonych w ramach prezentowanej pracy doktorskiej

wykazano możliwość ich tworzenia w dozowniku GC, w przypadku kiedy TFA wykorzystuje się jako czynnik wytrącający białka osocza. Szczegóły tych badań zawiera praca [D4].

Równie interesujące jak badania dotyczące TFA są wyniki otrzymane podczas stosowania TCA. Zostały one opublikowane w pracy [D6]. Dowiedziono w niej nie tylko tworzenia się analogicznych pochodnych Δ9-THC i Δ8-THC (tj. trichloroacylowych estrów Δ9-THC i Δ8-THC) w trakcie analizy tych kannabinoidów w próbkach osocza, ale wykazano również, że w przypadku użycia TCA jako czynnika wytrącającego białko, w znacznie większych ilościach powstają dichloroacylowe pochodne Δ9-THC i Δ8-THC w stosunku do trichloroacylowych. W celu udowodnienia niespodziewanej możliwości tworzenia się dichloroacylowych pochodnych Δ9-THC i Δ8-THC podczas analizy CBD i Δ9-THC techniką GC mimo, że do wytrącania białek osocza użyto TCA, a nie kwasu dichlorooctowego, otrzymano te pochodne w skali mikropreparatywnej. Otrzymane pochodne przebadano technikami spektroskopowymi, uzupełniając tym samym literaturę naukową i wskazując na pewne nieprawidłowości w bazie widm masowych NIST14.

Jedną z istotnych kwestii dotyczących oznaczania ksenobiotyków w złożonych matrycach biologicznych jest czułość ich analizy. Najprostszym sposobem wykorzystywanym w celu zwiększenia czułości analizy określonego związku jest jego derywatyzacja. Najpopularniejsze sposoby derywatyzacji związków to sililacja oraz acylacja. W drugim z wymienionych procesów stosuje się głównie halogenki i bezwodniki kwasowe, które przyczyniają się do obniżenia pH środowiska prowadzonej reakcji. Transformacja CBD do Δ9-THC i izomeryzacja tego drugiego kannabinoidu do Δ8-THC w kwasowym środowisku w istotny sposób utrudnia oznaczenie ilościowe CBD, Δ9-THC, jak i ich mieszanin. Z tego powodu sporo uwagi podczas realizacji badań prowadzonych w ramach pracy doktorskiej poświęcono eksperymentom, które miały na celu opracowanie sposobu blokady wymienionych przemian podczas acylacji tych kannabinoidów. W efekcie badań wykazano, że przemiany te można zablokować wykorzystując w trakcie derywatyzacji kannabinoidów trzeciorzędowe aminy i azyny. Ten nowatorski sposób oraz warunki jego prowadzenia zostały przedstawione w pracy [D5].

Jak wynika z literatury [33,34] oraz doświadczeń nabytych podczas badań realizowanych w ramach pracy doktorskiej, czułość analitycznego oznaczania CBD, CBN i Δ9-THC za pomocą GC-MS jest stosunkowo wysoka i wystarczająca do oznaczeń tych kannabinoidów w próbkach osocza, które są badane z powodów kryminalistycznych czy medycznych. Odmiennie przedstawia się sytuacja w odniesieniu do CBG. Przeprowadzone

eksperymenty wskazują, iż niska odpowiedź detektora MS na CBG wynika z jego transformacji w komorze jonizacyjnej, w trakcie której znaczna ilości cząsteczek tego związku przekształca się w cząsteczki piranowej pochodnej CBG. Jak wynika z badań, czułość oznaczania CBG można znacznie zwiększyć poprzez jego cyklizację do formy piranowej na etapie przygotowania próbki do analizy. Należy jednak zauważyć, że proces cyklizacji tego kannabinoidu powoduje zakwaszenie środowiska, w którym transformacji ulega nie tylko CBG, ale także CBD (do Δ9-/Δ8-THC) i Δ9-THC (do Δ8-THC). Skutkiem tego opracowany sposób zwiększania czułości analizy CBG techniką GC-MS powoduje, że ilościowe oznaczenie CBD i Δ9-THC jest niezbyt dokładne, gdy wszystkie trzy kannabinoidy są obecne w badanej próbce. Procedura cyklizacji CBG jest prosta i możliwa do przeprowadzenia w złożonych matrycach, takich jak próbki krwi/osocza i ekstrakty roślinne. Praca [D7] zawiera szczegóły opisanej procedury oraz ukazuje niepublikowane dotąd widma MS i NMR różnych derywatów CBG oraz jego piranowej pochodnej. Dane te stanowią istotne wzbogacenie literatury naukowej dotyczącej analizy CBG.

Zjawiskiem komplikującym ilościową ocenę analitu techniką GC w złożonych matrycach, np. we krwi i osoczu lub materiałach roślinnych, jest efekt matrycowy. Konsekwencją obecności w badanej próbce substancji wywołujących efekt matrycowy jest zwiększenie sygnału oznaczanego analitu w odniesieniu do tego, jaki uzyskuje się dla niego w przypadku próbki wolnej od substancji wywołujących ten efekt. Jednym z dodatków wykorzystywanych często w produkcji polipropylenowych naczyń laboratoryjnych (w tym strzykawek jednorazowych), który kontaminuje próbkę podczas czynności laboratoryjnych z wykorzystaniem takich naczyń, jest oleamid (nazwa systematyczna: (Z)-oktadek-9-enamid). W ramach badań opublikowanych w pracy [D2] udowodniono, że oleamid indukuje efekt matrycowy, który wpływa na wzrost wielkości sygnału CBD i wielu innych związków. Jednak efekt ten w przypadku oleamidu ma charakter przejściowy. Przedstawione wyniki dowodzą, że zidentyfikowany przejściowy efekt matrycowy może z powodzeniem być wykorzystany do poprawy czułości oznaczeń ilościowych ksenobiotyków, w tym kannabinoidów. Sposób ten został również uwidoczniony w pracy [D3], w której potwierdzono jego użyteczność także w analizie z obszaru kryminalistyki, pokazując wzrost czułości analizy Δ9-THC i jego metabolitów we krwi/osoczu prowadzonej z wykorzystaniem techniki GC-MS.

Jak zaznaczono wyżej, jedną z istotnych kwestii dotyczących oznaczania ksenobiotyków w złożonych matrycach biologicznych jest czułość ich analizy. Czułość złożonych procedur analitycznych zależy nie tylko od czułości zastosowanego instrumentu

analitycznego, ale także od stopnia odzysku badanego analitu przez zastosowaną metodę przygotowania próbki. Mając to na względzie w prowadzonych w ramach pracy doktorskiej badaniach sporo uwagi poświęcono ocenie użyteczności czterech metod przygotowania próbki do analizy chromatograficznej, które są najczęściej wykorzystywane w procedurach analitycznych oznaczania ksenobiotyków – precypitacji białek, ekstrakcji w układzie ciecz-ciecz (LLE), ekstrakcje do fazy stałej (SPE) oraz metodzie QuEChERS (ang. *Quick, Easy, Cheap, Effective, Rugged, and Safe*). Z przeprowadzonych badań wynika, że najwyższą czułość analizy chromatograficznej kannabinoidów we krwi/osoczu uzyskuje się przy zastosowaniu SPE jako metody przygotowania próbki. Wniosek ten potwierdzają zarówno wyniki badań przeprowadzonych z wykorzystaniem technik LC, jak i GC w końcowej fazie analizy. Stopnie odzysku poszczególnych kannabinoidów metodą SPE są podobne i bliskie 100%. W przypadku trzech pozostałych metod przygotowania próbek, stopnie odzysku poszczególnych kannabinoidów w ramach danej metody są również podobne, jednak ich wartości są wyraźnie niższe niż uzyskiwane przy użyciu SPE. Ponadto supernatanty otrzymywane w trakcie precypitacji białek, LLE oraz QuEChERS zawierają interferenty wywołujące efekt matrycowy, co utrudnia wiarygodną ocenę ilościową badanych kannabinoidów za pomocą techniki GC. Szczegóły przedstawionych badań zawarte są w pracy [D8].

Wnioski

- Analiza kannabinoidów w złożonych matrycach biologicznych powinna być prowadzona z wykorzystaniem techniki LC. Możliwe jest wówczas zastosowanie dowolnej metody przygotowania próbki, które są powszechnie wykorzystywane w procedurach analitycznych substancji nielotnych i trudnolotnych. Poza tym użycie LC w analizie końcowej ogranicza błędy w ilościowej ocenie CBD i Δ9-THC, tj. związków podatnych na przekształcenia (transformację i izomeryzację) w trakcie ich oznaczeń techniką GC.
- W przypadku konieczności użycia techniki GC do analizy kannabinoidów (brak chromatografu LC, zbyt niska czułość aparatury LC czy też wyższe koszty analizy) zalecanym sposobem przygotowania próbki do analizy jest SPE.
- W przypadku zastosowania precypitacji białek, LLE czy QuEChERS, jako metody przygotowania próbki w procedurze analizy kannabinoidów z wykorzystaniem GC, może wystąpić efekt matrycowy, który utrudnia rzetelność oznaczania ilościowego tych związków.
- Zastosowanie kwasowych odczynników wytrącających białka osocza w trakcie przygotowania próbki do analizy kannabinoidów z użyciem techniki GC wywołuje, poza efektem matrycowym, transformację CBD do Δ9-THC oraz izomeryzację Δ9-THC do Δ8-THC. Poza wymienionymi związkami w trakcie przemian tworzą się jeszcze 9α-OH-HHC, 8-OH-iso-HHC oraz CBN.
- Wykorzystanie popularnie stosowanych odczynników strącających białka osocza, tj. TFA i TCA, w oznaczaniu CBD i Δ9-THC prowadzi do powstania (oprócz wymienionych wyżej produktów transformacji) także trifluoroacylowych (w przypadku TFA) oraz trichloroacylowych i dichloroacylowych (w przypadku TCA) estrów Δ9-THC i Δ8-THC.
- Czułość analizy kannabinoidów można zwiększyć poprzez ich derywatyzację. Acylacja, jako jeden ze sposobów derywatyzacji, prowadzi do przekształceń CBD i Δ9-THC analogicznych jak opisane powyżej. Przekształcenia te mogą zostać zablokowane poprzez wykorzystanie w trakcie derywatyzacji trzeciorzędowych amin i azyn.
- Sygnał detektora MS w odpowiedzi na CBG można zwiększyć poprzez klasyczną derywatyzację tego kannabinoidu (sililacja, acylacja) oraz jego cyklizację.

Udowodniono, że drugi z wymienionych sposobów jest znacznie bardziej efektywny.

- Wykorzystanie w czynnościach laboratoryjnych (także tych dotyczących analizy kannabinoidów) naczyń polipropylenowych wykonanych z użyciem oleamidu jako czynnika poślizgowego, może być powodem nierzetelnych wyników wynikających z efektu matrycowego wywoływanego przez ten związek.
- Do zwiększenia czułości analizy kannabinoidów techniki GC można wykorzystać przejściowy efekt matrycowy. Korzystne w tym celu jest kontrolowane użycie oleamidu, który indukuje takie zjawisko.

Zdecydowana większość zagadnień zawartych w powyższych wnioskach stanowi nowość naukową i nie była wcześniej poruszana w literaturze dotyczącej analizy chromatograficznej związków chemicznych, a w szczególności kannabinoidów.

Bibliografia

[1] M.A. ElSohly, D. Slade, Chemical constituents of marijuana: The complex mixture of natural cannabinoids, Life Sci, 2005. https://doi.org/10.1016/j.lfs.2005.09.011

[2] A.W. Zuardi, History of cannabis as a medicine: A review, Rev. Bras. Psiquiatr. 28 (2006) 153–157. https://doi.org/10.1590/S1516-44462006000200015

[3] R.G. Pertwee, Cannabidiol as a potential medicine, in: R. Mechoulam (Ed.), Cannabinoids as Ther., Birkhäuser-Verlag, Basel, 2005: pp. 47–65. https://doi.org/10.1007/3-7643-7358-x_3

[4] R. Adams, M. Hunt, Structure of Cannabidiol, a Product Isolated from the Marihuana Extract of Minnesota Wild Hemp. I, J. Am. Chem. Soc. 62 (1940) 196–200. https://doi.org/10.1021/ja01858a058

[5] R.G. Pertwee, Cannabinoid pharmacology: The first 66 years, Br. J. Pharmacol. 147 (2006). https://doi.org/10.1038/sj.bjp.0706406

[6] Y. Gaoni, R. Mechoulam, Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish, J. Am. Chem. Soc. 86 (1964) 1646–1647. https://doi.org/10.1021/ja01062a046

[7] R.G. Pertwee, The central neuropharmacology of psychotropic cannabinoids, Pharmacol. Ther. 36 (1988) 189–261. https://doi.org/10.1016/0163-7258(88)90106-4

[8] A.C. Howlett, F. Barth, T.I. Bonner, G. Cabral, P. Casellas, W.A. Devane, C.C. Felder, M. Herkenham, K. Mackie, B.R. Martin, R. Mechoulam, R.G. Pertwee, International Union of Pharmacology. XXVII. Classification of cannabinoid receptors, Pharmacol. Rev. 54 (2002) 161–202. https://doi.org/10.1124/pr.54.2.161

[9] R.G. Pertwee, The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin, Br. J. Pharmacol. 153 (2008) 199–215. https://doi.org/10.1038/SJ.BJP.0707442

[10] M.A. Huestis, J.E. Henningfield, E.J. Cone, Blood cannabinoids. I. Absorption of THC and formation of 11-OH-THC and THCCOOH during and after smoking marijuana, J. Anal. Toxicol. 16 (1992) 276–282. https://doi.org/10.1093/JAT/16.5.276

[11] K.W. Hillig, P.G. Mahlberg, A chemotaxonomic analysis of cannabinoid variation in Cannabis (Cannabaceae), Am. J. Bot. 91 (2004) 966–975. https://doi.org/10.3732/ajb.91.6.966

[12] A. Jastrząb, I. Jarocka-Karpowicz, E. Skrzydlewska, The Origin and Biomedical Relevance of Cannabigerol, Int. J. Mol. Sci. 23 (2022) 7929. https://doi.org/10.3390/ijms23147929

[13] M.R. Amin, D.W. Ali, Pharmacology of Medical Cannabis, Adv. Exp. Med. Biol. 1162 (2019) 151–165. https://doi.org/10.1007/978-3-030-21737-2_8

[14] C. Duchateau, J.M. Kauffmann, M. Canfyn, C. Stévigny, K. De Braekeleer, E. Deconinck, Discrimination of legal and illegal Cannabis spp. according to European legislation using near infrared spectroscopy and chemometrics, Drug Test. Anal. 12 (2020) 1309–1319. https://doi.org/10.1002/DTA.2865

[15] P.S. Fasinu, S. Phillips, M.A. ElSohly, L.A. Walker, Current Status and Prospects for Cannabidiol Preparations as New Therapeutic Agents, Pharmacother. J. Hum. Pharmacol. Drug Ther. 36 (2016) 781–796. https://doi.org/10.1002/PHAR.1780

[16] M.P. Barnes, Sativex®: Clinical efficacy and tolerability in the treatment of symptoms of multiple sclerosis and neuropathic pain, Expert Opin. Pharmacother. 7 (2006) 607–615. https://doi.org/10.1517/14656566.7.5.607

[17] O. Devinsky, M.R. Cilio, H. Cross, J. Fernandez-Ruiz, J. French, C. Hill, R. Katz, V. Di Marzo, D. Jutras-Aswad, W.G. Notcutt, J. Martinez-Orgado, P.J. Robson, B.G. Rohrback, E. Thiele, B. Whalley, D. Friedman, Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders, Epilepsia. 55 (2014) 791–802. https://doi.org/10.1111/epi.12631

[18] V. Brighenti, M. Licata, T. Pedrazzi, D. Maran, D. Bertelli, F. Pellati, S. Benvenuti, Development of a new method for the analysis of cannabinoids in honey by means of high-performance liquid chromatography coupled with electrospray ionisation-tandem mass spectrometry detection, J. Chromatogr. A. 1597 (2019) 179–186. https://doi.org/10.1016/J.CHROMA.2019.03.034

[19] M. Prud'Homme, R. Cata, D. Jutras-Aswad, Cannabidiol as an intervention for addictive behaviors: A systematic review of the evidence, Subst. Abus. Res. Treat. 9 (2015) 33–38. https://doi.org/10.4137/SART.S25081

[20] A.L. Osborne, N. Solowij, K. Weston-Green, A systematic review of the effect of cannabidiol on cognitive function: Relevance to schizophrenia, Neurosci. Biobehav. Rev. 72 (2017) 310–324. https://doi.org/10.1016/j.neubiorev.2016.11.012

[21] M.M. Caffarel, C. Andradas, E. Pérez-Gómez, M. Guzmán, C. Sánchez, Cannabinoids: A new hope for breast cancer therapy?, Cancer Treat. Rev. 38 (2012) 911–918. https://doi.org/10.1016/j.ctrv.2012.06.005

[22] E.M. Rock, D. Bolognini, C.L. Limebeer, M.G. Cascio, S. Anavi-Goffer, P.J. Fletcher, R. Mechoulam, R.G. Pertwee, L.A. Parker, Cannabidiol, a nonpsychotropic component of cannabis, attenuates vomiting and nausea-like behaviour via indirect agonism of 5-HT 1A somatodendritic autoreceptors in the dorsal raphe nucleus, Br. J. Pharmacol. 165 (2012) 2620–2634. https://doi.org/10.1111/j.1476-5381.2011.01621.x

[23] J.M. McPartland, Cannabis sativa and cannabis indica versus "sativa" and "indica," in: Cannabis Sativa L. - Bot. Biotechnol., 2017: pp. 101–121. https://doi.org/10.1007/978-3-319-54564-6_4

[24] S. Deiana, Potential Medical Uses of Cannabigerol: A Brief Overview, in: V.R. Preedy (Ed.), Handb. Cannabis Relat. Pathol., Elsevier, Amsterdam, 2017: pp. 958–967. https://doi.org/10.1016/B978-0-12-800756-3.00115-0

[25] E.B. Russo, C. Cuttler, Z.D. Cooper, A. Stueber, V.L. Whiteley, M. Sexton, Survey of Patients Employing Cannabigerol-Predominant Cannabis Preparations: Perceived Medical Effects, Adverse Events, and Withdrawal Symptoms, Cannabis Cannabinoid Res. 7 (2022) 706–716. https://doi.org/10.1089/can.2021.0058

[26] H. Clarke, P. Roychoudhury, S.N. Narouze, Other Phytocannabinoids, in: Cannabinoids Pain, Springer, Cham, 2021: pp. 87–92. https://doi.org/10.1007/978-3-030-69186-8_12

[27] C. Maioli, D. Mattoteia, H.I.M. Amin, A. Minassi, D. Caprioglio, Cannabinol: History, Syntheses, and Biological Profile of the Greatest "Minor" Cannabinoid, Plants. 11 (2022) 2896. https://doi.org/10.3390/plants11212896

[28] W. Peschel, Quality control of traditional Cannabis tinctures: Pattern, markers, and stability, Sci. Pharm. 84 (2016) 567–584. https://doi.org/10.3390/scipharm84030567

[29] A. Hazekamp, J.T. Fischedick, M.D. Llano, A. Lubbe, R.L. Ruhaak, Chemistry of Cannabis, Compr. Nat. Prod. II Chem. Biol. 3 (2010) 1033–1084. https://doi.org/10.1016/B978-008045382-8.00091-5

[30] R. Kaufmann, Use of a water-soluble form of cannabinol for the treatment of sleeplessness, Int. J. Complement. Altern. Med. Volume 14 (2021) 186–190. https://doi.org/10.15406/IJCAM.2021.14.00558

[31] E.B. Russo, J. Marcu, Cannabis Pharmacology: The Usual Suspects and a Few Promising Leads, in: Adv. Pharmacol., Academic Press, 2017: pp. 67–134. https://doi.org/10.1016/bs.apha.2017.03.004

[32] K. Watanabe, Y. Itokawa, S. Yamaori, T. Funahashi, T. Kimura, T. Kaji, N. Usami, I. Yamamoto, Conversion of cannabidiol to Δ9-tetrahydrocannabinol and related cannabinoids in artificial gastric juice, and their pharmacological effects in mice, Forensic Toxicol. 25 (2007) 16–21. https://doi.org/10.1007/s11419-007-0021-y

[33] M. Antunes, M. Barroso, E. Gallardo, Analysis of Cannabinoids in Biological Specimens: An Update, Int. J. Environ. Res. Public Health. 20 (2023) 2312. https://doi.org/10.3390/ijerph20032312

[34] U. Mareck, G. Fusshöller, H. Geyer, M.A. Huestis, A.B. Scheiff, M. Thevis, Preliminary data on the potential for unintentional antidoping rule violations by permitted cannabidiol (CBD) use, Drug Test. Anal. 13 (2021) 539–549. https://doi.org/10.1002/dta.2959

Streszczenie w języku polskim

Na rozprawę doktorską pt.: „Problemy w ilościowej ocenie kannabidiolu, kannabigerolu i kannabinolu w matrycach biologicznych” składa się cykl ośmiu powiązanych ze sobą tematycznie artykułów w czasopismach naukowych, posiadających wskaźnik cytowań (ang. *impact factor*). Siedem z nich jest już opublikowanych, a jeden przyjęty do druku.

Procedura analizy kannabinoidów w matrycach biologicznych jest procesem złożonym, w trakcie którego pojawiają się problemy utrudniające rzetelne oznaczanie ilościowe analizowanych związków. Dotyczą one zarówno etapu przygotowania próbki do analizy, jak i samej analizy.

Jednym z problemów, na których skupiły się badania przeprowadzone w ramach pracy doktorskiej jest potencjalna transformacja kannabinoidów w pierwszym i drugim etapie procedury analitycznej. Wykazano [D1], że CBD, w trakcie analizy wykonywanej techniką GC po wykorzystaniu precypitacji białek odczynnikiem o charakterze kwasowym jako metody przygotowania próbki, przekształca się do CBN, Δ9-THC, Δ8-THC, 9α-hydroksyheksahydrokannabinolu oraz 8-hydroksy-izo-heksahydrokannabinolu. Poza tym wykazano, że zastosowanie kwasu trifluorooctowego, jako odczynnika wytrącającego białka w procesie przygotowania próbki do analizy CBD i Δ9-THC techniką GC, powoduje dodatkowo powstanie trifluoroacylowych pochodnych Δ9-THC i Δ8-THC, oprócz wymienionych wyżej produktów transformacji [D4]. Dowiedziono również, że zastosowanie w identycznej procedurze kwasu trichlorooctowego prowadzi do powstania dichloroacylowych pochodnych Δ9-THC i Δ8-THC [D6], co było trudnym do przewidzenia efektem.

Czułość analizy kannabinoidów to kolejny problem badawczy poruszany w doktoracie. Najprostszym sposobem zwiększenia czułości analizy związków jest ich derywatyzacja, która w pewnych przypadkach prowadzona jest w warunkach kwasowych. W przypadku CBD i Δ9-THC skutkuje to ich przekształceniem i spadkiem sygnału. W wyniku prowadzonych badań opracowano sposób blokady przemian tych kannabinoidów w środowisku kwasowym [D5].

Sporo uwagi w badaniach poświęcono niskiej czułości oznaczania CBG przy wykorzystaniu GC-MS. Wyjaśniono przyczyny i zaproponowano nowy sposób transformacji tego związku, który pozwala zwiększyć odpowiedź detektora względem CBG, a tym samym poprawić czułość jego analizy [D7].

Zjawiskiem komplikującym ilościową ocenę analitów w technice GC jest efekt matrycowy. Udowodniono, że jednym z czynników indukujących przejściowy efekt matrycowy, który znacząco wpływa na dokładność oznaczeń kannabinoidów i innych ksenobiotyków, jest oleamid [D2]. W badaniach potwierdzono również użyteczność wykorzystania oleamidu, ponieważ wywołuje on przemijający i kontrolowany efekt matrycowy, umożliwiając tym samym zwiększenie czułości analizy Δ9-THC i jego metabolitów we krwi/osoczu prowadzonej przy użyciu techniki GC-MS [D3].

Istotnym zagadnieniem w oznaczaniu kannabinoidów jest dobór metody przygotowania próbki do ich analizy. Przeprowadzono badania poświęcone ocenie użyteczności czterech metod przygotowania próbki do analizy chromatograficznej, które są najczęściej wykorzystywane w procedurach analitycznych ksenobiotyków – precypitacji białek, ekstrakcji w układzie ciecz-ciecz (LLE), ekstrakcji do fazy stałej (SPE) oraz metodzie QuEChERS. Ustalono, że najwyższą czułość analizy kannabinoidów otrzymuje się stosując SPE [D8].

Streszczenie w języku angielskim

The doctoral dissertation entitled: "Issues related to the quantitative assessment of cannabidiol, cannabigerol, and cannabinol in biological matrices" consists of a series of eight thematically related articles in scientific journals with an impact factor. Seven of them have already been published and one has been accepted for publication.

The procedure of analyzing cannabinoids in the biological matrices is a complex process during which problems arise and hinder the possibility to quantify the analyzed compounds. It concerns both the stage of sample preparation for analysis and the analysis itself.

One of the problems that the research focused on is the possible transformation of cannabinoids in the first and second stages of the analytical procedure. It was shown [D1] that CBD during the GC analysis, after using protein precipitation with an acidic reagent as a sample preparation method, is transformed into CBN, Δ9-THC, Δ8-THC, 9α-hydroxyhexahydrocannabinol, and 8-hydroxy-iso-hexahydrocannabinol. Moreover, the research indicates that the use of trifluoroacetic acid as a protein-precipitating reagent in the sample preparation method for CBD and Δ9-THC prior the GC analysis, additionally causes the formation of trifluoroacyl derivatives of Δ9-THC and Δ8-THC in addition to the above-mentioned transformation products [D4]. It was also proven that the use of trichloroacetic acid in an identical procedure leads to the formation of dichloroacyl derivatives Δ9-THC and Δ8-THC [D6], which was a difficult-to-predict effect.

The sensitivity of cannabinoid analysis is another issue addressed in the doctoral dissertation. The most straightforward method to increase the sensitivity of the analysis of compounds is their derivatization, which in some cases is conducted under acidic conditions. In the case of CBD and Δ9-THC, this results in their transformation and a decrease in their signal. As a result of the research, a method to block the transformation of these cannabinoids in an acidic environment was developed [D5].

Much attention has been paid to the low sensitivity of CBG determination using GC-MS. The reasons were explained and a new method of transforming this compound was proposed, which allows to increase the detector's response to CBG, and thus improve the sensitivity of its analysis [D7].

A phenomenon that complicates the quantitative assessment of analytes in the GC technique is the matrix effect. It has been proven that one of the factors inducing the transient matrix effect, which significantly affects the accuracy of the determination

of cannabinoids and other xenobiotics, is oleamide [D2]. The studies also confirmed the usefulness of the application of oleamide as it causes a transient and controlled matrix effect, thus enabling increased sensitivity of the analysis of Δ9-THC and its metabolites in blood/plasma using the GC-MS technique [D3].

An important issue in the determination of cannabinoids is the selection of the sample preparation method for their analysis. Research was conducted to assess the utility of four sample preparation methods for chromatographic analysis that are most often used in xenobiotic analytical procedures – protein precipitation, liquid-liquid extraction (LLE), solid phase extraction (SPE) and the QuEChERS method. It has been established that the highest sensitivity of cannabinoid analysis is obtained using SPE [D8].

Życiorys naukowy

Urodziłem się 19 lipca 1995 r. w Łukowie w województwie lubelskim. W 2014 r. ukończyłem I Liceum Ogólnokształcące im. Bolesława Prusa w Siedlcach.

W październiku 2014 r. rozpocząłem studia I stopnia na kierunku Chemia, specjalność chemia kryminalistyczna na Wydziale Chemii Uniwersytetu Marii Curie-Skłodowskiej w Lublinie. W 2017 r. obroniłem pracę licencjacką pt. „Zastosowanie spektroskopii FTIR do badań nanoporowatych materiałów krzemionkowych". W październiku 2017 r. rozpocząłem studia II stopnia na kierunku Chemia, specjalność chemia kryminalistyczna na Wydziale Chemii Uniwersytetu Marii Curie-Skłodowskiej w Lublinie. W 2019 r. ukończyłem studia ze średnią 4,73 i otrzymałem tytuł magistra chemii broniąc pracę dyplomową pt. „Zastosowanie matrycy SBA-15 otrzymanej z użyciem haloizytu i diatomitu do syntezy nanocząstek tlenku magnezu". W roku akademickim 2014/2015 otrzymywałem stypendium w ramach projektu „Od studenta do eksperta – ochrona środowiska w praktyce", a następnie w latach 2016/2017 oraz 2017/2018 stypendium Rektora dla najlepszych studentów Uniwersytetu Marii Curie-Skłodowskiej w Lublinie. W latach 2017 i 2019 uczestniczyłem w projekcie „WIZA na rynku pracy" organizowanym na Uniwersytecie Marii Curie-Skłodowskiej w Lublinie biorąc udział w dwóch trzymiesięcznych stażach, odpowiednio w Instytucie Technologii Bezpieczeństwa "MORATEX" w Łodzi oraz Wytwórni Surowic i Szczepionek „Biomed-Lublin" w Lublinie.

W październiku 2019 r. rozpocząłem studia w Szkole Doktorskiej Nauk Ścisłych i Przyrodniczych Uniwersytetu Marii Curie-Skłodowskiej w Lublinie przygotowując rozprawę doktorską pt. „Problemy w ilościowej ocenie kannabidiolu, kannabigerolu i kannabinolu w matrycach biologicznych" pod opieką promotora prof. dr. hab. Andrzeja L. Dawidowicza. Badania prowadziłem w Katedrze Chromatografii w Instytucie Nauk Chemicznych na Wydziale Chemii Uniwersytetu Marii Curie-Skłodowskiej w Lublinie.

Mój dorobek naukowy obejmuje współautorstwo ośmiu publikacji naukowych w czasopismach o łącznym IF 32,1 i sumarycznej liczbie punktów MEiN 800. Ponadto brałem udział w trzech konferencjach naukowych: dwóch o wymiarze międzynarodowym oraz jednej o wymiarze krajowym.

Udział w konferencjach naukowych

Międzynarodowe konferencje naukowe:

- ✓ Chemistry World Conference**:** Chemistry 2021, Magnus Group, 6-7 września 2021 r., Rzym (online), Poster: *"The influence of protein precipitation methods on uncontrolled conversion of cannabidiol to 9-tetrahydrocannabinol during analysis of plasma samples"*
- ✓ III Kongres Młodej Nauki – Międzynarodowa Konferencja Naukowa, Uniwersytet Gdański, 6-8 lipca 2023 r., Gdańsk, Poster: *„Wzrost czułości w analizie GC kannabinoidów i innych ksenobiotyków poprzez wywołanie efektu matrycowego oleamidem"*

Krajowe konferencje naukowe:

- ✓ 64. Zjazd Naukowy Polskiego Towarzystwa Chemicznego w Lublinie, 11-16 września 2022 r., Lublin, Poster: "*Formation of trifluoroacetic artefacts in gas chromatograph injector during cannabidiol analysis*"

Publikacje wchodzące w skład rozprawy doktorskiej

Publikacja D1

M.P. Dybowski, A.L. Dawidowicz, R. Typek, **M. Rombel**

Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – Troubles with reliable CBD quantitation when acidic precipitation agents are applied

Talanta 220 (2020) 121390

https://doi.org/10.1016/j.talanta.2020.121390

IF 2-letni: 6,1; MEiN: 100 pkt

Talanta 220 (2020) 121390

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – Troubles with reliable CBD quantitation when acidic precipitation agents are applied

Michal P. Dybowski *, Andrzej L. Dawidowicz, Rafal Typek, Michal Rombel

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie Sklodowska University in Lublin, 20-031, Lublin, Poland

ARTICLE INFO

Keywords:
CBD transformation
Δ9-THC
Plasma precipitation
GC–MS/MS
LC-MS

ABSTRACT

The growing popularity of supplements containing cannabidiol (CBD), mainly CBD oils, in self-medication of humans and the increased interest in this compound in different preclinical and clinical trials stimulates the development of procedures of CBD analysis in plasma for the study of CBD pharmacology in people and animals or in establishing dose–therapeutic effect relationships of this compound. Preliminary removal of protein by its precipitation from plasma is still one of the willingly applied plasma sample preparation methods in many analytical procedures estimating plasma drug concentration, including CBD. The present paper shows that a significant amount of CBD transforms to Δ9-tetrahydrocannabinol (Δ9-THC) in a hot GC injection system when acidic precipitation agents, such as TFA, TCA, $HClO_4$, H_2SO_4, $ZnSO_4$ or $CHCl_3$, are used for plasma protein precipitation. The transformation degree depends on the temperature of the GC injector, the concentration of the precipitation agent and the incubation time of plasma with the precipitating agent. At the CBD plasma concentration equal to 50 ng/ml, which is approximately the mean level for patients treated for epileptic syndromes, the CBD transformation degree can exceed 20%. For a reliable estimate of CBD in blood plasma, neutral precipitation agents (e.g. ACN, MeOH, acetone) should be used when plasma deproteinization precedes GC analysis. The presented results are important not only for analysts cooperating with pharmacologists and for medicine doctors examining the activity of CBD-containing drugs in the therapeutic process, but also for forensic scientists who may erroneously find innocent people guilty of using marijuana or its preparations.

1. Introducion

Cannabidiol (CBD), 2-[(1R,6R)-3-metylo-6-(prop-1-en-2-ylo)cyklo-heks-2-enylo]-5-pentylobenzeno-1,3-diol, is the second after delta-9-tetrahydrocannabinol (Δ9-THC) most abundant cannabinoid among the nearly 120 identified ones in marijuana and hemp plants [1–4]. Although it was discovered as early as in 1940 by R. Adams et al. [5], CBD has become a major area of widespread researches only in recent years. In particular, its biological activities are a topic of many interesting reports that suggest probable therapeutic benefits of CBD. Potential activity of CBD in the treatment of epileptic syndromes is especially emphasized [6,7]. In consequence of many available reports supporting the utility of CBD as an effective remedy in antiepileptic therapy [8], the United States FDA approved Epidiolex, the first drug with CBD authorized for use in epilepsy treatment. As results from the literature, a positive effect of CBD is wider. It is being espoused as potential treatment for immune dysfunctions [9], diabetes [10], addictive behavior [11,12] and cancer [13–15]. Studies also have suggested positive outcomes in patients with schizophrenia [16,17]. Preclinical studies demonstrate also its ant-nausea and analgesic effects [18,19]. Due to CBD's numerous biological activities and its potential therapeutic benefit, research is pursued with a view toward clinical applications as the absence of psychotropic effects and other adverse events gives CBD a major advantage over other cannabinoids.

In the face of the increased wave of CBD popularity in preclinical, clinical, and observational trials, effective methods for the analysis of CBD in plasma are critical for the study of its pharmacology in humans and animals and in establishing dose–therapeutic effect relationships of

* Corresponding author.
E-mail address: michal.dybowski@poczta.umcs.lublin.pl (M.P. Dybowski).

https://doi.org/10.1016/j.talanta.2020.121390
Received 28 April 2020; Received in revised form 4 July 2020; Accepted 6 July 2020
Available online 15 July 2020

the compound. Several analytical procedures have been developed for measuring CBD and other cannabinoids together with their metabolites in plasma or whole blood applying GC-MS [20–23], two-dimensional GC-MS [24], and HPLC-MS/MS [14,25–29] equipments. Although most of them involve classical or automated liquid-liquid extraction (LLE) or solid-phase extraction (SPE) as sample preparation method for CBD analysis in plasma, some of them recommend the older method, protein precipitation [14,30,31]. The last approach is not totally wrong as CBD is a highly hydrophobic molecule and strongly binds with plasma proteins [31,32]. Moreover, this sample preparation procedure is very simple, quick to perform and does not require special equipment. As recently reported [30], its application with HPLC-MS/MS allows to quantify CBD in plasma with sensitivity lower than 0.1 ng/mL.

There are many compounds and mixtures which can be used as protein precipitation agents in the analytical procedures of drug assay in plasma. In general, they can be divided into two groups: neutral (e.g. acetonitrile, methanol, acetone) and acidic (e.g. H_2SO_4, CF_3COOH, $ZnSO_4$ CCl_3COOH, $HClO_4$, $CHCl_3$) [31,33–39]. As demonstrated in Ref. [40], CBD, in artificial gastric juice which is strongly acidic medium, converts to 9α-hydroxyhexahydrocannabinol (9α-OH-HHC), 8-hydroxy-*iso*-hexahydrocannabinol (8-OH-*iso*-HHC), cannabinol (CBN) and Δ9-THC. In this context, the question arises whether the mentioned compounds can also be formed during the protein precipitation process frequently applied as sample preparation procedure for CBD analysis in plasma by GC or HPLC, and if so, how they influence the accuracy of CBD concentration estimation in plasma samples.

2. Experimental

2.1. Materials

Acetonitryle and methanol (LC/MS grade) were purchased from Merck (Warszawa, Poland). Formic acid (99%), trichloroacetic acid (TCA) (>99%), trifluoroacetic acid (TFA) (>99%) and phosphate buffered saline (PBS), human serum albumin (HSA) (97%) were acquired from Sigma-Aldrich (Poznan, Poland). Perchloric acid (60%), zinc sulfate heptahydrate (99% powder), sulfuric acid (96%) n-hexan (99%), acetone (99%) and chloroform (99.9%) were obtained from POCH (Gliwice, Poland). Deionized water was purified by the Milli-Q system (Millipore Sigma, Bedford, MA, USA). The standards (certified reference materials) of Δ9-THC (1.0 mg/mL in methanol -Cerilliant) and CBD (1.0 mg/mL in methanol - Cerilliant) were acquired from Sigma-Aldrich (Poznan, Poland).

2.2. Collecting plasma samples

Two types of blood plasma were applied in the experiments: human plasma and plasma from pig blood. The first one was used in the experiments examining the probability of CBD transformation to its derivatives during plasma analysis procedure involving protein precipitation (see Figs. 1–2, Fig. 2S in supplementary material and Table 1). The estimation of the influence of some experimental parameters on the CBD transformation degree requires statistical analysis and, in consequence, more measurements using up a large volume of plasma. Hence, this part of the experiments was performed with plasma from pig blood (see Figs. 2S–6S in supplementary material).

Human plasma samples were obtained by the centrifugation of human blood samples. The blood samples were collected by a registered nurse from volunteers, after obtaining their informed consent, using a single closed system containing an S-Monovette coagulation activator, according to the manufacturer instructions (Sarstedt AG, Nümbrecht, Germany), and thoroughly mixed in order to maintain their homogeneity. Pig plasma was separated from pig blood donated by a local meat processing plant (CIOCZEK, Lublin, Poland).

2.3. Plasma protein precipitation procedure

An appropriate volume of the precipitation agent was added to 500 μL of human plasma or pig plasma or HSA solution or pig plasma fortified in protein by HSA addition or egg white protein solution, each containing CBD. The samples were vortex mixed, incubated for a given time and centrifuged for 5 min at 18600 x g. The separated supernatants were subjected to GC-MS and LC-MS analysis. The following precipitation agents were applied in the experiments: ACN, MeOH, ACN/MeOH (10/1 v/v) mixture, MeOH+1 %HCOOH, 0.2 M $ZnSO_4$ in MeOH/H_2O (7/3 v/v) mixture, $CHCl_3$, H_2SO_4, TFA, TCA and $HClO_4$. The volumes of the individual precipitating agents, in the form of the volume ratio of precipitating agent to plasma, are given in Tables 1 and 2, and in captions to the figures. In the same places are given information concerning the CBD plasma concentration, the concentration of the applied precipitation agents and the incubation times of plasma with precipitating agent before supernatant centrifugation.

2.4. GC–MS/MS measurements

Qualitative and quantitative analyses of CBD and its transformation products were conducted using a gas chromatograph hyphenated with a

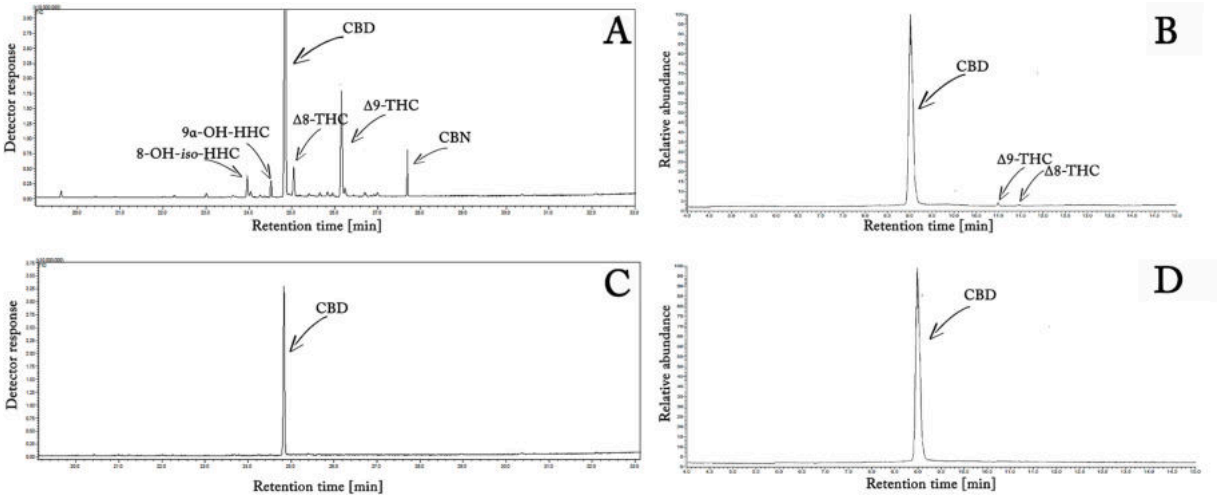


Fig. 1. Exemplary GC-MS (A, C) and LC-MS (B, D) chromatograms (in TIC mode) of supernatants centrifuged from human plasma samples containing CBD (5 μg/ml) after 1 h incubation with 50% TFA (A, B) and ACN (C, D). In the case of TFA and ACN, precipitation agent/sample volume ratio equals 1/5 and 1/3, respectively; temperature of GC injector - 300 °C.

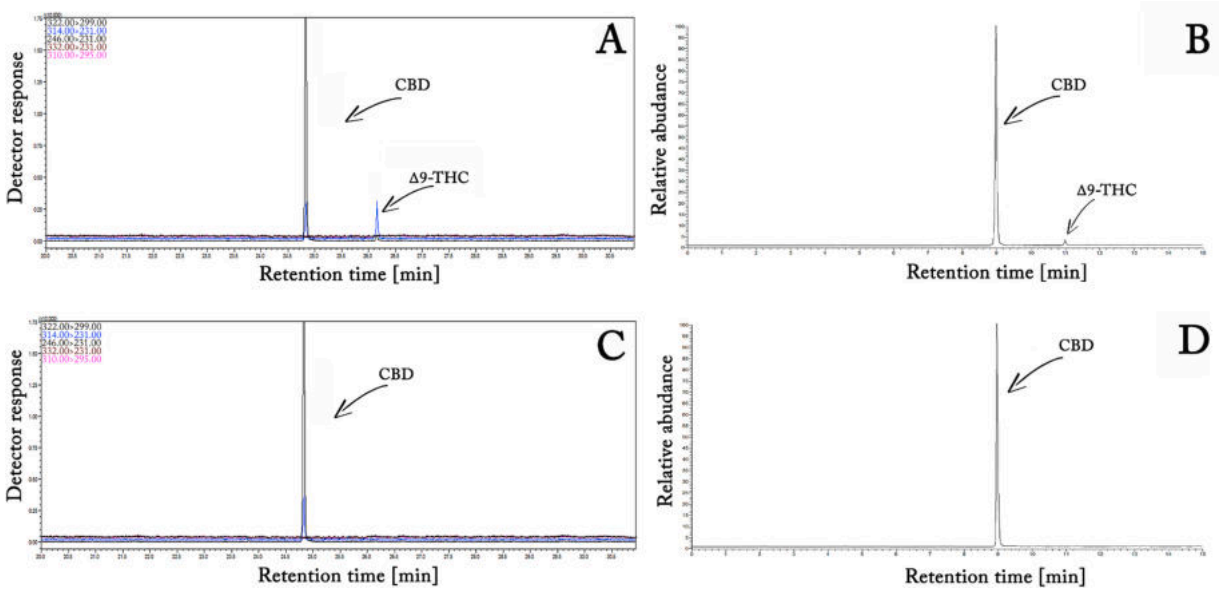


Fig. 2. MRM chromatograms from GC-MS/MS (A, C) and SIM chromatograms from LC-MS (B, D) of supernatants centrifuged from human plasma samples containing CBD (50 ng/ml) after 1 h incubation with 50% TFA (A, B) and ACN (C, D). In the case of TFA and ACN, precipitation agent/sample volume ratio equals 1/5 and 1/3, respectively; temperature of GC injector - 300 °C.

Table 1
The presence (+) and absence (−) of Δ9-THC peak on GC-MS and LC-MS chromatogram of supernatant isolated from CBD containing plasma after its preliminary deproteinization by typically used precipitation agent.CBD concentration in plasma sample - 50 ng/ml; Incubation time of plasma sample with precipitation agent – 1 h.

Precipitating agent	Sample	Precipitation agent/ sample volume ratio	Appearance of Δ9-THC peak on chromatogram		Lit.
			GC-MS	LC-MS	
Acetone	plasma/ PBS 1/4	2.4/1	–	–	[33]
ACN	plasma	2/1	–	–	[34]
ACN	plasma	3/1	–	–	[34]
MeOH	plasma	2/1	–	–	[34]
MeOH	plasma	3/1	–	–	[34]
ACN/MeOH (10/1 v/v)	plasma	11/1	–	–	[35]
MeOH + 1% HCOOH	plasma	3/1	–	–	[36]
0,2 M $ZnSO_4$ in MeOH/H_2O 7/3 v/v	plasma	4/1	+	–	[31]
0,2 M $ZnSO_4$ in MeOH/H_2O 7/3 v/v	plasma	8/1	+	–	[31]
0,2 M $ZnSO_4$ in MeOH/H_2O 7/3 v/v	plasma	80/1	+	–	[31]
Chloroform	plasma/ PBS 1/16	2.4/1	+	–	[33]
50% H_2SO_4	plasma	1/5	+	–	[37]
50% TFA	plasma	1/5	+	–	[38]
50% TCA	plasma	1/5	+	–	[38]
10% $HClO_4$	plasma	1/1	+	–	[39]

Table 2
CBD concentration values estimated by GC/MS in human plasma samples spiked with the examined compound after their preliminary deproteinization by neutral precipitation agents. Incubation time with precipitating agent - 1 h incubation; temperature of GC injector - 300 °C; precipitation agent/sample volume ratio equals 3/1 in the case of ACN, MeOH and acetone/PBS and 11/1 for ACN/MeOH.

CBD concentration in spiked plasma sample [ng/mL]	Precipitation agent			
	ACN	MeOH	ACN/ MeOH	Acetone/ PBS
	CBD concentration estimated in the spiked plasma sample [ng/mL ± SD]			
25	24.23 ± 0.87	24.56 ± 0.98	24.61 ± 1.02	24.49 ± 1.01
50	49.61 ± 1.79	49.72 ± 1.98	49.67 ± 2.05	49.88 ± 2.05
75	74.93 ± 2.70	74.88 ± 2.99	74.89 ± 3.10	74.67 ± 3.07

triple quadruple tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan). GC–MS/MS conditions were as follows:

- capillary column: Zebron ZB5-MSi (30 m × 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Torrance, CA, USA)
- carrier gas: helium (grade 5.0);
- flow rate: 1.56 ml/min;
- high-pressure injection mode: 250.0 kPa for 1.5 min;
- injector temperature: 225, 250, 275; 300 and 325 °C;
- injection volume: 1 μL;
- temperature program: initial temperature 60 °C held for 2 min, I ramp at a rate of 9 °C/min to the temperature 220 °C, I ramp at a rate of 5 °C/min to the temperature 280 °C, III ramp at a rate of 10 °C/min to the final temperature and held for 15 min;
- tandem mass spectrometer operated with normalized electron energy of 70 eV;
- ion source temperature: 225 °C;
- collision gas: argon (grade 5.0);

For qualitative purposes the full SCAN mode with range 40–550 m/z

was employed and for quantitative analyses the Multiple Reaction Monitoring (MRM) mode was used.

2.5. Optimization of MRM transitions for MS/MS detection

Quantitative analysis of CBD and Δ9-THC with a GC–MS/MS instrument was operated in the MRM mode. For this reason, the drug fragmentation pathways for CBD and Δ9-THC were established and energies for MRM transitions were optimized. To find the optimal collision energies (CE) for MRM transitions, the standard solutions of CBD and Δ9-THC were used. Four characteristic MRM transitions for CBD and five for Δ9-THC were monitored. The collision cell was operated at 5, 9, 12, 15, 18, 21, 24, 27 and 30 eV. The results of those experiments are summarized in Fig. 1S A,B and Table 1S (supplementary materials). Three MRM transitions of the highest intensity were selected for further experiments:

- 314 > 193 (CE = 21eV), 314 > 231(CE = 24eV) and 246 > 231 (CE = 21eV) for CBD,
- 314 > 299 (CE = 12eV), 314 > 271(CE = 15eV) and 314 > 231 (CE = 18eV) for Δ9-THC.

Qualitative analysis of CBD transformation products (9α-OH-HHC; Δ8-THC; 8-OH-*iso*-HHC and CBN) was also performed on the GC–MS/MS instrument operated in the SCAN and MRM mode. The following MRM transitions (of the highest intensity) were selected for the experiment:

- 332 > 299 (CE = 15eV) for 9α-OH-HHC,
- 314 > 231 (CE = 18eV) for Δ8-THC,
- 332 > 231(CE = 15eV) for 8-OH-*iso*-HHC and
- 310 > 295 (CE = 21eV) for CBN.

2.6. LC-MS measurements

LC-MS system composed of an UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) and a linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) was applied for the chromatographic analyses of the examined supernatants. ESI ionization source operating in the positive polarization mode at needle potential equal to 4.5 kV was employed. Nitrogen (>99.98%) was used as sheath gas (at 40 arbitrary units), auxiliary gas (at 10 arbitrary units) and sweep gas (at 10 arbitrary units). Capillary temperature equalled 320 °C. The scan cycle used a full-scan event at the resolution of 60,000. Chromatographic separations were performed on Gemini C18 column (4.6 × 100 mm, 3 μm; Phenomenex, USA). The mobile phase components were: A - 25 mM formic acid in water and B - 25 mM formic acid in acetonitrile. Isocratic elution was applied using mobile phase containing 40% of B component. The total run time was 15 min at the mobile phase flow rate 0.5 mL/min.

Analyzing supernatants from plasma samples containing 5 μg/ml of CBD, MS spectra in the range of 100–1000 *m/z* were continuously collected in the course of each run. In the case of supernatants from plasma samples containing 50 ng/ml of CBD, the SIM function was used to better visualize the chromatographic separation and to remove the signal connected with the plasma components and the precipitation agent. The monitored ions were as follows:

311 *m/z* for CBN;
315 *m/z* for CBD, Δ9-THC and Δ8-THC, and.
333 *m/z* for 9α-OH-HHC and 8-OH-*iso*-HHC.

2.7. Calibration

The linearity of the assay was calculated by the least square method and expressed as the coefficient of determination (R^2). Calibration plots were prepared using:

- blank pig plasma samples spiked with CBD at the concentration levels of 0.5, 1, 2.5, 5, 10, 25 and 50 ng/mL
- blank pig plasma samples spiked with Δ9-THC at the concentration levels of 0.5, 1, 2.5, 5, 10 and 20 ng/mL
- blank human plasma samples spiked with CBD at the concentration levels of 0.10, 0.25, 0.5, 1.0, 2.5 and 5 μg/mL
- blank human plasma samples spiked with Δ9-THC at the concentration levels of 0.05, 0.10, 0.25, 0.5, 1.0 and 2.5 μg/mL.

Each solution was prepared in triplicate.

2.8. Statistical analysis

All data are presented as the mean of three independent measurements (n = 3). Variance analysis (ANOVA) was applied to compare differences in the amounts of the remaining CBD and the formed Δ9-THC by examining:

- the influence of incubation time of plasma with 10% TFA
- the influence of protein concentration.

Differences in the tested groups were considered significant for p values lower than 0.05 and F values higher than F_{crit}. Variance analysis showed statistically significant differences for the examined groups.

3. Results and discussion

As stated in Introduction, CBD converts in artificial gastric juice to four CBD derivatives: 9α-OH-HHC, 8-OH-*iso*-HHC, Δ9-THC and CBN [40]. To answer the question if the same compounds are formed when protein precipitation process is applied as sample preparation procedure for the estimation of CBD concentration in plasma, the supernatants centrifuged from human plasma samples spiked with CBD after their preliminary protein precipitation by TFA or ACN were examined. The results of their GC-MS and LC-MS analyses (TIC chromatograms) are shown in Fig. 1 A-D. It is important that the CBD concentration in plasma samples used for the experiments was 5 μg/ml (i.e. 10 times lower than in the experiments examining CBD transformation in gastric juice [40] and about 100 times higher than the average CBD concentration in the plasma of patients taking CBD due to drug-resistant epilepsy [41]). As results from the chromatograms, the presence of all the mentioned CBD derivatives is confirmed only in the case of the supernatant obtained from plasma sample after its preliminary deproteinization by the acidic precipitation agent TFA, and only by GC-MS equipment (Fig. 1 A). In addition to the peaks representing the CBD derivatives mentioned in Refs. [40], GC-MS chromatogram contains still one more peak (peak 4) identified as Δ8-tetrahydrocannabinol (Δ8-THC). This cannabinoid was not reported as a transformation product of CBD in gastric juice [40]. The LC-MS chromatogram of the same supernatant (after protein precipitation by TFA) (Fig. 1 B) shows the presence of only two CBD derivatives: Δ9-THC and Δ8-THC. As the CBD concentration in the human plasma sample was high, 9α-OH-HHC, 8-OH-*iso*-HHC and CBN should be formed in sufficient amount to be registered by LC-MS equipment. Hence, the absence of the last mentioned CBD derivatives in this supernatant looks puzzling. This data suggests that the pathway of CBD transformation during protein precipitation by an acidic agent is different than that in gastric juice and/or that CBD, during protein precipitation by an acidic agent, either does not transform to some CBD derivatives or it does but the kinetics of these transformations are very slow. The last supposition is in agreement with the results of the report dealing with the formation of CBD derivatives in artificial gastric juice of pH = 1.2 [40]. The CBD transformation process described in Ref. [40] was performed in a highly concentrated solution (50 μg/mL) at 37 °C during 20 h and the resulting CBD conversion degree to Δ9-THC, CBN, 9α-OH-HHC and 8-OH-*iso*-HHC was very low - 2.9, 1.1, 1.4 and 10%, respectively [40]. The plasma proteins precipitation is a relatively

short-lasting process. As the kinetics of CBD transformation at room temperature is very slow, the absence of some CBD derivatives peaks on the LC-MS chromatogram (Fig. 1 B) should not be surprising. It results from the LC-MS data (Fig. 1 B) that about 0,32% and 0,03% of CBD transforms to Δ9-THC and Δ8-THC, respectively, during 1 h incubation of a plasma sample with TFA.

The analysis of the GC-MS and LC-MS chromatograms corresponding to the supernatant after protein precipitation by TFA (Fig. 1 A and B) and the differences in the chromatographic separation conditions indicate that most of the discussed CBD derivatives are formed in the GC injector. The conditions prevailing in the GC injector, i.e. high temperature and slightly increased pressure, favor and accelerates the transformation of CBD to its derivatives. The CBD transformation in the hot GC injector does not occur when the non-acidic precipitation agent ACN is applied for plasma deproteinization (see Fig. 1C).

The results presented in Fig. 1 were obtained using human plasma samples containing very high CBD concentration with respect to that estimated in the plasma of an epilepsy patient treated with CBD-containing drugs or supplements [41]. As the kinetics of any chemical reaction, including CBD transformation, strongly depends on the concentration of the reacting components, it was decided to repeat the above experiments using a human plasma sample with CBD concentration at 50 ng/ml, the level most frequently found in patients' plasma [41]. Exemplary GC-MS and LC-MS chromatograms of appropriately prepared supernatants are presented in Fig. 2 A-D. In this case the MRM function in GC-MS and the SIM function in LC-MS measurements were applied. As can be seen, only one among the discussed CBD transformation products, Δ9-THC, is registered only by GC-MS in the supernatant obtained from the human plasma sample after its preliminary deproteinization by TFA (see Fig. 2 A). The chromatograms from Fig. 2 partly confirm the conclusions from Fig. 1 and indicate that the kinetics and, consequently, also the main pathways of CBD transformation in high temperature of the GC injector are different than those in gastric juice at 37 °C [40] and that only Δ9-THC is formed.

MRM chromatograms of supernatants obtained after protein precipitation by TFA from samples of human plasma, pig plasma, HSA solution and egg white protein solution, all containing CBD with the same concentration (50 ng/ml), are presented in Fig. 2S A-D (see supplementary materials). Their comparison indicates that the type of precipitated protein does not have any essential influence on the amount of the formed Δ9-THC, as the magnitudes of the corresponding peaks are very similar.

There are many compounds and mixtures recommended for protein precipitation during analytical procedures of drug assay in plasma [31, 33–39]. Table 1 lists the protein precipitation agents used in the experiments to check which of them catalyze the transformation of CBD to Δ9-THC. These experiments were performed with human plasma samples containing 50 ng/ml CBD. As results from the table, Δ9-THC peak appears only on GC-MS chromatograms when an acidic agent was applied for protein precipitation. The other CBD derivatives in acidic supernatants were not detected. The information contained in Table 1 refers to the supernatants separated from plasma samples after their 1 h incubation with the precipitation agent. These results show again that in high temperature of the GC injection system a part of CBD is transformed to Δ9-THC when an acidic participation agent is used for plasma deproteinization. The transformation occurs even at weak acidification of plasma environment - see the results for $ZnSO_4$ (lately a willingly used plasma protein precipitation agent) or for $CHCl_3$ (easily decomposing with formation of HCl in high temperature of the GC injection system).

Examining analogous samples incubated with precipitation agent for longer time (more than 4 h), it appeared that Δ9-THC peak began to be registered on the LC-MS chromatogram when strong acidic precipitation agents such as 50% H_2SO_4, 50% TCA and 50% TFA were applied. It confirms the transformation of CBD to Δ9-THC at room temperature, however, the process is very slow.

The results presented above are important both from the pharmacological as well as forensic points of view. The application of GC and an acidic precipitation agent for plasma deproteinization in analytical estimation of CBD concentration in plasma can, for example:

- lead to improper establishing dose–therapeutic effect relationships for this compound or,
- cause erroneous accusation of marijuana abuse by a person taking CBD, the legal cannabinoid not exhibiting psychotropic effects.

Most important for the analysts is the magnitude of analytical error caused by the application of the discussed procedure for the estimation of CBD concentration in a plasma sample. To illustrate this problem a number of examinations were performed by GC-MS of supernatants obtained from CBD containing plasma samples deproteinized by TFA. In those experiments pig plasma samples were applied. As the transformation degree of CBD can depend on hydrogen ion concentration, different TFA/sample volume ratio were applied for protein precipitation. Temperature is another important factor influencing reaction kinetics, including CBD transformation. For this reason the GC-MS measurements were performed at three different temperatures of the GC injection system. In all of them plasma samples of CBD concentration equal to 50 ng/ml were applied. The obtained experimental data allowed to estimate the percentage of residual CBD after its transformation and the concentration of formed Δ9-THC, which would be the concentration estimated in plasma by a forensic investigator in real practice. It should be additionally pointed out that CBD and Δ9-THC are poorly volatile compounds and the magnitude of the detector's response to these compounds strongly depends on the temperature of the GC injection system. A case like that is shown in Fig. 3S (see supplementary materials). This is why separate calibration curves prepared at given injector temperatures were required to perform the above estimations.

The influence of TFA concentration on the percentage of CBD after its transformation during the applied procedure and on Δ9-THC plasma concentration which would probably be detected in the plasma of a person tested for marijuana abuse are shown in Fig. 4S A and B, respectively (see supplementary materials). As results from the plots, the transformation degree of CBD to Δ9-THC depends both on the concentration of the acidic precipitation agent and the temperature of the GC injection system: the higher the concentration of hydrogen ions (= the stronger the acidification of a plasma sample by an acidic precipitation agent) and/or the higher the GC injector temperature, the greater the amount of CBD transforming to Δ9-THC during the high temperature GC injection.

The discussed procedure of estimating CBD concentration by GC using plasma deproteinization by an acidic precipitation agent as sample preparation method also has important criminological implications. CBD users may erroneously be accused of the abuse of marijuana and its products. The plots in Fig. 4S B demonstrate that using the discussed procedure in the analysis of plasma samples with a CBD concentration of 50 ng/ml, high Δ9-THC concentration level can be found even up to 8 ng/ml. Such hypothetical concentration significantly exceeds the value assumed in criminology as "the state under the influence of the psychomotor action of Δ9-THC", i.e. 2.5 ng/ml in EU directives [42].

In the discussion of the content of Table 1 it is noted that Δ9-THC can appear in the supernatant from plasma with 50 ng/ml CBD concentration, but only after a long incubation of the plasma sample with strong acidic precipitation agents like H_2SO_4, TCA or TFA. As the extended incubation period may lead to the decrease of CBD concentration in supernatant, it can be expected that the incubation time should also have impact on further transformation of the remaining (non-transformed) CBD to Δ9-THC, i.e. on the process occurring in a hot GC injector. This possibility also seems worth considering as the prepared samples can wait a long time for analysis, for instance in the injection system. The influence of the incubation time of plasma with 50% TFA on the percentage of remaining CBD and on the Δ9-THC plasma concentration is illustrated by Fig. 5S A and B (see supplementary materials). As

appears from the diagram, the impact of incubation time on the analytical results is considerable when the time is shorter than 4 h. The differences between the percentage of remaining CBD or Δ9-THC concentrations at longer incubation times are statistically insignificant. The last statement is valid in the experimentally examined system, i.e. for plasma samples of CBD concentration equal to 50 ng/ml and 50% TFA as precipitating agent.

It is generally known that CBD is a hydrophobic compound strongly binding with plasma proteins [31,32], particularly with their main component, albumin. The range of protein concentration in normal human plasma is quite wide and extends from 66 to 87 g/l [43]. However, plasma containing significantly less protein fraction is not rare. The level 45 g/l is considered as critical concentration of total protein concentration. The percentage of the remaining CBD and the Δ9-THC concentrations estimated by GC-MS in supernatants from the pig plasma samples reinforced in protein (by adding different amounts of HSA) and having the same CBD concentration (50 ng/ml) are illustrated in Fig. 6S A and B, respectively (see supplementary materials). The presented data does not show any essential influence of protein concentration on the transformation degree of CBD to Δ9-THC, what results from the fact that the CBD transformation process mainly occurs in a hot GC injector system. This finding is additionally supported by the data presented in Fig. 2S showing the influence of protein type on the CBD transformation degree as the matrices applied in those experiments differed in protein concentrations, too.

As results from Fig. 1C and 2C, CBD transformation in the hot GC injector does not occur when the non-acidic precipitation agent ACN is applied for plasma deproteinization. The transformation also does not occur when other non-acidic precipitation agents are used for the deproteinization of CBD containing plasma.

Table 2 shows the values of CBD concentrations estimated by GC/MS in three differently spiked human plasma samples when preliminary protein precipitation was performed with the most frequently used neutral precipitation agents: ACN, MeOH, ACN/MeOH mixture or acetone/PSB mixture. As can be seen, at each examined concentration level:

- the estimated values of CBD concentration are very close to the real CBD concentration in a given plasma sample;
- the type of the applied precipitation agent has no essential influence on the estimated value of CBD concentration.

The presented results prove that for a reliable analysis of CBD in blood plasma neutral precipitation agents should be used when plasma deproteinization process is one of the analytical steps.

4. Conclusions

Most chromatographers believe that if a sample can be analyzed GC or HPLC, it is advisable to choose GC, because GC separation is easier and cheaper, the equipment is less expensive and, most importantly, detection is more sensitive than in HPLC. That is why GC is very often the method of choice in the case of CBD analysis. If so and if the applied analytical procedure employs protein precipitation as a sample preparation method before GC quantitation, then neutral precipitating agents (e.g. ACN, MeOH, acetone) should be used. Precipitation of plasma proteins is one of the oldest methods of simplifying plasma composition employed for sample preparation in analytical procedures estimating the drug concentration in this complex biological matrix. Due its easy performance, no special requirements concerning laboratory equipment and low costs of precipitating agents, the method is willingly used also in recently developed analytical procedures.

As results from the presented data, the application of acidic agents, such as TFA, TCA, $HClO_4$, H_2SO_4, $ZnSO_4$, $CHCl_3$, for protein precipitation from plasma samples containing CBD causes the transformation of the compound to Δ9-THC. Although at room temperature the transformation process occurs very slowly, its speed increases significantly at elevated temperatures. When GC is applied for the analysis of plasma supernatant, the degree of CBD transformation to Δ9-THC in a hot GC injector can exceed even 20%. The magnitude of the transformation degree depends on the GC injector's temperature, hydrogen ion concentration in sample containing the precipitation agent and the incubation time of the examined plasma sample with precipitation agent.

The presented results are important not only for the accuracy of clinical trials examining CBD pharmacokinetics and dose–therapeutic effect relationships of CBD in different disease entities but also for forensic investigations. The growing popularity of CBD-containing supplements, mainly CBD oils, in self-treatment of humans can lead to the increase of erroneous accusations of innocent people of using marijuana or its preparations when the GC-MS system is applied in forensic laboratories.

Credit author statement

Andrzej L. Dawidowicz has conceived and designed the experiments. Michal P. Dybowski, Rafal Typek, Michal Rombel have performed the experiments and analyzed the data. Michal P. Dybowski has prepared a graphic elaboration of the results. All of the authors have participated in manuscript writing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.121390.

References

[1] M.M. Delgado-Povedano, C. Sánchez-Carnerero Callado, F. Priego-Capote, C. Ferreiro-Vera, Untargeted characterization of extracts from Cannabis sativa L. cultivars by gas and liquid chromatography coupled to mass spectrometry in high resolution mode, Talanta 208 (2019) 120384, https://doi.org/10.1016/j.talanta.2019.120384.
[2] H. Li, Y. Liu, D. Tian, L. Tian, X. Ju, L. Qi, Y. Wang, C. Liang, Overview of cannabidiol (CBD) and its analogues: structures, biological activities, and neuroprotective mechanisms in epilepsy and Alzheimer's disease, Eur. J. Med. Chem. 192 (2020) 112163, https://doi.org/10.1016/j.ejmech.2020.112163.
[3] W. Peschel, M. Politi, 1H NMR and HPLC/DAD for Cannabis sativa L. chemotype distinction, extract profiling and specification, Talanta 140 (2015) 150–165, https://doi.org/10.1016/j.talanta.2015.02.040.
[4] K. Leiman, L. Colomo, S. Armenta, M. de la Guardia, F.A. Esteve-Turrillas, Fast extraction of cannabinoids in marijuana samples by using hard-cap espresso machines, Talanta 190 (2018) 321–326, https://doi.org/10.1016/j.talanta.2018.08.009.
[5] R. Adams, M. Hunt, Structure of cannabidiol, a product isolated from the marihuana extract of Minnesota wild hemp. I, J. Am. Chem. Soc. 62 (1940) 196–200, https://doi.org/10.1021/ja01858a058.
[6] C. Sánchez-Carnerero Callado, N. Núñez-Sánchez, S. Casano, C. Ferreiro-Vera, The potential of near infrared spectroscopy to estimate the content of cannabinoids in Cannabis sativa L.: a comparative study, Talanta 190 (2018) 147–157, https://doi.org/10.1016/j.talanta.2018.07.085.
[7] O. Devinsky, M.R. Cilio, H. Cross, J. Fernandez-Ruiz, J. French, C. Hill, R. Katz, V. Di Marzo, D. Jutras-Aswad, W.G. Notcutt, J. Martinez-Orgado, P.J. Robson, B. G. Rohrback, E. Thiele, B. Whalley, D. Friedman, Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders, Epilepsia 55 (2014) 791–802, https://doi.org/10.1111/epi.12631.
[8] K. Sekar, A. Pack, Epidiolex as adjunct therapy for treatment of refractory epilepsy: a comprehensive review with a focus on adverse effects [version 1; referees: 3 approved], F1000Research 8 (2019), https://doi.org/10.12688/f1000research.16515.1.
[9] A.L. Osborne, N. Solowij, K. Weston-Green, A systematic review of the effect of cannabidiol on cognitive function: relevance to schizophrenia, Neurosci. Biobehav. Rev. 72 (2017) 310–324, https://doi.org/10.1016/j.neubiorev.2016.11.012.

[10] A.B. El-Remessy, Y. Khalifa, S. Ola, A.S. Ibrahim, G.I. Liou, Cannabidiol protects retinal neurons by preserving glutamine synthetase activity in diabetes, Mol. Vis. 16 (2010) 1487–1495.
[11] S. Shannon, J. Opila-Lehman, Cannabidiol oil for decreasing addictive use of marijuana: a case report, Integr. Med. A Clin. J. 14 (2015) 31.
[12] M. Prud'Homme, R. Cata, D. Jutras-Aswad, Cannabidiol as an intervention for addictive behaviors: a systematic review of the evidence, Subst. Abuse Res. Treat. 9 (2015) 33–38, https://doi.org/10.4137/SART.S25081.
[13] M.M. Caffarel, C. Andradas, E. Pérez-Gómez, M. Guzmán, C. Sánchez, Cannabinoids: a new hope for breast cancer therapy? Canc. Treat Rev. 38 (2012) 911–918, https://doi.org/10.1016/j.ctrv.2012.06.005.
[14] W.H. Abd-Elsalam, M.A. Alsherbiny, J.Y. Kung, D.W. Pate, R. Löbenberg, LC–MS/MS quantitation of phytocannabinoids and their metabolites in biological matrices, Talanta 204 (2019) 846–867, https://doi.org/10.1016/j.talanta.2019.06.053.
[15] R.G. Pertwee, Cannabidiol as a potential medicine, in: Cannabinoids as Ther., Birkhäuser-Verlag, 2005, pp. 47–65, https://doi.org/10.1007/3-7643-7358-x_3.
[16] C. Scuderi, D. De Filippis, T. Iuvone, A. Blasio, A. Steardo, G. Esposito, Cannabidiol in medicine: a review of its therapeutic potential in CNS disorders, Phyther. Res. 23 (2009) 597–602, https://doi.org/10.1002/ptr.2625.
[17] P. McGuire, P. Robson, W.J. Cubala, D. Vasile, P.D. Morrison, R. Barron, A. Taylor, S. Wright, Cannabidiol (CBD) as an adjunctive therapy in schizophrenia: a multicenter randomized controlled trial, Am. J. Psychiatr. 175 (2018) 225–231, https://doi.org/10.1176/appi.ajp.2017.17030325.
[18] A.A. Izzo, F. Borrelli, R. Capasso, V. Di Marzo, R. Mechoulam, Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb, Trends Pharmacol. Sci. 30 (2009) 515–527, https://doi.org/10.1016/j.tips.2009.07.006.
[19] E.M. Rock, D. Bolognini, C.L. Limebeer, M.G. Cascio, S. Anavi-Goffer, P.J. Fletcher, R. Mechoulam, R.G. Pertwee, L.A. Parker, Cannabidiol, a nonpsychotropic component of cannabis, attenuates vomiting and nausea-like behaviour via indirect agonism of 5-HT 1A somatodendritic autoreceptors in the dorsal raphe nucleus, Br. J. Pharmacol. 165 (2012) 2620–2634, https://doi.org/10.1111/j.1476-5381.2011.01621.x.
[20] R. Andrews, S. Paterson, A validated method for the analysis of cannabinoids in post-mortem blood using liquid-liquid extraction and two-dimensional gas chromatography-mass spectrometry, Forensic Sci. Int. 222 (2012) 111–117, https://doi.org/10.1016/j.forsciint.2012.05.007.
[21] T. Nadulski, F. Sporkert, M. Schnelle, A.M. Stadelmann, P. Roser, T. Schefter, F. Pragst, Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract, J. Anal. Toxicol. 29 (2005) 782–789, https://doi.org/10.1093/jat/29.8.782.
[22] R. Brenneisen, P. Meyer, H. Chtioui, M. Saugy, M. Kamber, Plasma and urine profiles of Δ9-tetrahydrocannabinol and its metabolites 11-hydroxy-Δ9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol after cannabis smoking by male volunteers to estimate recent consumption by athletes, Anal. Bioanal. Chem. (2010) 2493–2502, https://doi.org/10.1007/s00216-009-3431-3.
[23] R.A. Gustafson, E.T. Moolchan, A. Barnes, B. Levine, M.A. Huestis, Validated method for the simultaneous determination of Δ 9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy- THC in human plasma using solid phase extraction and gas chromatography-mass spectrometry with positive chemical ionization, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 798 (2003) 145–154, https://doi.org/10.1016/j.jchromb.2003.09.022.
[24] E.L. Karschner, A.J. Barnes, R.H. Lowe, K.B. Scheidweiler, M.A. Huestis, Validation of a two-dimensional gas chromatography mass spectrometry method for the simultaneous quantification of cannabidiol, Δ9- tetrahydrocannabinol (THC), 11-hydroxy-THC, and 11-nor-9-carboxy-THC in plasma, Anal. Bioanal. Chem. (2010) 603–611, https://doi.org/10.1007/s00216-010-3599-6.
[25] S. Pichini, G. Mannocchi, M. Gottardi, A.P. Pérez-Acevedo, L. Poyatos, E. Papaseit, C. Pérez-Mañá, M. Farré, R. Pacifici, F.P. Busardò, Fast and sensitive UHPLC-MS/MS analysis of cannabinoids and their acid precursors in pharmaceutical preparations of medical cannabis and their metabolites in conventional and non-conventional biological matrices of treated individual, Talanta 209 (2020) 120537, https://doi.org/10.1016/j.talanta.2019.120537.
[26] O. Aizpurua-Olaizola, I. Zarandona, L. Ortiz, P. Navarro, N. Etxebarria, A. Usobiaga, Simultaneous quantification of major cannabinoids and metabolites in human urine and plasma by HPLC-MS/MS and enzyme-alkaline hydrolysis, Drug Test. Anal. 9 (2017) 626–633, https://doi.org/10.1002/dta.1998.
[27] K.B. Scheidweiler, M.N. Newmeyer, A.J. Barnes, M.A. Huestis, Quantification of cannabinoids and their free and glucuronide metabolites in whole blood by disposable pipette extraction and liquid chromatography-tandem mass spectrometry, J. Chromatogr., A 1453 (2016) 34–42, https://doi.org/10.1016/j.chroma.2016.05.024.
[28] D.M. Schwope, K.B. Scheidweiler, M.A. Huestis, Direct quantification of cannabinoids and cannabinoid glucuronides in whole blood by liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 401 (2011) 1273–1283, https://doi.org/10.1007/s00216-011-5197-7.
[29] A.J. Pandopulos, R. Bade, J.W. O'Brien, B.J. Tscharke, J.F. Mueller, K. Thomas, J. M. White, C. Gerber, Towards an efficient method for the extraction and analysis of cannabinoids in wastewater, Talanta 217 (2020) 121034, https://doi.org/10.1016/j.talanta.2020.121034.
[30] M.J. Roslawski, R.P. Remmel, A. Karanam, I.E. Leppik, S.E. Marino, A.K. Birnbaum, Simultaneous quantification of 13 cannabinoids and metabolites in human plasma by liquid chromatography tandem mass spectrometry in adult epilepsy patients, Ther. Drug Monit. 41 (2019) 357–370, https://doi.org/10.1097/FTD.0000000000000583.
[31] J. Klawitter, C. Sempio, S. Mörlein, E. De Bloois, J. Klepacki, T. Henthorn, M. A. Leehey, E.J. Hoffenberg, K. Knupp, G.S. Wang, C. Hopfer, G. Kinney, R. Bowler, N. Foreman, J. Galinkin, U. Christians, J. Klawitter, An atmospheric pressure chemical ionization MS/MS assay using online extraction for the analysis of 11 cannabinoids and metabolites in human plasma and urine, Ther. Drug Monit. 39 (2017) 556–564, https://doi.org/10.1097/FTD.0000000000000427.
[32] M.W. Elmes, M. Kaczocha, W.T. Berger, K.N. Leung, B.P. Ralph, L. Wang, J. M. Sweeney, J.T. Miyauchi, S.E. Tsirka, I. Ojima, D.G. Deutsch, Fatty acid-binding proteins (FABPs) are intracellular carriers for Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), J. Biol. Chem. 290 (2015) 8711–8721, https://doi.org/10.1074/jbc.M114.618447.
[33] L. Jiang, L. He, M. Fountoulakis, Comparison of protein precipitation methods for sample preparation prior to proteomic analysis, J. Chromatogr., A 1023 (2004) 317–320, https://doi.org/10.1016/j.chroma.2003.10.029.
[34] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 785 (2003) 263–275, https://doi.org/10.1016/S1570-0232(02)00914-5.
[35] F. de L. Moreira, M.P. Marques, G. Duarte, V.L. Lanchote, Determination of raltegravir and raltegravir glucuronide in human plasma and urine by LC–MS/MS with application in a maternal-fetal pharmacokinetic study, J. Pharmaceut. Biomed. Anal. 177 (2020), https://doi.org/10.1016/j.jpba.2019.112838.
[36] S. Gao, Z. Wang, X. Xie, C. You, Y. Yang, Y. Xi, W. Chen, Rapid and sensitive method for simultaneous determination of first-line anti-tuberculosis drugs in human plasma by HPLC-MS/MS: application to therapeutic drug monitoring, Tuberculosis 109 (2018) 28–34, https://doi.org/10.1016/j.tube.2017.11.012.
[37] I. Andersen, W. Torjussen, H. Zachariasen, Analysis for nickel in plasma and urine by electrothermal atomic absorption spectrometry, with sample preparation by protein precipitation, Clin. Chem. 24 (1978) 1198–1202, https://doi.org/10.1093/clinchem/24.7.1198.
[38] I. Nemet, L. Varga-Defterdarović, Z. Turk, Preparation and quantification of methylglyoxal in human plasma using reverse-phase high-performance liquid chromatography, Clin. Biochem. 37 (2004) 875–881, https://doi.org/10.1016/j.clinbiochem.2004.05.024.
[39] S. Souverain, S. Rudaz, J.-L. Veuthey, Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures, J. Chromatogr., A 1058 (2004) 61–66, https://doi.org/10.1016/j.chroma.2004.08.118.
[40] K. Watanabe, Y. Itokawa, S. Yamaori, T. Funahashi, T. Kimura, T. Kaji, N. Usami, I. Yamamoto, Conversion of cannabidiol to Δ9-tetrahydrocannabinol and related cannabinoids in artificial gastric juice, and their pharmacological effects in mice, Forensic Toxicol. 25 (2007) 16–21, https://doi.org/10.1007/s11419-007-0021-y.
[41] D. Sulak, R. Saneto, B. Goldstein, The current status of artisanal cannabis for the treatment of epilepsy in the United States, Epilepsy Behav. 70 (2017) 328–333, https://doi.org/10.1016/j.yebeh.2016.12.032.
[42] H. Schulze, M. Schumacher, R. Urmeew, K. Auerbach, Driving Under the Influence of Drugs, Alcohol andMedicines in Europe — findings from the DRUID project. DRUID - final Report: work performed, main results and recommendations, Druid (2012) 1–58. ISSN 1725-5767.
[43] D.C. Howlett, N. Gainsborough, 100 Cases for Medical Data Interpretation, CRC Press, 2013.

Supplementary materials

Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – Troubles with reliable CBD quantitation when acidic precipitation agents are applied

Michał P. Dybowski, Andrzej L. Dawidowicz, Rafał Typek, Michał Rombel

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031, Lublin, Poland

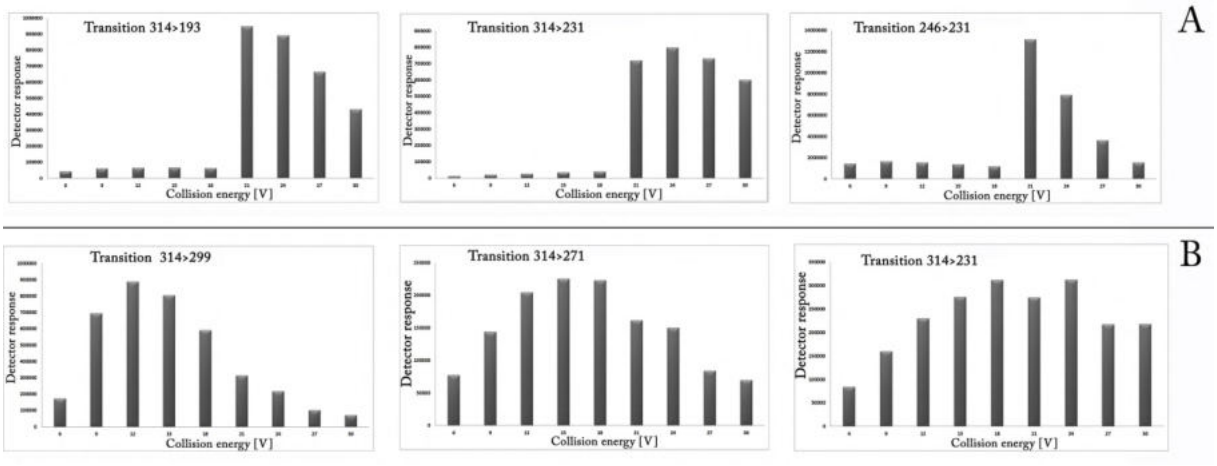


Fig. 1S. Optimization of collision energies of MRM transitions for CBD (A) and Δ9-THC (B).

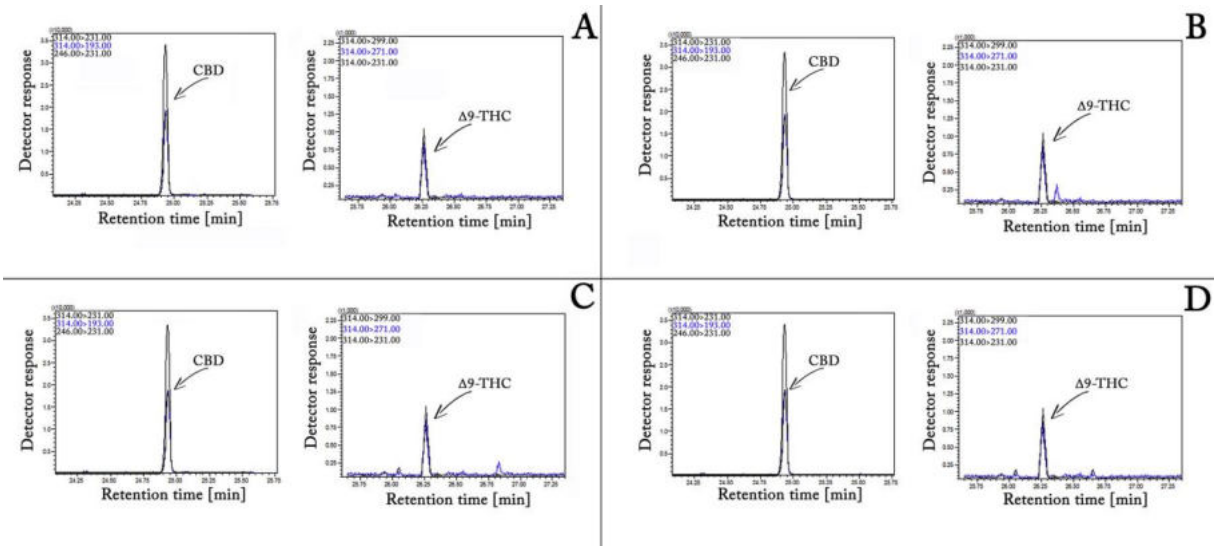


Fig. 2S. MRM chromatograms of supernatants after protein precipitation by TFA from samples of human plasma (A), pig plasma (B), HSA solution (C) and egg white protein solution (D) containing 50 ng/ml CBD. Incubation time with 50 % TFA - 1 h incubation; Precipitation agent/sample volume ratio equals 1/5; temperature of GC injector - 300 ^{o}C.

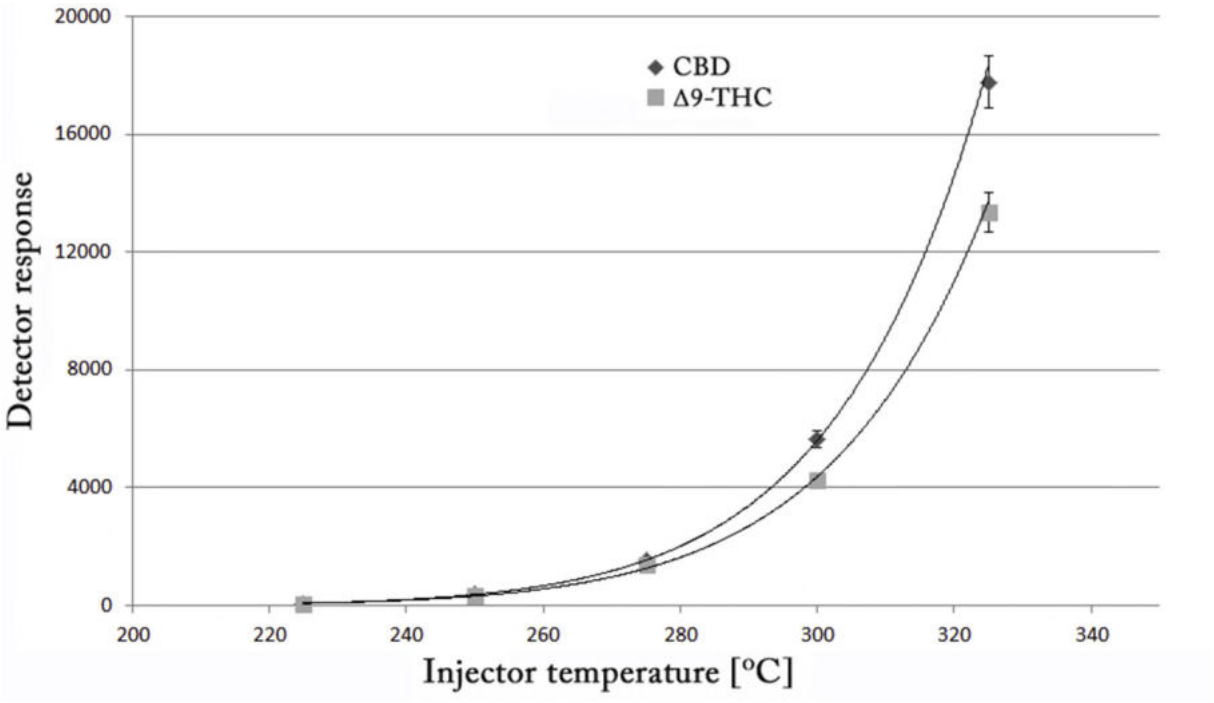


Fig. 3S. Exemplary plots showing the influence of the injector temperature on the signal magnitude of CBD (A) and Δ9-THC (B).

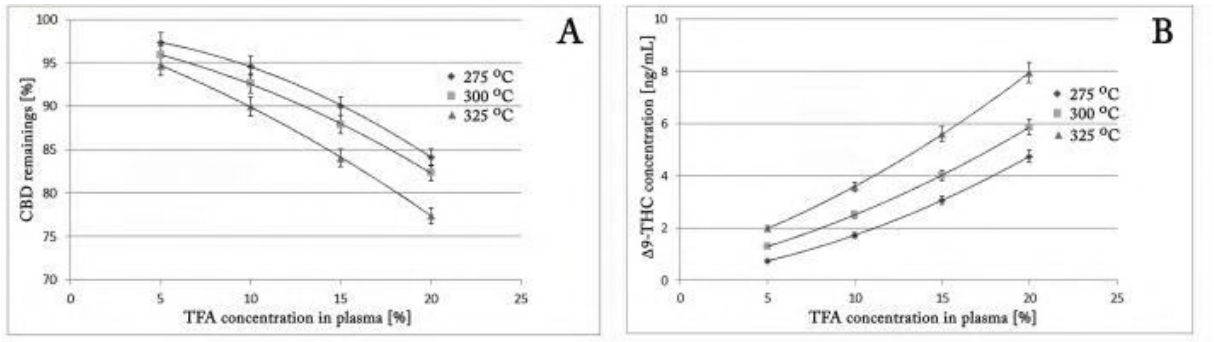


Fig. 4S. The influence of TFA concentration in plasma sample on:(A) - percentage of untransformed CBD, and (B) - Δ9-THC concentration (in ng/ml) which could be accidently detected in plasma of a person not abusing marijuana or its preparations but treated with drugs or supplements containing CBD, e.g. CBD oil. CBD plasma concentration - 50 ng/ml; TFA/plasma volume ratio – 1/5; In this experiments pig plasma and 25, 50, 75 and 100 % TFA were applied.

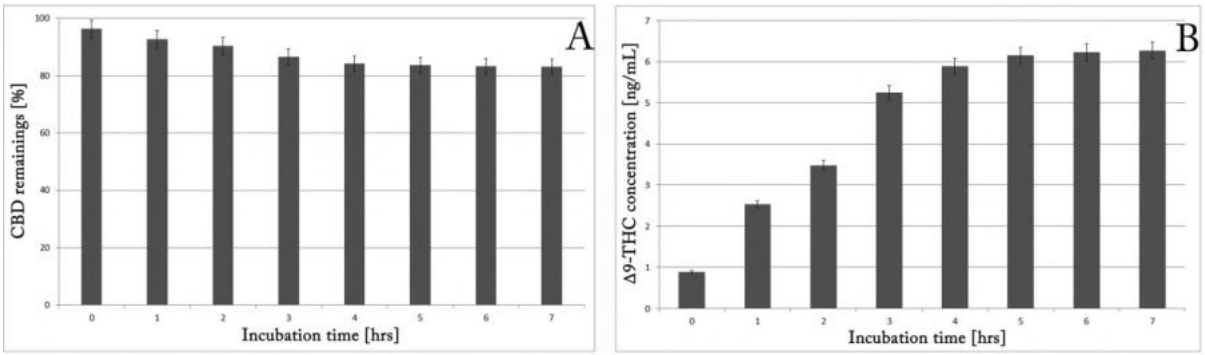


Fig. 5S. The influence of incubation time of plasma with 50% TFA (TFA/plasma volume ratio – 1/5) on (A) - percentage of untransformed CBD, and (B) - the Δ9-THC plasma concentration Injector temperature – 300 ºC; CBD plasma concentration - 50 ng/ml; In this experiments pig plasma was applied.

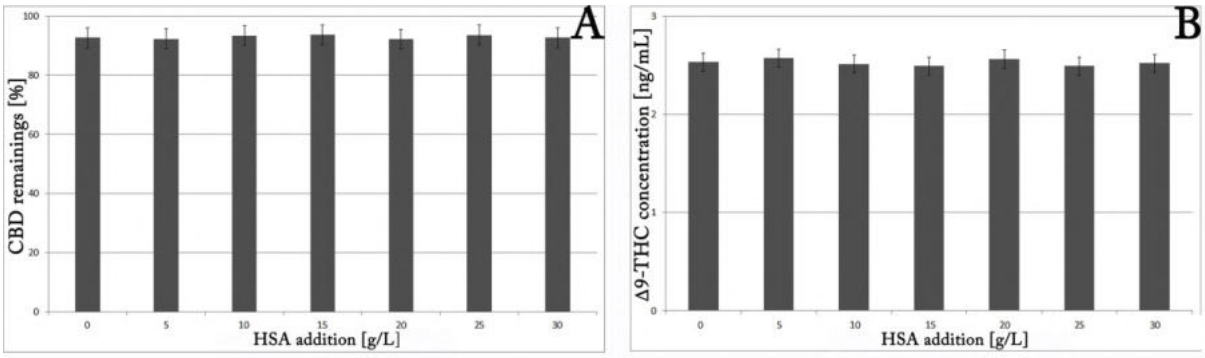


Fig. 6S. The percentage of untransformed CBD (A) and Δ9-THC concentration (B) estimated by GC-MS in supernatants from pig plasma samples reinforced with HSA.CBD sample concentration - 50 ng/ml; Injector temperature – 300 ºC; 50 % TFA/sample volume ratio – 1/5.

Table 1S. Multiple reaction monitoring (MRM) transitions and collision voltages of CBD and Δ9-THC for gas chromatography – tandem mass spectrometry.

Compound	**Retention time [min]**	**Qualitative MRM transition [mass > product mass]**	**Collision voltage [V]**	**Quantitative MRM transition [mass > product mass]**
CBD	24.94	314>193	21	246>231
		314>231	24	
		246>231	21	
Δ9-THC	26.27	314>299	12	214>299
		314>271	15	
		314>231	18	

Publikacja D2

A.L. Dawidowicz, M.P. Dybowski, **M. Rombel**, R. Typek

Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: Oleamide-induced transient matrix effect

J. Pharm. Biomed. Anal. 204 (2021) 114265

https://doi.org/10.1016/J.JPBA.2021.114265

IF 2-letni: 3,4; MEiN: 100 pkt

Journal of Pharmaceutical and Biomedical Analysis 204 (2021) 114265

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: Oleamide-induced transient matrix effect

Andrzej L. Dawidowicz, Michal P. Dybowski*, Michal Rombel, Rafal Typek

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie Sklodowska University in Lublin, 20-031, Lublin, Poland

ARTICLE INFO

Article history:
Received 7 June 2021
Received in revised form 6 July 2021
Accepted 9 July 2021
Available online 17 July 2021

Keywords:
Transient matrix effect
Oleamide effect
Analytical sensitivity increase
Cannabidiol analysis
GC analysis

ABSTRACT

The paper discusses the matrix effect evoked by oleamide (OLA), a compound frequently found in samples processed and/or stored in lab polypropylene vials or disposable syringes. In the case of many substances a higher response for their samples containing OLA than for net solutions is observed. The analyte signal gain resulting from OLA presence in the examined sample depends on the ratio of OLA concentration to analyte concentration. A characteristic feature of the matrix effect evoked by oleamide is its short duration, which makes the chromatographic data (retention value and signal magnitude of examined compound) repeatable and reproducible. The identified "transient matrix effect" may significantly increase the sensitivity of many analytical procedures employing GC. Evoking the transient matrix effect by means of OLA in the experimental analytical quantitation of cannabidiol in plasma allowed to lower its limit of detection (LOD) by more than 50 %.

1. Introduction

Plasma protein precipitation, liquid-liquid extraction and solid phase extraction are the most frequently applied sample preparation methods in analytical procedures of blood/plasma components [1,2]. In recent attempts to create the best procedure of sample preparation for the chromatographic analysis of xenobiotics in blood/plasma, more and more attention has been paid also to QuEChERS [3,4]. Due to the small volume of blood/plasma samples, all the mentioned sample preparation procedures are usually carried out in plastic, mostly polypropylene, low volume vessels like for instance the Eppendorf tube. Polypropylene (PP) used for the production of the Eppendorf tubes, disposable syringes and other laboratory auxiliary vessels contains in their structure a number of necessary additives (plasticizers, antioxidants, emulsifiers, etc.), which, during the activities required by the analytical procedure, may migrate to the examined samples and contaminate them. One of the additives frequently used in the production of polypropylene laboratory vessels is oleamide (OLA) [IUPAC: (Z)-Octadec-9-enamide]. This compound, plying the role of lubricant in the production of polypropylene vessels, very easily migrates to and contaminates samples during their transport, storage and/or laboratory activities performed using PP vessels. The negative influence of OLA on the result of biochemical assays of samples stored in PP vessels is known [5]. According to our preliminary studies, the presence of OLA in blood/plasma samples causes an signal increase of xenobiotics during their GC analysis. A specific nature of this OLA-induced effect indicate that it can be used to increase the sensitivity of xenobiotics analysis in blood/plasma by GC, which is novelty of the presented study.

The paper discusses the positive and negative impact of OLA presence in samples containing cannabidiol (CBD), 2-[(1R,6R)-3-metylo-6-(prop-1-en-2-ylo)cykloheks-2-enylo]-5-pentylobenzeno-1,3-diol, on CBD quantitative estimation. It is worth mentioning that this cannabinoid is one of the most frequently mentioned component of marijuana and hemp plants in the literature [6,7]. Considerable interest in the bioactive properties of CBD resulting from a number of preclinical and clinical studies and observations, as well as a marked increase in the use of dietary supplements containing CBD in the human self-treating process [8], stimulates the development of reliable and sensitive analytical procedures of its quantitation in blood/plasma samples [9] (e.g. to establish dose-therapeutic effect relationship) or in plant material [10] (e.g. to determine its profitability in the production of CBD and supplements containing this compound).

* Corresponding author.
E-mail address: michal.dybowski@poczta.umcs.lublin.pl (M.P. Dybowski).

https://doi.org/10.1016/j.jpba.2021.114265

2. Materials and methods

2.1. Materials

Acetonitrile (LC—MS grade), anhydrous magnesium sulfate (99.5 % powder; $MgSO_4$) and sodium chloride were purchased from Merck (Darmstadt, Germany). The Sepra C18-E sorbent (50 μm, 65 Å) used in the QuEChERS process was obtained from Phenomenex (Torrance, CA, USA). Formic acid (99 %), anthracene, fluoranthene, biphenyl, bisphenol A, testosterone, cholesterol, human serum albumin (HSA) and phosphate buffered saline, pH 7.2 (PBS) were acquired from Sigma-Aldrich (Poznan, Poland). Glycerol was obtained from Avantor Performance Materials Poland (Gliwice, Poland). Synthetic cannabinoid called as CP-47,497 {IUPAC: 2-[(1R,3S)-3-hydroksycykloheksylo]-5-(2-metylooktan-2-ylo)phenol} was purchased from LGC Standards Group (Łomianki, Poland). The standards of cannabidiol (CBD), cannabidiol-D3 (CBD-D_3), cannabigerol (CBG), cannabinol (CBN) all of 1.0 mg/mL in methanol (Cerilliant) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purified by the Milli-Q system (Millipore Sigma, Bedford, MA, USA).

Human plasma samples were obtained by the centrifugation of human blood samples. The blood samples were collected by a registered nurse from volunteers, after obtaining their informed consent, using a single closed system containing an S-Monovette coagulation activator, according to the manufacturer instructions (Sarstedt AG, Nümbrecht, Germany), and thoroughly mixed in order to maintain their homogeneity.

2.2. Test solutions

The preliminary studies showing the effect of OLA presence on the CBD signal magnitude (see Fig. 1) were performed in PP Eppendorf test tubes without knowledge about OLA migration to examined samples and about OLA concentration in samples. More detailed studies showing the effect of OLA presence on the signal magnitude of selected substances (mainly of CBD) were carried out in glass vessels using solutions of these compounds without OLA addition and with known OLA concentration. Their concentrations are given in the captions to the figures. To avoid the influence of interfering matrix components existing in a natural sample (e.g. plasma components) on the analyte signal magnitude, it was decided to perform a part of experiments applying plasma simulant instead of human plasma. 7% HSA solution in PBS was used in this purpose.

2.3. Eppendorf tube extraction

In order to confirm OLA emissions from the Eppendorf tubes (Eppendorf Tubes® - Eppendorf AG, Germany) used in the experiments, a 1.5 mL test tube was filled with acetonitrile, sealed tightly and left for 30 min at room temperature. After that time, the contents of the test tube was analyzed by GC–MS/MS.

2.4. QuEChERS

To plasma or HSA solution (700 μL) spiked properly with CBD and placed in a PP Eppendorf tube or in glass vial, the internal standard (10 μL of CP-47,497 solution, concentration 10 μg/mL) was added. The mixture was hand-shaken for 2 min, whereupon $MgSO_4$ (200 mg) and NaCl (50 mg) were added. After vortexing for 1 min, acetonitrile (700 μL) was introduced to the tube and the whole suspension was vortexed again and next centrifuged at 12000 rpm for 3 min. Finally, the aliquot (600 μL) was cleaned-up by d-SPE with 20 mg of C18, centrifuged at 12000 rpm for 3 min and subjected to chromatographic analysis.

When examining the influence of OLA addition on the analyte signal magnitude in the analytical procedure involving QuEChERS, OLA solution was added to the final QuEChERS extract before its GC analysis.

In order to estimate the detection limit (LOD) for CBD using this method, QuEChERS extracts from CBD spiked plasma samples were injected. The LOD was considered to be signalto-noise ratios equal to 3.

2.5. GC–MS/MS measurements

Quantitative analyses of CBD and other examined compounds were conducted using a gas chromatograph hyphenated with a triple quadruple tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan). GC separations were performed employing capillary column Zebron ZB5-MSi (30 m x 0.25 mm i.d., 0.25 μm film thickness- Phenomenex, Torrance, CA, USA) and helium (grade 5.0) as carrier gas with flow rate 1.56 mL/min. The 1 μL samples were injected into the column using injection system working at 310 °C in high-pressure splitless injection mode (150.0 kPa for 1.5 min).

The chromatographic process was performed using the following temperature program:

- initial temperature 60 maintained for 2 min, I ramp at a rate of 9° °C/min to the temperature 220 °C, II ramp at a rate of 5 °C/min to the temperature 280 °C, III ramp at a rate of 10 °C/min to the final temperature 310 °C and maintained for 15 min.

The MS conditions were as follows:

- tandem mass spectrometer operated with normalized electron energy of 70 eV;
- ion source temperature: 225 °C;
- collision gas: argon (grade 5.0);

For qualitative purposes the full SCAN mode with range 40–550 *m/z* was employed and for quantitative analyses the Multiple Reaction Monitoring (MRM) mode was used.

2.6. MRM transitions for MS/MS detection

GC–MS/MS quantitation of all the examined compounds was performed in the MRM mode. For this reason, the fragmentation pathways for each compound were established and energies for MRM transitions were as follows:

314 > 193 (CE = 21eV), 314 > 231(CE = 24eV) and 246 > 231 (CE = 21eV) for CBD,

178 > 152 (CE = 15eV), 178 > 89 (CE = 15eV) and 152 > 89 (CE = 15eV) for Anthracene,

154 > 128 (CE = 15eV), 154 > 76 (CE = 15eV) and 128 > 76 (CE = 15eV) for Biphenyl,

202 > 174 (CE = 21eV), 202 > 101(CE = 24eV) and 174 > 101 (CE = 21eV) for Fluoranthene,

264 > 132 (CE = 21eV), 264 > 118(CE = 24eV) and 132 > 118 (CE = 21eV) for BAP,

310 > 295 (CE = 21eV), 310 > 238(CE = 30eV) and 295 > 238 (CE = 27eV) for CBN,

316 > 231 (CE = 15eV), 316 > 193(CE = 15eV) and 316 > 123 (CE = 6eV) for CBG,

318 > 215 (CE = 15eV), 300 > 215(CE = 15eV) and 233 > 215 (CE = 15eV) for CP-47,497,

228 > 213 (CE = 15eV), 228 > 135(CE = 15eV) and 213 > 135 (CE = 15eV) for Bisphenol-A,

288 > 246 (CE = 15eV), 288 > 124(CE = 15eV) and 246 > 124 (CE = 15eV) for Testosterone,

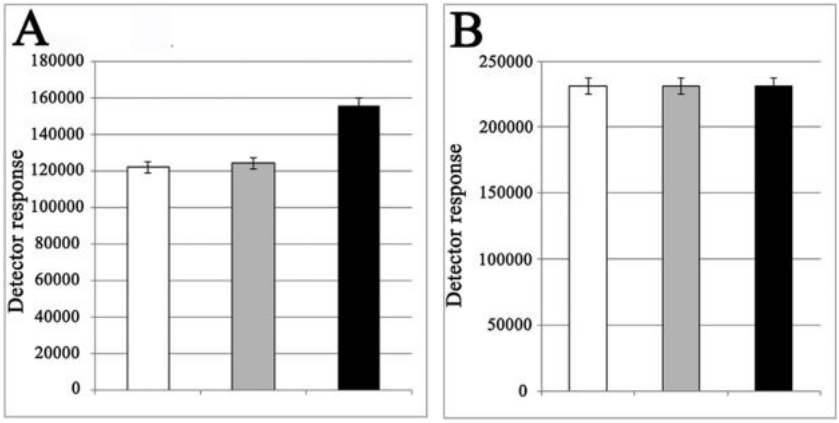


Fig. 1. CBD signal magnitudes from GC–MS/MS (A) and LC–MS (B) data for CBD solution in acetonitrile (white bars); QuEChERS extract obtained in glass tube from CBD spiked HSA sample (gray bars); QuEChERS extract obtained in PP Eppendorf tube from CBD spiked HSA sample (black bars). CBD concentrations in samples examined by GC–MS/MS were the same and equaled 600 ng/mL. CBD concentration in samples examined by LC–MS equaled 6 μg/mL.

386 > 301 (CE = 15eV), 386 > 275(CE = 15eV) and 368 > 301 (CE = 15eV) for Cholesterol,

92 > 74 (CE = 15eV), 74 > 61(CE = 15eV) and 74 > 43 (CE = 15eV) for Glycerol.

2.7. LC–MS

An LC–MS system composed of an UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) and a linear trap quadrupole- Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) was applied for the chromatographic analyses of the examined CBD solutions. ESI ionization source operating in the positive polarization mode at needle potential equal to 4.5 kV was employed. Nitrogen (>99.98 %) was used as sheath gas (at 40 arbitrary units), auxiliary gas (at 10 arbitrary units) and sweep gas (at 10 arbitrary units). Capillary temperature equalled 320 °C. Chromatographic separations were performed on Gemini C18 column (4.6 × 100 mm, 3 μm; Phenomenex, USA). The mobile phase components were: A – 25 mM formic acid in water and B –25 mM formic acid in acetonitrile. Isocratic elution was applied using mobile phase containing 40 % of B component. The total run time was 15 min at the mobile phase flow rate 0.5 mL/min. The SIM function was used for analyzing CBD solutions. The 315 *m/z* ion was monitored.

2.8. Statistical analysis

All results are presented as the mean value of five independent measurements (n = 5) ± SD. The magnitude of chromatographic signals were compared using analysis of variance (ANOVA). The differences in the signal magnitudes were considered as significant for $p \leq 0.05$ and $F_{crit} < F_{exp}$.

3. Results and discussion

Fig. 1 shows the CBD signal values obtained in GC–MS/MS (Fig. 1A) and LC–MS (Fig. 1B) measurements for the supernatant prepared from an exemplary HSA sample spiked with CBD following QuEChERS procedure in a glass test tube (gray bars) and PP Eppendorf tube (black bars). White bars in Fig. 1A and B represents CBD signal values obtained in GC–MS and LC–MS analyzes after the injection of acetonitrile CBD solution of the same CBD concentration as in the HSA sample. Although the presented results clearly indicate nearly 100 % recovery of CBD from the HSA sample by the QuEChERS technique, more important for the paper's research question is the exceptionally large magnitude of the CBD signal

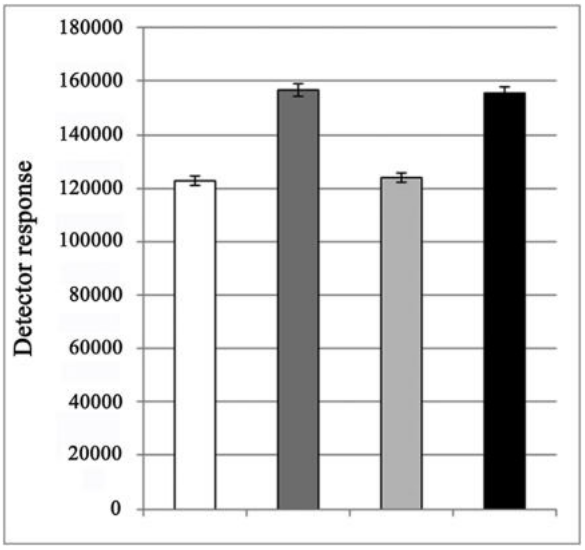


Fig. 2. CBD signal magnitudes from GC–MS/MS data obtained for CBD solution in OLA-free acetonitrile (white bar); CBD solution in acetonitrile containing OLA (dark gray bar); QuEChERS extract obtained from CBD spiked OLA-free HSA sample (light gray bar); QuEChERS extract obtained from HSA sample spiked with CBD and OLA (black bar). CBD concentration – 600 ng/mL, OLA concentration – 5 μg/mL. All samples prepared in glass vials.

obtained in the GC–MS/MS measurements for the HSA sample processed in a PP Eppendorf tube. It is about 28 % higher than that obtained for the same sample processed in a glass vessel (compare gray and black bars in Fig. 1A). This suggests that during the QuEChERS procedure some components of PP vial could be extracted and may have produced the so-called "matrix effect" [11]. This phenomenon causes a higher response in sample solution containing matrix components than in net solutions [12,13], which was discussed a few times in the literature, especially in the context of pesticides analysis in samples containing co-extracted matrix components [11,14–17]. The data presented in Fig. 1A suggest that it also occurs during the GC analysis of CBD (and other compounds - see below) in samples contaminated with the low-volatile components of PP vessels. The possibility of sample contamination with the components of PP used for the production of different laboratory equipment is strongly suggested by Fig. 1S (see supplementary materials) showing an exemplary chromatogram of the acetonitrile extract from PP Eppendorf tubes. As can be seen, the main volatile contaminant of the samples processed in the applied Eppendorf

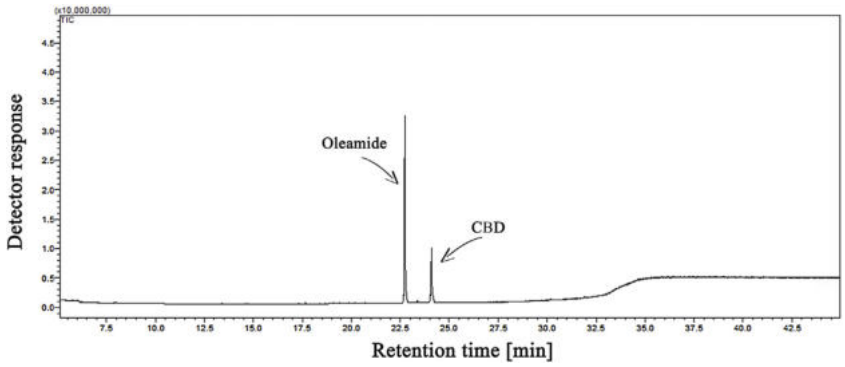


Fig. 3. Exemplary GC–MS chromatogram (Scan) of CBD solution in acetonitrile with OLA addition.

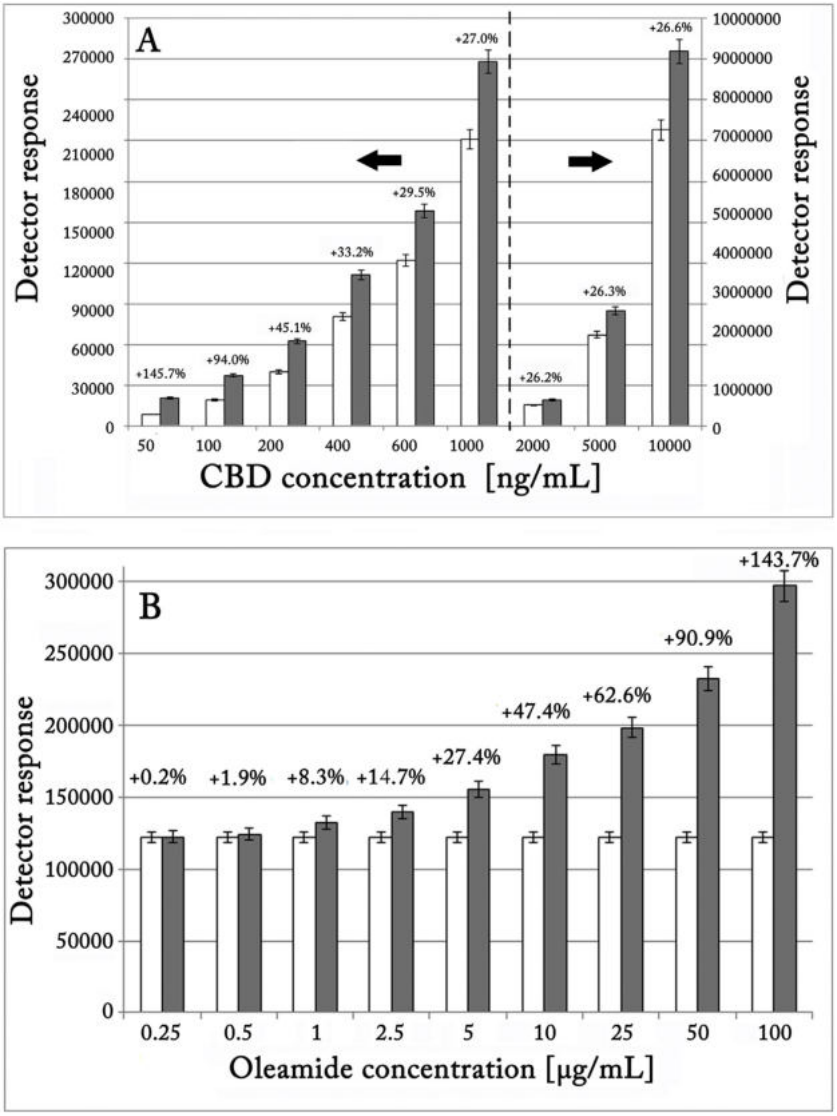


Fig. 4. The effect of CBD concentration increase on the CBD signal magnitude when CBD solutions in OLA-free acetonitrile (white bars) and CBD solutions in acetonitrile with OLA in constant concentration (5 µg/mL) (gray bars) were used (Fig. part A), and OLA concentration increase on the CBD signal magnitude when CBD solutions in OLA-free acetonitrile (white bars) and CBD solutions in acetonitrile containing OLA in escalating concentrations (gray bars) were used (Fig. part B). CBD concentration - 600 ng/mL.

tubes is OLA, which evokes the observed matrix effect. The diagrams in Fig. 2 confirm this supposition. The CBD signal magnitudes for the OLA-added samples (dark gray and black bars) are clearly larger than those for the OLA-free ones (white and light gray bars). Thus, the presence of OLA not only influences the results of the biochemical tests [5] but also the results of the GC chromatographic analyses.

As results from the literature [11], the matrix effect evoking the increase in the analyte signal magnitude is caused by the blockade of the existing active centers or by the creation of new active centers

resulting from the accumulation of low-volatile matrix compounds in the GC system. If so, the increase in the analyte signal magnitude in subsequent injections of the sample containing low-volatile sample contaminants (e.g. OLA) should be higher and higher. The diagram in Fig. 2S (see supplementary materials) presenting the CBD signal magnitudes obtained in five subsequent injections of CBD solutions of the same CBD concentration in acetonitrile containing OLA and free from OLA, injected alternately, contradicts this expectation. In both CBD solutions, the CBD signal magnitude and its retention are constant and repeatable. It is also worth noting at this point that the data in Figs. 1 and 2 are characterized by a small spread (small SD values), which also indicate the reproducibility of the quantitative data. Thus, the matrix effect discussed in this paper cannot be related to the permanent accumulation of OLA in the chromatographic system. Taking into account small differences in vapor pressure of both OLA and CBD and their boiling temperatures (ca. 394 and 425 °C, respectively), it is probable that OLA co-condenses with CBD at the beginning of the column and modifies the stationary phase, increasing the solubility of CBD in it. After reaching the appropriate temperature in the column operation program, OLA starts to migrate and leaves the column in due time in the form of a separate peak (see Fig. 3). Hence, the observed matrix effect should rather be specified as "transient matrix effect".

The magnitude of the CBD signal increase due to the identified transient matrix effect depends on the mutual quantitative ratio between the analyte and OLA. This is confirmed by the diagram in Fig. 4 illustrating (1) the effect of the CBD concentration increase on the magnitude of its signal established using solutions with ever-increasing CBD concentration in acetonitrile without OLA (white bars) and with OLA in constant concentration (gray bars) (see Fig. 4A), and (2) the effect of the OLA concentration increase on the magnitude of the CBD signal determined using solutions with a constant concentration of CBD in acetonitrile without OLA (white bars) and with OLA in increasing concentrations (gray bars) (see Fig. 4 B).

A decrease of the increment in the CBD signal magnitude for the first case (see the percentage increment of signal magnitude in Fig. 4A) and an increase of the increment in the CBD signal magnitude for the second case (see the percentage increment of the signal magnitude in Fig. 4B) is observed. The results from Fig. 4 are consistent with the description of transient matrix effect presented above and confirm that the increase in the magnitude of the analyte signal depends on the relative amount of the analyte and OLA in examined sample.

The question appears whether transient matrix effect induced by OLA applies only to CBD or to other compounds, too. Fig. 3S (see supplementary materials) shows the signal magnitudes of several exemplary compounds injected to GC–MS in the form of their acetonitrile solutions with and without OLA. The concentrations of individual substances in the corresponding solutions were the same (600 ng/mL). As the presented examples demonstrate, the presence of OLA does not alter the signal magnitude of aromatic hydrocarbons (anthracene, fluoranthene, biphenyl). A positive influence of OLA presence on the change of signal magnitude is observed for medium polar substances (CBG, CBD, CBN, CP-47,497, bisphenol A, testosterone, cholesterol, glycerol). This is understandable since the OLA is mid-polar component and its co-condensation with analytes at the beginning of the column favors the solubility growth of those analytes which strongly interact with OLA or exhibit a similar nature to OLA. Among the examined polar compounds the greatest signal gain resulting from OLA presence in the sample is observed for bisphenol A, and the smallest for the synthetic cannabinoid CP-47,497 and glycerol.

When analyzing the results presented above, the reproducibility of the analyte signal increase (exemplified CBD) resulting from the transient matrix effect deserves particular emphasis. It indicates

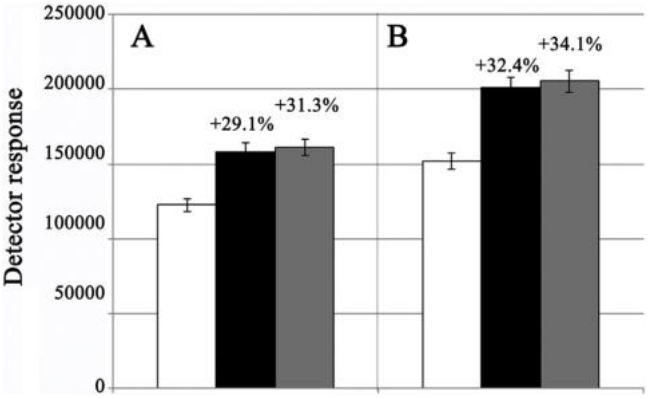


Fig. 5. CBD signal magnitudes from GC–MS/MS data obtained for CBD solution prepared in glass vial using OLA-free acetonitrile (white bar in part A); CBD solution prepared in glass vial using acetonitrile containing OLA (black bar in part A); CBD solution prepared in PP vial using OLA-free acetonitrile (gray bar in part A) and for QuEChERS extract prepared in glass vial using CBD spiked OLA-free human plasma sample (white bar in part B); QuEChERS extract prepared in PP vial using CBD spiked OLA-free human plasma sample (gray bar in part B); QuEChERS extract prepared in glass vial using CBD spiked human plasma sample with OLA addition (black bar in part B). CBD concentration – 200 ng/mL, CBD-D_3 concentration – 200 ng/mL, OLA concentration – 5 μg/mL.

the possibility of using the effect to increase the sensitivity of GC analysis (to increase the analyte signal magnitude i.e. to lower LOD). Taking onto account such a possibility in the context of quantifying trace amounts of xenobiotics, it should be remembered that natural samples like plasma very often contain low volatile substances, which can also cause matrix effect increasing signal magnitude of analyzed substances. Fig. 5 shows the CBD signal values obtained in GC–MS/MS measurements for CBD solutions prepared in glass (white and black bars) and PP vials (gray bar) (Fig. 5A) and for supernatants prepared from human plasma samples spiked with CBD, in accordance with QuEChERS procedure performed in glass (white and black bars) and PP vials (gray bar) (Fig. 5B). Its analysis confirms the previously formulated conclusions and leads to the following additional ones that (1) the CBD signal increase is caused not only by OLA but also by plasma matrix components, and (2) the increase of CBD signal resulting from the presence of plasma matrix components is additionally magnified by OLA.

A few methods can be used to counteract the matrix effect [11,12,14,15,18,19,20]. They are either labour-intensive [11], or require the usage of previously modified GC equipment [20], or cause quick column deterioration, or are of limited effectiveness [11,12,14,18,19]. Their application can eliminate also the transient matrix effect evoked by OLA, intentionally added to the sample to increase the signal magnitude of the quantified analyte. To avoid the probability of losing the OLA effect of heightening the sensitivity of xenobiotic analysis in plasma by GC an appropriate internal standard should be used. Its losses in the process of sample preparation for analysis and its signal gain due to the presence of OLA should be the same as for an analyte. Hence, the usage of deuterated analyte as internal standard in the analytical procedure seems to be the only option.

As results from Fig. 4, the increase of analyte signal magnitude (exemplified by CBD) resulting from the presence of OLA in the sample depends on the concentration ratio of the two components. Consequently, by injecting calibration solutions with different analyte concentrations and a constant concentration of the internal standard and a constant concentration of the substance causing transient matrix effect (OLA), a calibration curve will be obtained, which will have to be approximated by a polynomial equation. Its course will depend on the OLA concentration used in preparing calibration solutions, and the accuracy of its course will depend

on the number of points defining the calibration plot (i.e. the number of calibration solutions). Fig. 4S (see supplementary materials) shows exemplary calibration curves for CBD quantification in plasma samples using QuEChERS as sample preparation technique. The calibration plots were prepared using plasma solutions without OLA and adding OLA to QuEChERS extract. Deuterated CBD was used as an internal standard. In this experiment, OLA reduced LOD of QuEChERS procedure for CBD estimation by 53 % (LOD lowered from 1.03 ng/mL in the case of OLA-free samples to 0.48 ng/mL after OLA addition).

4. Conclusions

The presented results show that the presence of OLA in samples examined by GC causes the signal magnitude increase of CBD and many other compounds in relation to their signal magnitude in OLA-free samples. The reason for this is matrix effect, which in the case of OLA should be referred to more accurately as transient matrix effect. It makes the magnitude of the signal gain for an examined analyte reproducible. The presented results prove that the identified transient matrix effect can be used to improve the sensitivity of a number of analytical quantitation of xenobiotics in plasma by GC. For this purpose, internal calibration with a deuterated analyte as an internal standard should be used. Moreover, OLA should be added to the sample just before its injection.

Authors' contributions

Andrzej L. Dawidowicz: Conceptualization, Writing- Original draft preparation, Investigation. **Michal P. Dybowski:** Writing- Original draft preparation, Writing- Reviewing and Editing, Investigation, Methodology, Data curation, Visualization. **Rafal Typek:** Writing- Original draft preparation, Investigation, Methodology, Data curation. **Michal Rombel:** Writing- Original draft preparation, Investigation, Data curation

Authorship statement

All authors have read the journal's authorship statement and agree to it.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114265.

Declaration of Competing Interest

The authors report no declarations of interest.

References

[1] L.A. Resende, P.H.R. da Silva, C. Fernandes, Quantitative determination of the antimalarials artemether and lumefantrine in biological samples: a review, J. Pharm. Biomed. Anal. 165 (2019) 304–314, http://dx.doi.org/10.1016/j.jpba.2018.12.021.

[2] J.S. Millership, L.G. Hare, M. Farry, P.S. Collier, J.C. McElnay, M.D. Shields, D.J. Carson, The use of hydrophilic lipophilic balanced (HLB) copolymer SPE cartridges for the extraction of diclofenac from small volume paediatric plasma samples, J. Pharm. Biomed. Anal. 25 (2001) 871–879, http://dx.doi.org/10.1016/S0731-7085(01)00367-3.

[3] M.P. Dybowski, A.L. Dawidowicz, Application of the QuEChERS procedure for analysis of Δ9-tetrahydrocannabinol and its metabolites in authentic whole blood samples by GC–MS/MS, Forensic Toxicol. 36 (2018) 415–423, http://dx.doi.org/10.1007/s11419-018-0419-8.

[4] H. Wang, X. Zhao, Y. Shang, H. Wang, P. Wang, X. Yu, Quantitative analysis of 20 fentanyl analogues by modified QuEChERS-LC–MS/MS in health products and transdermal patches, J. Pharm. Biomed. Anal. 201 (2021) 114100, http://dx.doi.org/10.1016/j.jpba.2021.114100.

[5] G.R. McDonald, A.L. Hudson, S.M.J. Dunn, H. You, G.B. Baker, R.M. Whittal, J.W. Martin, A. Jha, D.E. Edmondson, A. Holt, Bioactive contaminants leach from disposable laboratory plasticware, Science 80 (322) (2008) 917, http://dx.doi.org/10.1126/science.1162395.

[6] A. Zgair, J.C.M. Wong, A. Sabri, P.M. Fischer, D.A. Barrett, C.S. Constantinescu, P. Gershkovich, Development of a simple and sensitive HPLC-UV method for the simultaneous determination of cannabidiol and δ9-tetrahydrocannabinol in rat plasma, J. Pharm. Biomed. Anal. 114 (2015) 145–151, http://dx.doi.org/10.1016/j.jpba.2015.05.019.

[7] M.P. Dybowski, A.L. Dawidowicz, R. Typek, M. Rombel, Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – troubles with reliable CBD quantitation when acidic precipitation agents are applied, Talanta 220 (2020) 121390, http://dx.doi.org/10.1016/j.talanta.2020.121390.

[8] P. Berman, L. Sulimani, A. Gelfand, K. Amsalem, G.M. Lewitus, D. Meiri, Cannabinoidomics – an analytical approach to understand the effect of medical Cannabis treatment on the endocannabinoid metabolome, Talanta 219 (2020) 121336, http://dx.doi.org/10.1016/j.talanta.2020.121336.

[9] M.J. Roslawski, R.P. Remmel, A. Karanam, I.E. Leppik, S.E. Marino, A.K. Birnbaum, Simultaneous quantification of 13 cannabinoids and metabolites in human plasma by liquid chromatography tandem mass spectrometry in adult epilepsy patients, Ther. Drug Monit. 41 (2019) 357–370, http://dx.doi.org/10.1097/FTD.0000000000000583.

[10] B. Fodor, I. Boldizsár, I. Molnár-Perl, Alkylsilyl speciation and direct sample preparation of plant cannabinoids prior to their analysis by GC-MS, Anal. Chim. Acta 1021 (2018) 51–59, http://dx.doi.org/10.1016/j.aca.2018.03.049.

[11] T. Fujiyoshi, T. Ikami, T. Sato, K. Kikukawa, M. Kobayashi, H. Ito, A. Yamamoto, Evaluation of the matrix effect on gas chromatography - mass spectrometry with carrier gas containing ethylene glycol as an analyte protectant, J. Chromatogr. A 1434 (2016) 136–141, http://dx.doi.org/10.1016/j.chroma.2015.12.085.

[12] R. Rodríguez-Ramos, S.J. Lehotay, N. Michlig, B. Socas-Rodríguez, M.Á. Rodríguez-Delgado, Critical review and re-assessment of analyte protectants in gas chromatography, J. Chromatogr. A 1632 (2020), http://dx.doi.org/10.1016/j.chroma.2020.461596.

[13] F. Jesús, R. Hladki, N. Gérez, N. Besil, S. Niell, G. Fernández, H. Heinzen, M.V. Cesio, Miniaturized QuEChERS based methodology for multiresidue determination of pesticides in odonate nymphs as ecosystem biomonitors, Talanta 178 (2018) 410–418, http://dx.doi.org/10.1016/j.talanta.2017.09.014.

[14] A. Garrido Frenich, J.L. Martínez Vidal, J.L. Fernández Moreno, R. Romero-González, Compensation for matrix effects in gas chromatography-tandem mass spectrometry using a single point standard addition, J. Chromatogr. A 1216 (2009) 4798–4808, http://dx.doi.org/10.1016/j.chroma.2009.04.018.

[15] M. Anastassiades, K. Maštovská, S.J. Lehotay, Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides, in: J. Chromatogr. A, Elsevier, 2003, pp. 163–184, http://dx.doi.org/10.1016/S0021-9673(03)01208-1.

[16] C.F. Poole, Matrix-induced response enhancement in pesticide residue analysis by gas chromatography, J. Chromatogr. A 1158 (2007) 241–250, http://dx.doi.org/10.1016/j.chroma.2007.01.018.

[17] J. Hajšlová, K. Holadová, V. Kocourek, J. Poustka, M. Godula, P. Cuhra, M. Kempný, Matrix-induced effects: a critical point in the gas chromatographic analysis of pesticide residues, J. Chromatogr. A 800 (1998) 283–295, http://dx.doi.org/10.1016/S0021-9673(97)01145-X.

[18] M.M. Rahman, A.M. Abd El-Aty, J.H. Shim, Matrix enhancement effect: a blessing or a curse for gas chromatography?-A review, Anal. Chim. Acta 801 (2013) 14–21, http://dx.doi.org/10.1016/j.aca.2013.09.005.

[19] V. Veeravalli, H.S. Cheruvu, P. Srivastava, L.M. Vamsi Madgula, Three-dimensional aspects of formulation excipients in drug discovery: a critical assessment on orphan excipients, matrix effects and drug interactions, J. Pharm. Anal. 10 (2020) 522–531, http://dx.doi.org/10.1016/j.jpha.2020.02.007.

[20] K. Sugitate, H. Anazawa, S. Nakamura, N. Orikata, K. Mizukoshi, M. Nakamura, A. Toriba, K. Hayakawa, Decrease in the matrix effect of GC/MS by a gold-plated ion source, J. Pestic. Sci. 37 (2012) 148–155, http://dx.doi.org/10.1584/jpestics.D11-043.

Supplementary materials

Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: Oleamide-induced transient matrix effect

Andrzej L. Dawidowicz, Michał P. Dybowski, Michał Rombel, Rafał Typek

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031, Lublin, Poland

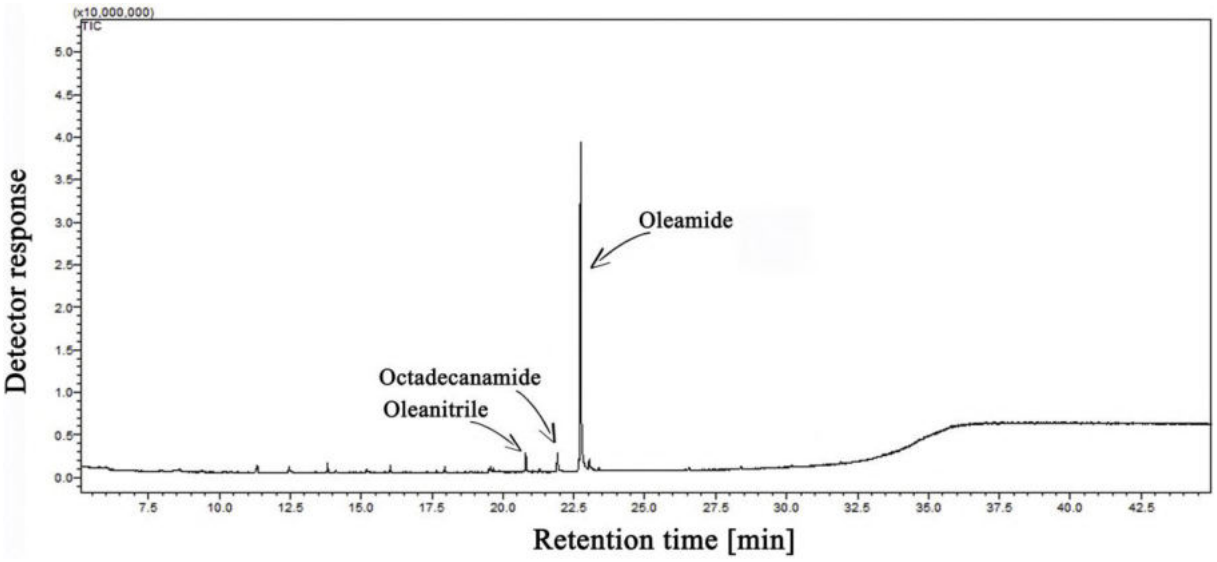


Fig. 1S. GC-MS (SCAN) chromatogram of acetonitrile extract from PP Eppendorf tube.

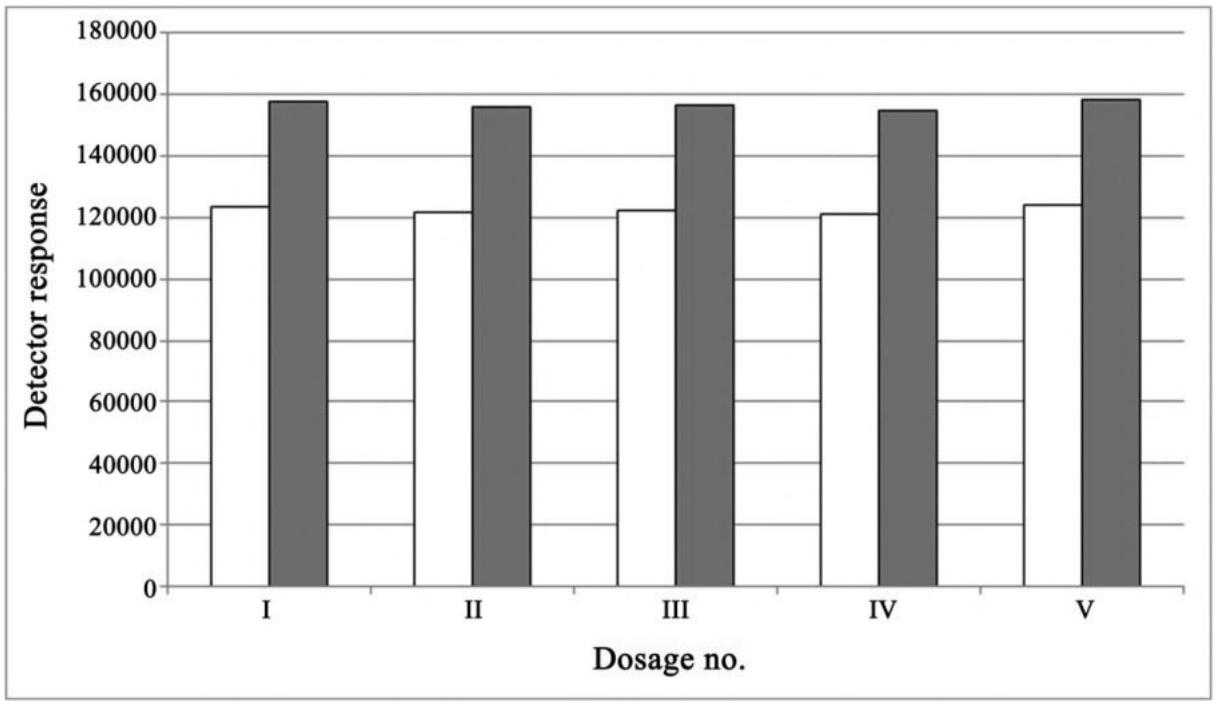


Fig. 2S. CBD signal magnitudes from GC-MS/MS data obtained in five consecutive injections of CBD solution in OLA-free acetonitrile (white bars) and CBD solution in acetonitrile containing OLA (gray bars). CBD concentration – 600 ng/mL, OLA concentration – 5 µg/mL.

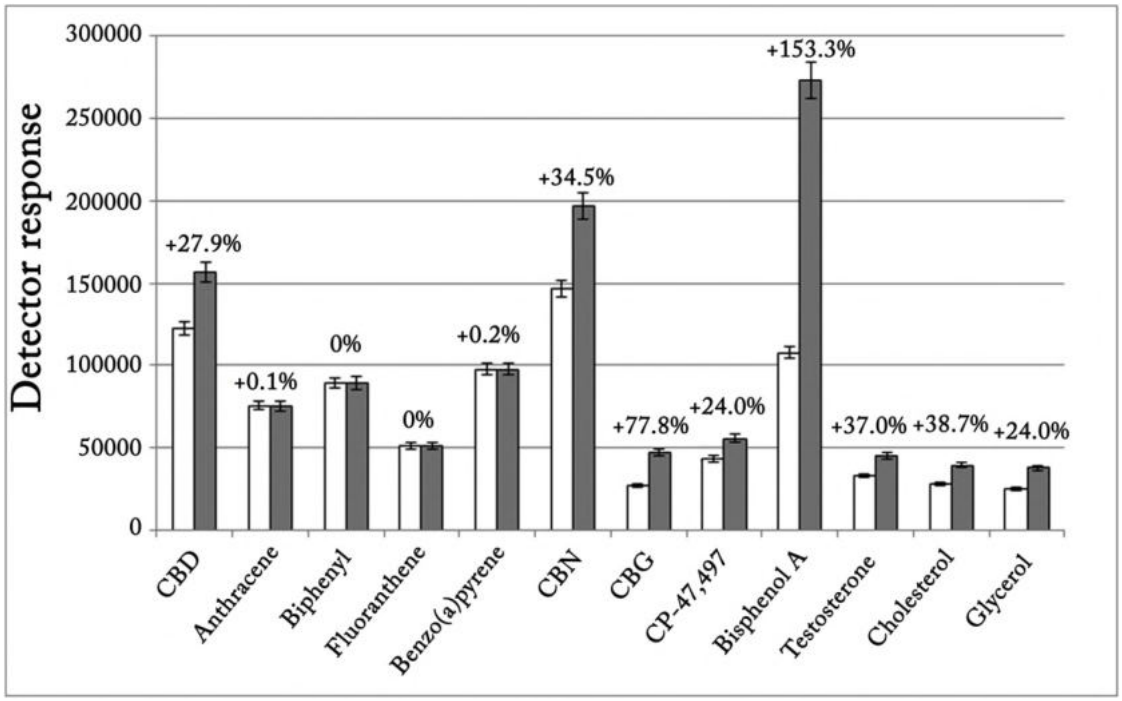


Fig. 3S. The effect of OLA addition on the change of GC-MS/MS signal magnitude for a few exemplary compounds. White bars – compound solution (600 ng/mL) in OLA-free acetonitrile; gray bars – compound solution (600 ng/mL) in acetonitrile containing OLA (5 µg/mL).

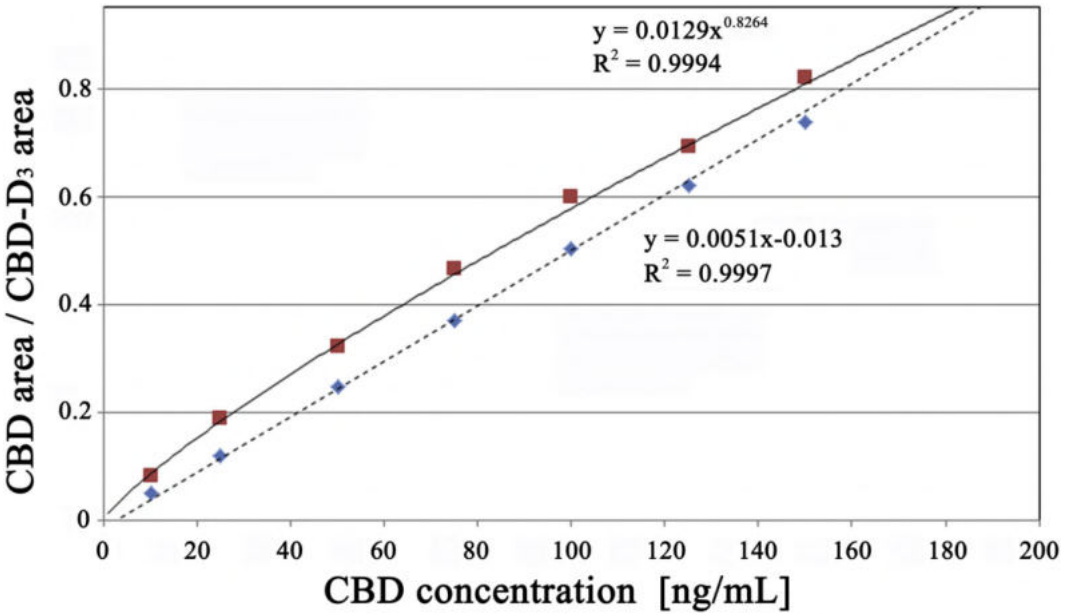


Fig. 4S. Internal standard calibration curves for CBD quantitation in OLA-free plasma samples (dashed line with diamonds) and in plasma samples with OLA (5 µg/mL) (solid line with squares) using QuEChERS as sample preparation method before GC analysis. Deuterated CBD (200 ng/mL) was applied as internal standard. All steps of analytical procedure were performed in glass vessels.

Publikacja D3

A.L. Dawidowicz, M.P. Dybowski, **M. Rombel**, R. Typek

Oleamide as analyte protectant in GC analysis of THC and its metabolites in blood

J. Pharm. Biomed. Anal. 215 (2022) 114800

https://doi.org/10.1016/J.JPBA.2022.114800

IF 2-letni: 3,4; MEiN: 100 pkt

Journal of Pharmaceutical and Biomedical Analysis 215 (2022) 114800

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Short communication

Oleamide as analyte protectant in GC analysis of THC and its metabolites in blood

Andrzej L. Dawidowicz, Michal P. Dybowski *, Michal Rombel, Rafal Typek

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie Sklodowska University, 20-031 Lublin, Poland

ARTICLE INFO

Keywords:
Oleamide-induced transient matrix effect
Analytical sensitivity increase
THC
THC-OH
THC-COOH

ABSTRACT

Methods for the analysis of cannabinoids in bio-matrices are continually being developed, to achieve a proper sensitivity required for their detection and accuracy in their quantification. The presented paper shows that the analytical sensitivity of GC–MS to THC and its metabolites in blood samples can be significantly increase by oleamide (OLA) addition to the examined sample, which evokes the matrix effect of transient character. The magnitude of signal increment resulting from oleamide presence in the examined sample is the greatest for THC metabolites and depends on oleamide concentration in the examined sample. The use of transient matrix effect to increase the sensitivity of the analysis can be applied not only in QuEChERS procedure, which is applied in the described experiments, but also in other blood sample preparation methods. Evoking the transient matrix effect by means of OLA in the experimental analytical quantitation of THC and its metabolites in blood allowed to lower limit of detection (LOD) approximately by 20.5%, 87.6% and 90.1% in the case of THC, THC-OH and THC-COOH, respectively.

1. Introduction

The matrix effect is a phenomenon that can complicate accurate quantification of an analyte by GC in complex matrices, e.g. in blood and plasma or plant materials [1–3]. It is caused by low-volatile matrix components condensing on the active sites of GC injection liner and/or GC column and preventing the irreversible adsorption and/or degradation of analytes due to their interaction with those sites. As a consequence, the signal magnitude of the analyte being determined is greater than that obtained for analyte samples free of substances causing the matrix effect. Since the quantity of matrix components in a given matrix type varies (e.g. blood from different individuals, plant material from different plantations), the signal magnitude change of the analyzed substance resulting from the matrix effect is different and, in consequence, a reliable quantitative estimation of analytes is problematic. A few ways to compensate for the matrix effect were described in the literature [3–8]. They are either labor-intensive [4], or require the usage of previously modified GC equipment [5], or cause quick column deterioration, or are of limited effectiveness [3,4,8].

Oleamide (OLA) [IUPAC: (*Z*)-Octadec-9-enamide] is one of the compounds in blood and plasma samples found by us to cause the matrix effect [9]. The compound, plying the role of a lubricant in the production of polypropylene vessels and disposable syringes, very easily migrates to and contaminates blood and plasma samples during their transport, storage and/or other activities performed using PP vessels. In the course of our research on the determination of xenobiotics in blood and plasma by GC method, it was observed that OLA caused the matrix effect significantly increasing the signal of the analyzed compound [9,10]. Further research showed that OLA-induced matrix effect did not cause permanent modification of the chromatographic system (the retention data of analytes in subsequent GC separations were the same); therefore it can be referred to as the transient matrix effect [9]. In view of the above, the idea appeared of using OLA-induced transient matrix effect to increase the sensitivity of the analysis. This idea seems to be particularly important in biomedical analyses, when not only the drug, but also its metabolites are determined in the biological material.

One of the most frequently detected substances in the blood of people suspected of using psychoactive substances is Δ9-tetrahydrocannabinol (THC) [11–13]. The paper discusses the effect of OLA addition on the increase in the signal size of THC and its major metabolites, 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) [13–15], and shows how to use this amide to increase the sensitivity of GC analysis of these compounds in derivatized blood and plasma samples. The analysis of THC

* Corresponding author.
E-mail address: m_dybowski@interia.pl (M.P. Dybowski).

https://doi.org/10.1016/j.jpba.2022.114800
Received 1 February 2022; Received in revised form 6 April 2022; Accepted 21 April 2022
Available online 26 April 2022

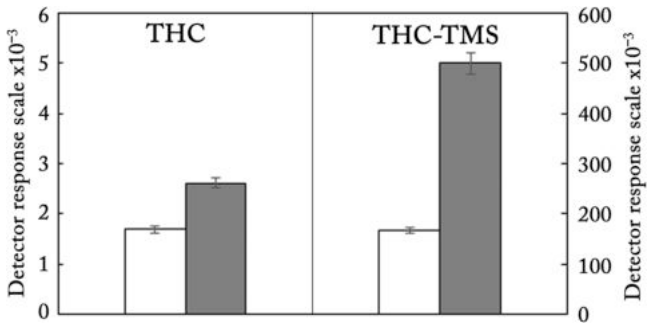


Fig. 1. The exemplary difference in GC–MS/MS signal magnitude of THC and THC-TMS observed after the injection of their solutions in acetonitrile without and with OLA addition - white and dark gray bars, respectively.

and its metabolites in blood and plasma samples generally requires the use of an appropriate sample preparation method. For this purpose, protein precipitation method, liquid-liquid extraction (LLE), solid phase extraction (SPE) and QuEChERS are most often used [13,16–20]. In this experiments QuEChERS was applied.

2. Materials and methods

2.1. Materials

Acetonitrile, n-hexane, ethyl acetate (all LC–MS grade), anhydrous magnesium sulfate (99.5% powder; $MgSO_4$), sodium chloride, iron (III) chloride, zinc (II) chloride, copper (II) chloride, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from Merck (Darmstadt, Germany). The Sepra C18-E sorbent (50 μm, 65 Å) used in the QuEChERS process was obtained from Phenomenex (Torrance, CA, USA). OLA and the standards (certified reference materials) of THC (1.0 mg mL-1 in methanol T-005 Cerilliant), THC-d3 (1.0 mg mL^{-1} in methanol T-011 Cerilliant), 11- THC-OH (1.0 mg mL^{-1} in methanol H-027 Cerilliant), THC-OH-d3 (100.0 μg mL^{-1} in methanol H-041 Cerilliant), THC-COOH (1.0 mg mL^{-1} in methanol T-010 Cerilliant) and THC-COOH-d3 (100.0 μg mL^{-1} in methanol T-004 Cerilliant) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Types of examined samples

In order to show the effect of OLA addition on the increase in the signal size of THC and its major metabolites, THC-OH and THC-COOH, the following test solutions and samples were prepared:

- THC, THC-OH and THC-COOH solutions in acetonitryl (concentration of each component equal 10.0 ng mL-1);
- THC solutions (10.0 ng mL-1) in methanol and methanol containing Fe (III), Zn (II) and Cu (II) ions (1.0 and 2.0 mg mL-1 of each ion);
- THC solution (10.0 μg mL-1) in methanol and methanol containing Fe (III) ions (2.0 mg mL-1);
- blood samples from voluntaries, which were spiked with THC, THC-OH and THC-COOH (concentration of each component equal 10.0 ng mL-1);
- blood samples taken from persons suspected of DUID (Driving Under the Influence of Drug) with positive cannabinoid test result.

All blood samples were collected by a qualified person from voluntaries and persons suspected of DUID with positive cannabinoid test results on a Dräger DrugTest 5000 tester (Dräger, Lübeck, Germany). The blood samples (2 ×5.0 mL) were collected using a single closed system from Sarstedt AG (Nümbrecht, Germany) containing an S-Monovette coagulation activator and subsequently mixed thoroughly in order to maintain their homogeneity and stored at − 20 °C (± 2 °C) before undergoing the analytical procedure involving QuEChERS and LLE.

2.3. QuEChERS

To a blood sample (750.0 μL) placed in a glass vial, the internal standards (10.0 μL of THC-d3, THC-OH-d3, THC-COOH-d3 solution, concentration 10.0 μg mL^{-1}) were added. The mixture was hand-shaken for 2 min, whereupon $MgSO_4$ (200.0 mg) and NaCl (50.0 mg) were added. After vortexing for 1 min, acetonitrile (1500.0 μL) was introduced to the tube and the whole suspension was vortexed again and centrifuged at 12,000 rpm for 3 min. Finally, the aliquot (1600.0 μL) was cleaned-up by d-SPE with 20.0 mg of C18, centrifuged at 12,000 rpm for 3 min and subjected to the derivatization procedure by silylation.

2.4. LLE

To a blood sample (1500.0 μL) placed in a glass vial, the internal

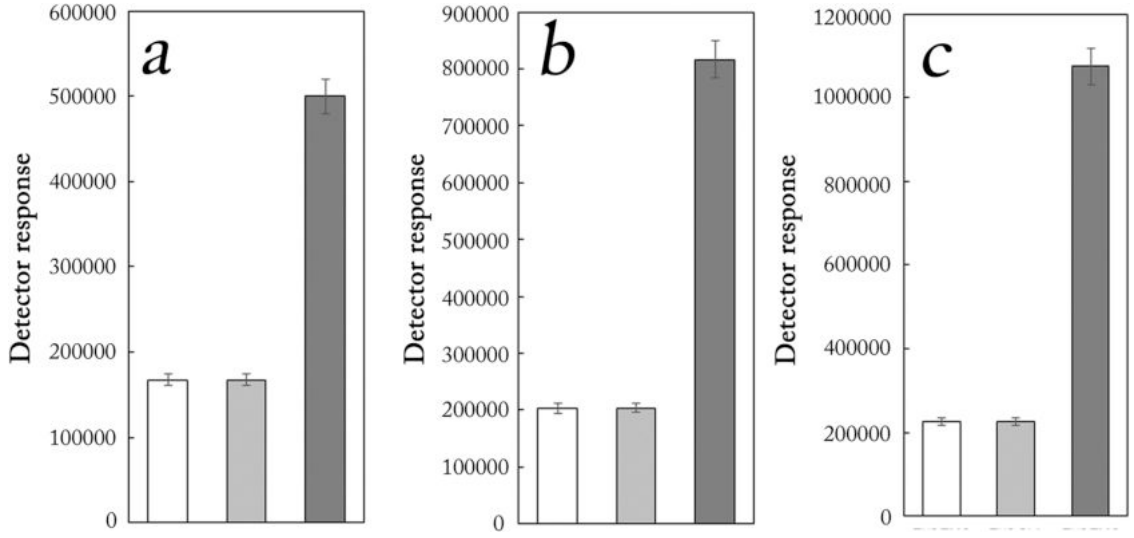


Fig. 2. The exemplary difference in GC–MS/MS signal magnitudes of ***a*** THC-TMS, ***b*** THC-OH-TMS and ***c*** THC-COOH-TMS observed after injection of their solution in acetonitrile without OLA addition (white bars) and with OLA addition before and after derivatization process - light gray and dark gray bars, respectively.

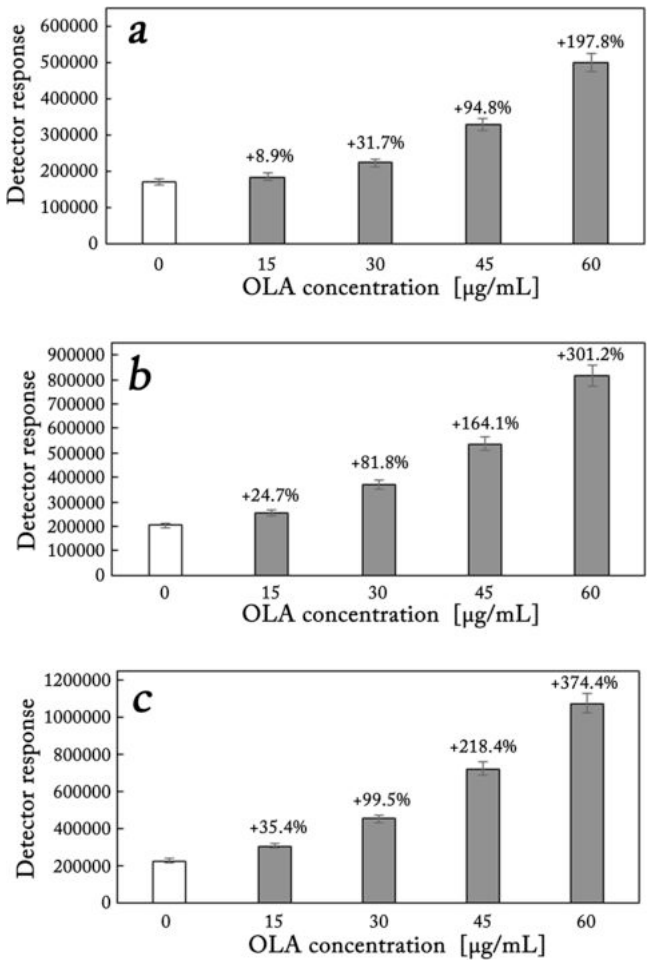


Fig. 3. The effect of OLA concentration increase on the increment of GC–MS/MS signal magnitude for ***a*** THC-TMS, ***b*** THC-OH-TMS and ***c*** THC-COOH-TMS. White bars - OLA-free analyte solution in acetonitrile; gray bars – acetonitrile solutions of the analytes containing different OLA concentration.

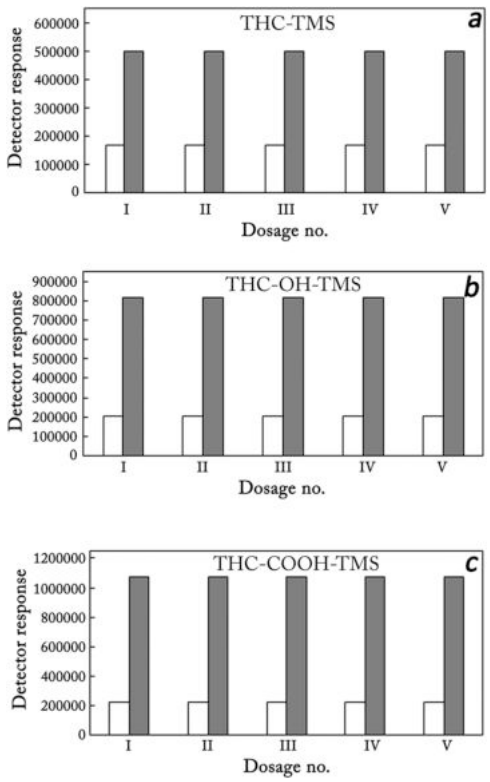


Fig. 4. GC–MS/MS signal magnitudes of ***a*** THC-TMS, ***b*** THC-OH-TMS and ***c*** THC-COOH-TMS obtained in five consecutive injections of their OLA-free samples (white bars) and samples with OLA addition after the termination of derivatization process (dark gray bars). OLA concentration - 60 µg mL^{-1}.

standards (20.0 µL of THC-d3, THC-OH-d3, THC-COOH-d3 solution, concentration 10.0 µg mL^{-1}) were added. Liquid-liquid extraction was carried out with 3000.0 µL n-hexane/ethyl acetate (9/1, v/v) for 2 min on a vortex shaker. After centrifugation (12,500 rpm for 3 min), the organic phase was withdrawn and subjected to the derivatization procedure by silylation.

2.5. Sample derivatization: silylation procedure

1500.0 µL of the examined sample (blood QuEChERS/LLE extract, or acetonitryl solution of THC, THC-OH and THC-COOH or acetonitryl/methanol solution of THC) was dried in nitrogen stream. The dried residue was reconstituted with 1500.0 µL of derivatization mixture consisting of HMDS/TMCS/acetonitryle (1:1:1 v/v) and mixed for 2 min. To terminate the derivatization process, the obtained mixture was heated at 60 °C for 15 min, and - after cooling - methanol (300.0 µL) was added. The resulting trimethylsilyl (TMS) derivatives were centrifuged at 12,000 rpm for 5 min. The individual TMS derivatives were designated in this paper as follows: THC-TMS, THC-OH-TMS and THC-COOH-TMS.

Three types of silylated samples were prepared for GC–MS/MS analysis:

1. samples without OLA
2. samples to which OLA solution was added before silylation, and
3. samples to which OLA solution was added after silylation

To exclude quantitative errors resulting from sample volume increase due to OLA addition, the OLA-free samples were supplemented with an appropriate volume of acetonitrile.

2.6. Thermal treatment of THC solution containing Fe (III) ions

THC solutions (50.0 µg in 5.0 mL) in methanol and methanol containing Fe (III) ions (2.0 mg mL^{-1}) were heated at 100 °C in tightly closed glass vials for 5 h. After cooling, the samples were analyzed using LC–MS.

2.7. GC–MS/MS measurements

Quantitative analyses of THC, THC-TMS, THC-OH-TMS and THC-COOH-TMS.were conducted using the GC–MS/MS TQ8040 (Shimadzu, Kyoto, Japan) equipped with a ZB5-MSi fused-silica capillary column (30.0 m × 0.25 mm i.d., 0.25 µm film thickness; Phenomenex). Helium (grade 5.0) as carrier gas and argon (grade 5.0) as collision gas were used. Column flow was 1.56 mL min^{-1}, and 1.0 µL of the sample was injected by an AOC-20i+s type autosampler (Shimadzu). The injector was working in high pressure mode (250.0 kPa for 1.5 min; column flow at initial temperature was 4.90 mL min^{-1}) at the temperature of 320 °C. The following temperature program was applied:

For 2 min 60 °C, then a linear temperature increase up to 320 °C at the rate of 10 °C min^{-1}, and hold at 320 °C for 15 min

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode using optimal conditions – see [13]. The ion source was operated at 220 °C.

In order to estimate the limit of detection (LOD) and the limit of quantitation (LOQ) of TMS derivatives, their acetonitrile solutions were injected. The LOD and LOQ were considered as signal-to-noise ratios equal to 3 and 10, respectively.

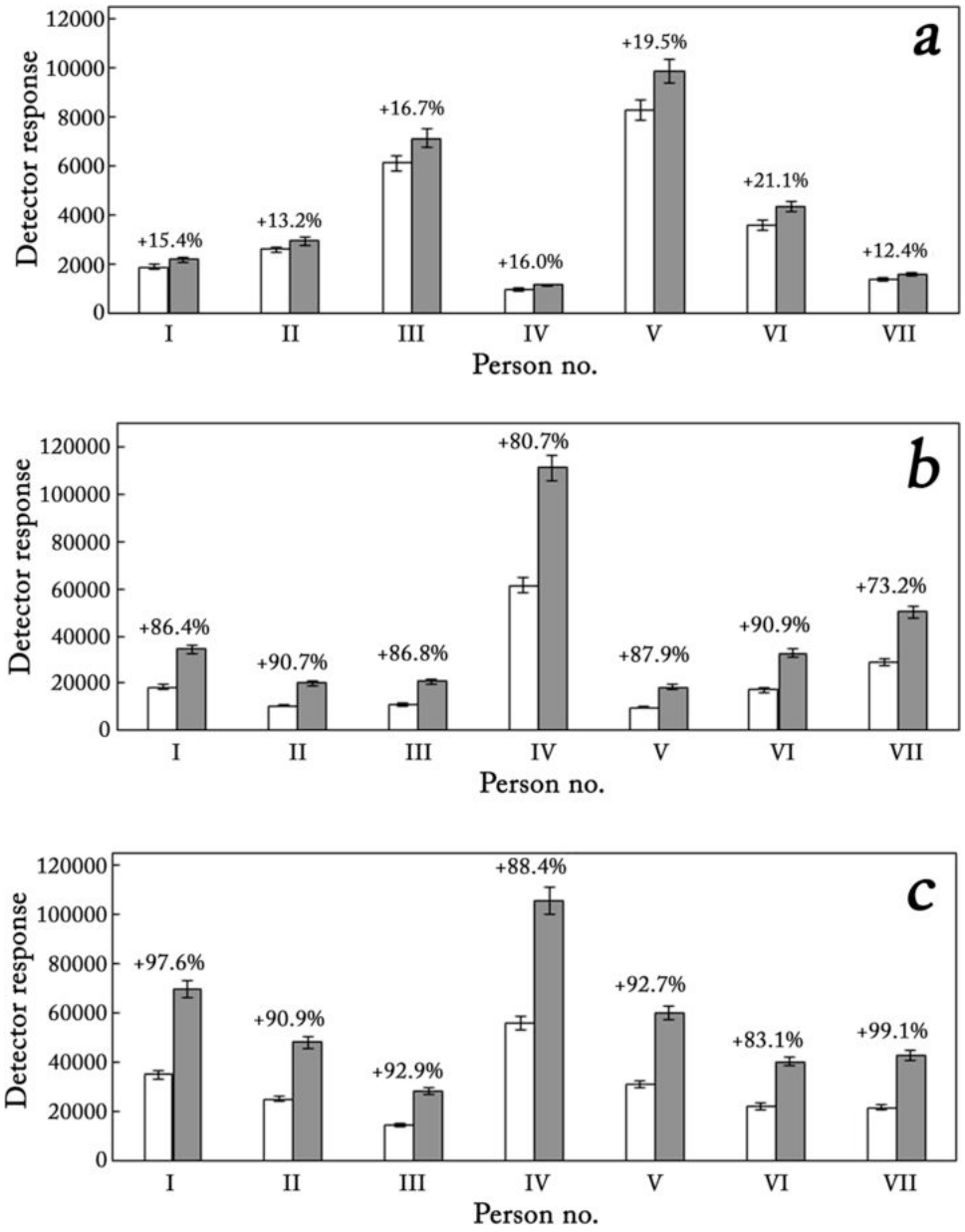


Fig. 5. The effect of the OLA addition to the derivatized supernatants from blood samples of marijuana smoking persons on the change of GC–MS/MS signal magnitude of ***a*** THC-TMS, ***b*** THC-OH-TMS and c THC-COOH-TMS. OLA-free samples - white bars; samples with OLA addition - dark gray bars. OLA concentration - 60 μg mL^{-1}.

2.8. LC/MS measurements

The HRMS analyses of the thermally treated THC solutions were carried out at conditions described in [21] using the LC–MS equipment. composed of an UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) and a linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) was applied for the chromatographic analyses of the examined samples. ESI ionization source operating in the positive polarization mode at needle potential equal to 4.5 kV was employed. Nitrogen (> 99.98%) was used as sheath gas (at 40 arbitrary units), auxiliary gas (at 10 arbitrary units) and sweep gas (at 10 arbitrary units). Capillary temperature equalled 320 °C. The scan cycle used a full-scan event at the resolution of 60,000. Chromatographic separations were performed on Gemini C18 column (4.6 × 100.0 mm, 3.0 μm; Phenomenex, USA). Mobile phase A was 25.0 mM formic acid in water; mobile phase B was 25.0 mM formic acid in acetonitrile. The gradient program was started at 35% B, increasing to 95% for 40 min, followed by isocratic elution (100% B) for 5 min. The total run time was 45 min at the mobile phase flow rate of 0.4 mL min^{-1}.

2.9. Statistical analysis

All results are presented as the mean values of five independent measurements (n = 5) ± SD. The magnitudes of chromatographic signals were compared using analysis of variance (ANOVA). Variance analysis (ANOVA) was applied to compare differences in the signal response of THC, THC-OH, THC-COOH and their silyl derivatives, THC-TMS, THC-OH-TMS and THC-COOH-TMS by examining the influence of:

- OLA presence in examined sample
- OLA concentration;
- metal ion concentration

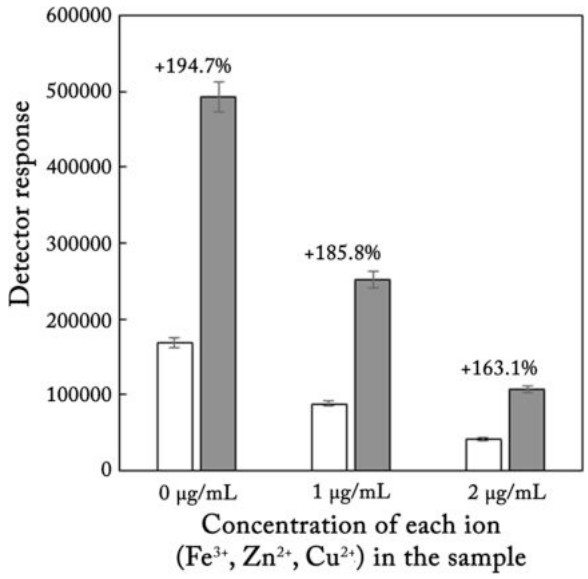


Fig. 6. The effect of OLA addition on GC–MS/MS the signal increment of THC-TMS in the case of its methanolic solutions differing in metal ions concentration.

OLA-free samples - white bars; samples with OLA addition - dark gray bars. OLA concentration - 60 μg mL^{-1}. F_{exp} = 1826,20 > F_{crit} = 5,32, p = 9,91 e-11, F_{exp} = 1552,85 > F_{crit} = 5,32, p = 1,89 e-10 and F_{exp} = 1299,01 > F_{crit} = 5,32, p = 3,85 e-10 for metal ion concentration equal 0, 1.0 and 2.0 μg mL^{-1}, respectively.

Differences in the signal magnitudes were considered significant for $p \leq 0.05$ and $F_{crit} < F_{exp}$.

3. Results and discussion

As noted in Introduction, the presence of OLA in the samples analyzed by GC may cause the matrix effect manifested by the increase of the signal magnitude of the analyzed substances. For the determination of THC in blood and plasma samples, the procedure with or without derivatization of this psychoactive compound can be used [13,22,23]. For THC derivatization, the silylation procedure is most often applied. Therefore, in the first stage of the presented experiments, the impact of OLA addition to samples containing THC and its silyl derivative (THC-TMS) on their signal size was checked. To the derivatized samples OLA was added after the termination of the derivatization process. The results of the experiments are presented in Fig. 1. Not only do they show positive influence of OLA presence in the examined samples on the increment of THC and THC-TMS signal size (F_{exp}>F_{crit}), but also demonstrate extremely high signal size increment for the silyl derivative of THC (F_{exp}=824,38>F_{crit}=5,32; p = 2,34 e-09).

As the addition of OLA to the analyzed sample significantly increases the signal magnitude of THC-TMS, next question concerns the effect of OLA presence on the signal magnitude of the main THC metabolites, THC-OH and THC-COOH, and more precisely of their silyl derivatives THC-OH-TMS and THC-COOH-TMS. Assuming the possibility of the signal increase for derivatives of THC metabolites due to OLA presence, a decision had to be made whether the amide should be added to the examined sample before or after its derivatization, remembering that THC metabolites can be analyzed by GC only after their prior derivatization. The solution is provided by the results presented in Fig. 2. The figure shows the signal magnitudes of silyl derivatives of THC and its two main metabolites THC-OH and THC-COOH obtained for the sample without OLA (white bars), for the sample with OLA added before its derivatization (light gray bars) and for the sample with OLA introduced after its derivatization and termination of the silylation process (dark gray bars). It is seen that the addition of OLA to the sample prior to its derivatization does not increase the signal corresponding to the silyl derivatives of THC and its metabolites. This is understandable as OLA contains an amino group which are derivatized during silylation. The silyl derivative of OLA does not evoke the matrix effect (i.e. it does not act as an analyte protectant). In Fig. 2, the greatest increase in the signal resulting from the addition of OLA to the sample after its derivatization is observed in the case of THC-COOH-TMS. The studies carried out with the use of the GC–MS/MS instruments show that a certain OLA concentration in the injected model sample, for instance 60.0 μg mL^{-1}, causes a 2.98-fold increase in the THC-TMS signal, 4.02-fold increase in the THC-OH-TMS signal and 4.76-fold increase in the THC-COOH–TMS signal in relation to signal of these individual compound in OLA-free samples. Statistical significance of signal increase for individual compounds is as follows: F_{exp}= 2557,60>F_{crit}= 5,32; p = 8,64 e-11 for THC-TMS, F_{exp}= 2557,60>F_{crit}= 5,32; p = 5,59 e-11 for THC-OH-TMS and F_{exp}= 2863,16>F_{crit}= 5,32; p = 1,65 e-11 for THC-COOH-TMS.

The results presented in Figs. 1 and 2 were obtained using samples containing the same concentrations of THC, THC-OH and THC-COOH. The same amount of 60.0 μg/mL OLA was introduced into them in required cases. It should be noted, however, that the increment of the signal size of silyl derivatives of THC and its metabolites depends on the OLA concentrations in the final sample. This is illustrated in Fig. 3. The greatest increase in the signal magnitude resulting from the increase in OLA concentration is observed for THC-COOH-TMS. Statistical significance of signal increase for individual compounds, due to different OLA concentration, is as follows: F_{exp}= 353,43>F_{crit}= 2,87; p = 3,03 e-18 for THC-TMS, F_{exp}= 753,89>F_{crit}= 2,87; p = 1,69 e-21 for THC-OH-TMS and F_{exp}= 945,33>F_{crit}= 2,87; p = 1,78 e-22 for THC-COOH-TMS.

In [9], the matrix effect evoked by OLA in relation to many non-silanized xenobiotics was recognized as the “transient matrix effect”. The diagram in Fig. 4 presenting the signal magnitudes of THC-TMS, THC-OH-TMS and THC-COOH-TMS obtained in five subsequent injections of their solutions of the same concentration in acetonitrile containing OLA (gray bars) and free from OLA (white bars), injected alternately, show that the matrix effect evoked by OLA is also “transient” in relation to the silyl derivatives of THC and its metabolites. In both types of solutions, the signal magnitudes of individual compounds (and their retention) are constant and repeatable.

The effect of OLA addition on GC–MS/MS signal magnitude of THC-TMS, THC-OH-TMS and THC-COOH-TMS examined in the blood samples of people suspected of smoking marijuana is seen in Fig. 5. The data demonstrate that OLA addition to the resultant blood samples causes a visible increase of the signal magnitude of the examined silyl derivatives (the greatest for THC-COOH-TMS); however, the observed signal increments for individual compounds are not as great as for their solutions in acetonitryle. The reason is that blood and plasma samples contain many Lewis acids [e.g. metal ions like Fe (III), Al(III), Zn(II), Cu(II) etc.], which, in GC injection conditions, catalyze the polymerization of some part of silyl derivatives of THC and its metabolites, and, in consequence, lower the increment of their signal resulting from the OLA-induced matrix effect. Statistical significance of signal increase for individual compounds, due to OLA addition, in the presence of Lewis acids in the sample, is as follows: F_{exp}= 172,58>F_{crit}= 4,01; p = 9,57 e-19 for THC-TMS, F_{exp}= 2180,38>F_{crit}= 4,01; p = 1,56 e-46 for THC-OH-TMS and F_{exp}= 3445,39>F_{crit}= 4,01; p = 5,49 e-52 for THC-COOH-TMS. The presence of Lewis acids can also lead to the conversion of Δ8-THC-TMS to Δ9-THC-TMS. This explanation is supported by the data in Figs. 6 and 7. Fig. 6 shows the increases of the GC–MS/MS signal for THC-TMS resulting from OLA addition to its solutions containing/and free of metal [Fe (III), Zn (II) and Cu (II)] ions, while Fig. 7 shows LC–MS chromatograms of THC solutions with and without metal ions. These solutions, before the chromatographic process, were subjected to the heat treatment. Fig. 6 proves that the increase in the THC-TMS signal size resulting from the transient matrix effect is considerably smaller for the sample with metal ions, whereas Fig. 7 indicates the appearance THC dimer in the THC solution containing these Lewis acids. As Lewis acids in

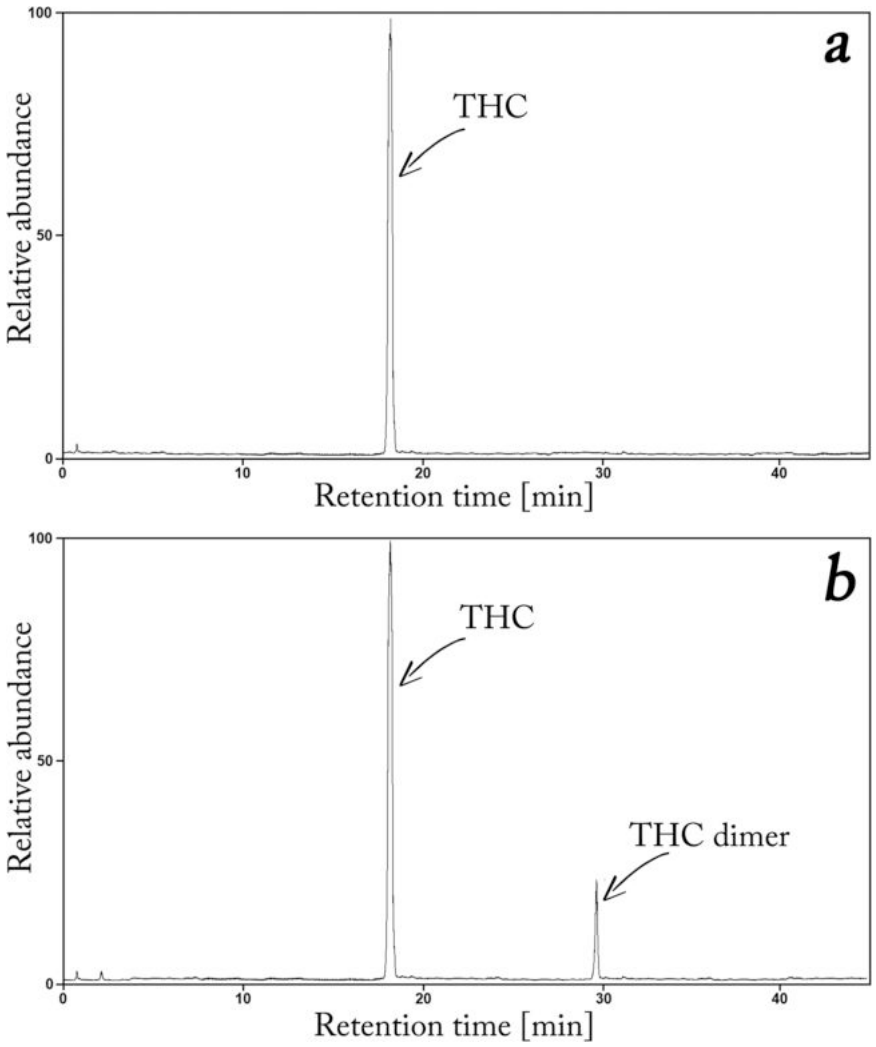


Fig. 7. LC–MS chromatograms (SIM mode) of THC-TMS solutions with (*b*) and without (*a*) the addition of Fe (III) ions, which before the chromatographic process were subjected to heat treatment.

blood and plasma lower the concentration of silyl derivatives of THC and its metabolites in the GC injection system, the OLA-evoked signal increment in the presence of Lewis acids is smaller than in the Lewis acid-free systems. However, the observed signal increment for these compounds, resulting from OLA addition to the analyzed blood sample/s is still considerable – see Fig. 8 which shows the data obtained with application of QuEChERS and LLE. The results of variance analysis for the OLA addition to QuEChERS and LLE supernatants form blood sample on the signal increase of individual compounds are collected in Table 1. As results from the table, the differences in signal magnitudes are statistically significant. Thus, the OLA-induced "transient matrix effect" can be used to increase the sensitivity of the analysis of THC and its metabolites. In this experiment, OLA addition (60.0 μg mL^{-1}) reduced LOD of QuEChERS procedure for estimation of:

- THC by 20.5% (LOD lowered from 0.010 ng mL^{-1} in the case of OLA-free samples to 0.008 ng mL^{-1} after OLA addition);
- THC-OH by 87.6% (LOD lowered from 0.12 ng mL^{-1} in the case of OLA-free samples to 0.015 ng mL^{-1} after OLA addition);
- THC-COOH by 90.1% (LOD lowered from 0.09 ng mL^{-1} in the case of OLA-free samples to 0.009 ng mL^{-1} after OLA addition);

4. Conclusions

The present study shows that the analytical sensitivity of GC–MS used for analyzing THC and its metabolites in blood and plasma samples can be significantly increased by OLA addition to the examined sample to evoke the transient matrix effect. Although some metal ions present in the blood and plasma catalyze the polymerization of THC and its metabolites, (and in consequence they reduce the analyte amount during their evaporation and transfer from the GC injector to the column), the increase in size of the analytes signals resulting from the addition of OLA is significant. Using OLA to increase the analysis sensitivity of THC and its metabolites in blood and plasma samples, the following need be taken into account:

1. OLA should be added at the end of the sample preparation procedure (just before sample injection) – see Fig. 9;
2. the magnitude of the signal increase due to the transient matrix effect depends on the quantitative ratio of the analyte to OLA;
3. the usage of deuterated analytes as internal standards is preferred when internal calibration is applied.

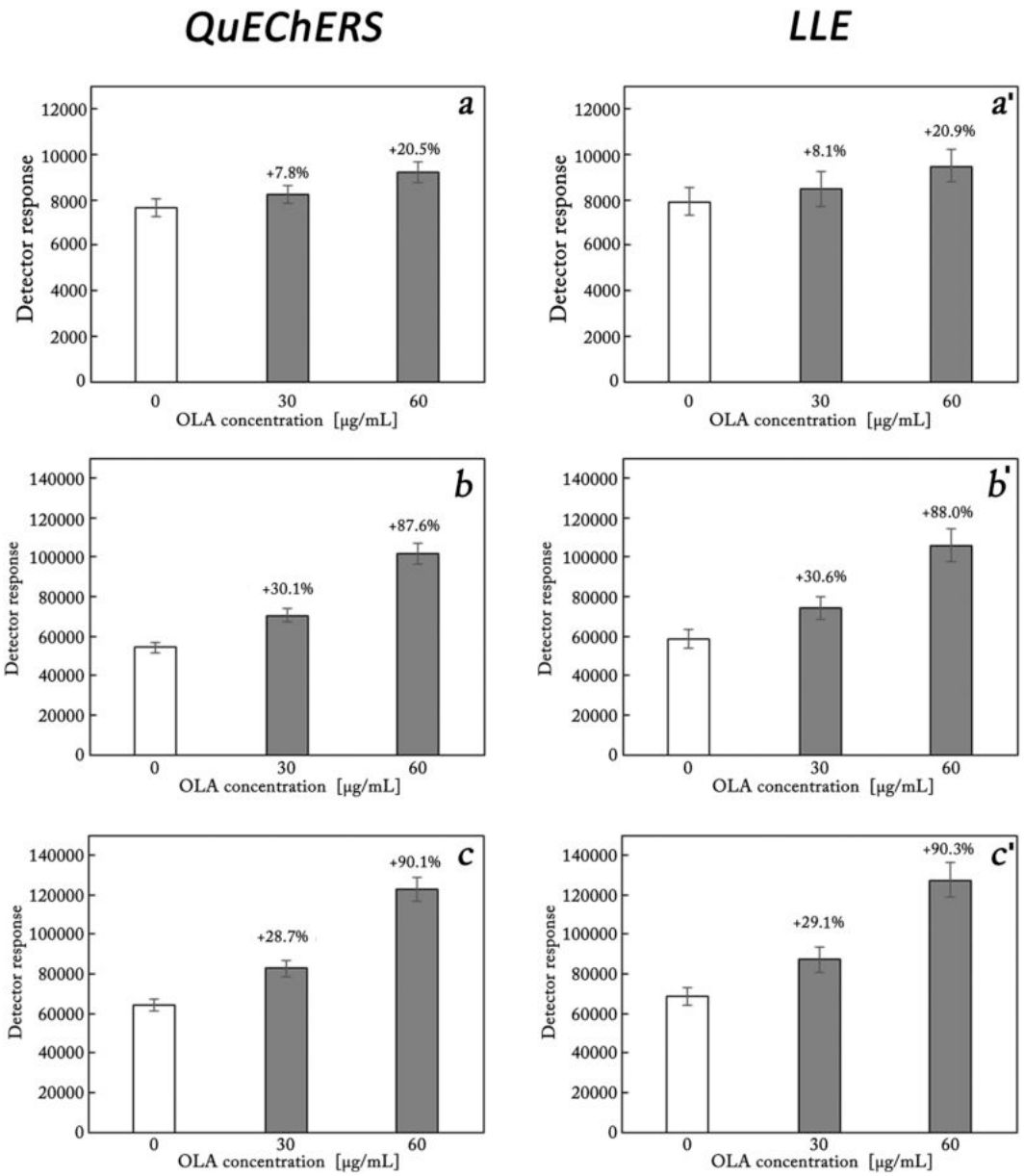


Fig. 8. The exemplary effect of OLA concentration increase in the derivatized QuEChERS and LLE supernatant from blood sample of marijuana smoking person on the change of GC–MS/MS signal magnitude of THC-TMS (***a*** and ***a'***), THC-OH-TMS (***b*** and ***b'***) and THC-COOH-TMS (***c*** and ***c'***).

Table 1
F-values and p-values obtained during variance analysis for the effect of OLA addition to QuEChERS and LLE blood supernatants on THC, THC-OH and THC-COOH signal magnitude increase.

Compound	Sample preparation method	
	QuEChERS	LLE
THC	$F_{exp} = 11{,}99 > F_{crit} = 3{,}89$ p = 1,38 e-03	$F_{exp} = 11{,}18 > F_{crit} = 3{,}89$ p = 1,82 e-03
THC-OH	$F_{exp} = 290{,}38 > F_{crit} = 3{,}89$ p = 6,88 e-11	$F_{exp} = 116{,}09 > F_{crit} = 3{,}89$ p = 1,41 e-08
THC-COOH	$F_{exp} = 346{,}36 > F_{crit} = 3{,}89$ p = 2,44 e-11	$F_{exp} = 150{,}97 > F_{crit} = 3{,}89$ p = 3,12 e-09

The use of the transient matrix effect to increase analysis sensitivity can also be made in other blood sample preparation methods [24], not only in the QuEChERS procedure reported in the present work.

CRediT authorship contribution statement

Andrzej L. Dawidowicz: Conceptualization, Writing – original draft, Investigation. **Michal P. Dybowski**: Writing – original draft, Writing – review & editing, Investigation, Methodology, Data curation, Visualization. **Rafal Typek**: Writing – original draft, Investigation, Methodology, Data curation. **Michal Rombel**: Writing – original draft, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

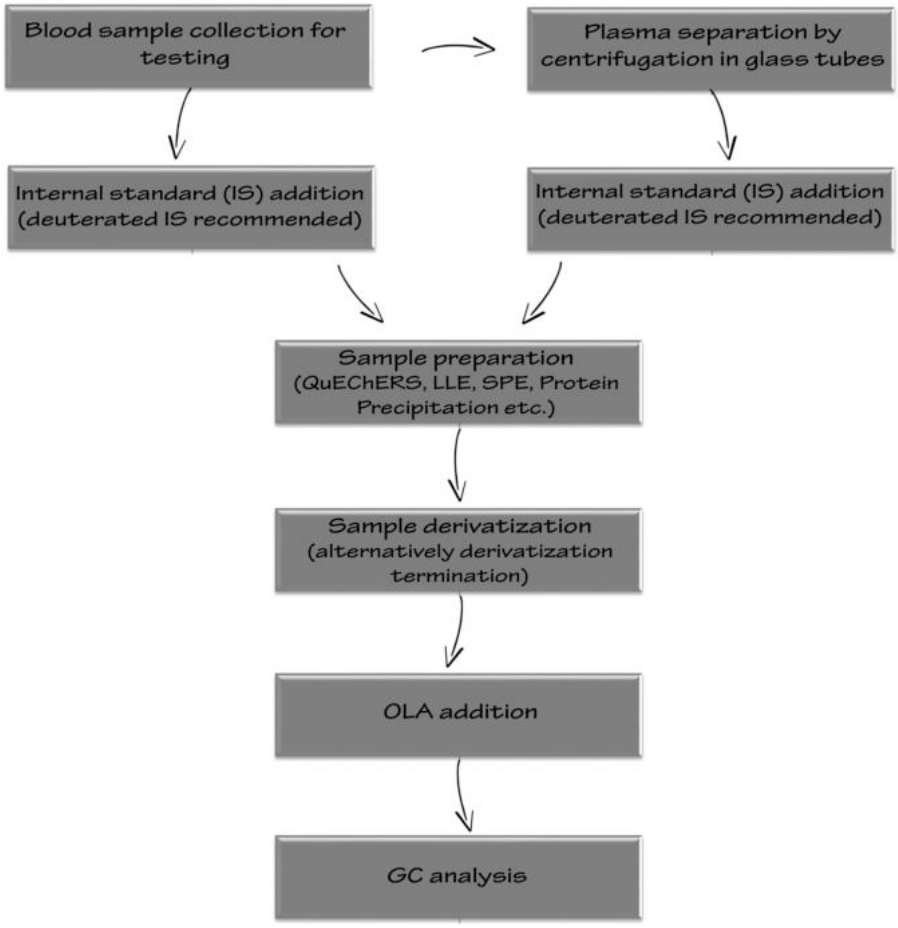


Fig. 9. Diagram of the procedure increasing the sensitivity of THC, THC-OH and THC-COOH analysis in blood and plasma using OLA-induced transient matrix effect.

Acknowledgments

We gratefully acknowledge the use of the facilities of the Ecotech-Complex in Lublin, under project number UDA-POIG.02.01.00-06-212/09-03.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

References

[1] M.M. Rahman, A.M. Abd El-Aty, J.H. Shim, Matrix enhancement effect: a blessing or a curse for gas chromatography?-A review, Anal. Chim. Acta 801 (2013) 14–21, https://doi.org/10.1016/j.aca.2013.09.005.

[2] M. Soliman, M.A. Khorshid, M.M. Abo-Aly, Combination of analyte protectants and sandwich injection to compensate for matrix effect of pesticides residue in GC–MS/MS, Microchem. J. 156 (2020), 104852, https://doi.org/10.1016/J.MICROC.2020.104852.

[3] R. Rodríguez-Ramos, S.J. Lehotay, N. Michlig, B. Socas-Rodríguez, M.Á. Rodríguez-Delgado, Critical review and re-assessment of analyte protectants in gas chromatography, J. Chromatogr. A 1632 (2020), https://doi.org/10.1016/j.chroma.2020.461596.

[4] T. Fujiyoshi, T. Ikami, T. Sato, K. Kikukawa, M. Kobayashi, H. Ito, A. Yamamoto, Evaluation of the matrix effect on gas chromatography - mass spectrometry with carrier gas containing ethylene glycol as an analyte protectant, J. Chromatogr. A 1434 (2016) 136–141, https://doi.org/10.1016/j.chroma.2015.12.085.

[5] K. Sugitate, H. Anazawa, S. Nakamura, N. Orikata, K. Mizukoshi, M. Nakamura, A. Toriba, K. Hayakawa, Decrease in the matrix effect of GC/MS by a gold-plated ion source, J. Pestic. Sci. 37 (2012) 148–155, https://doi.org/10.1584/jpestics.D11-043.

[6] K. Hasegawa, K. Minakata, M. Suzuki, O. Suzuki, The standard addition method and its validation in forensic toxicology, 2021 392, Forensic Toxicol. 39 (2021) 311–333, https://doi.org/10.1007/S11419-021-00585-8.

[7] N. Adachi, H. Kinoshita, M. Nishiguchi, M. Takahashi, H. Ouchi, T. Minami, K. Matsui, T. Yamamura, S. Yoshida, H. Nishio, Determination of acephate and methamidophos in tissues: appearance of matrix effect in gas chromatography–mass spectrometry, 2011 292, Forensic Toxicol. 29 (2011) 159–162, https://doi.org/10.1007/S11419-011-0110-9.

[8] A. Garrido Frenich, J.L. Martínez Vidal, J.L. Fernández Moreno, R. Romero-González, Compensation for matrix effects in gas chromatography-tandem mass spectrometry using a single point standard addition, J. Chromatogr. A 1216 (2009) 4798–4808, https://doi.org/10.1016/j.chroma.2009.04.018.

[9] A.L. Dawidowicz, M.P. Dybowski, M. Rombel, R. Typek, Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: oleamide-induced transient matrix effect, J. Pharm. Biomed. Anal. 204 (2021), 114265, https://doi.org/10.1016/J.JPBA.2021.114265.

[10] G.R. McDonald, A.L. Hudson, S.M.J. Dunn, H. You, G.B. Baker, R.M. Whittal, J. W. Martin, A. Jha, D.E. Edmondson, A. Holt, Bioactive contaminants leach from disposable laboratory plasticware, Science 322 (2008) 917, https://doi.org/10.1126/science.1162395.

[11] E.G. de Campos, B.R.B. da Costa, F.S. dos Santos, F. Monedeiro, M.N.R. Alves, W.J. R. Santos Junior, B.S. De Martinis, Alternative matrices in forensic toxicology: a critical review, Forensic Toxicol. 2021 (2021) 1–18, https://doi.org/10.1007/S11419-021-00596-5.

[12] F. Palazzoli, C. Citti, M. Licata, A. Vilella, L. Manca, M. Zoli, M.A. Vandelli, F. Forni, G. Cannazza, Development of a simple and sensitive liquid chromatography triple quadrupole mass spectrometry (LC–MS/MS) method for the determination of cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC) and its metabolites in rat whole blood after oral administration of a single high dose of CBD, J. Pharm. Biomed. Anal. 150 (2018) 25–32, https://doi.org/10.1016/J.JPBA.2017.11.054.

[13] M.P. Dybowski, A.L. Dawidowicz, Application of the QuEChERS procedure for analysis of Δ9-tetrahydrocannabinol and its metabolites in authentic whole blood samples by GC–MS/MS, Forensic Toxicol. 36 (2018) 415–423, https://doi.org/10.1007/s11419-018-0419-8.

[14] S. Hanisch, A. Paulke, S.W. Toennes, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide exhibits acyl-migration isomers, J. Pharm. Biomed. Anal. 146 (2017) 261–265, https://doi.org/10.1016/J.JPBA.2017.08.044.

[15] B. Fodor, I. Boldizsár, I. Molnár-Perl, Alkylsilyl speciation and direct sample preparation of plant cannabinoids prior to their analysis by GC-MS, Anal. Chim. Acta 1021 (2018) 51–59, https://doi.org/10.1016/j.aca.2018.03.049.

[16] M.J. Roslawski, R.P. Remmel, A. Karanam, I.E. Leppik, S.E. Marino, A.K. Birnbaum, Simultaneous quantification of 13 cannabinoids and metabolites in human plasma by liquid chromatography tandem mass spectrometry in adult epilepsy patients, Ther. Drug Monit. 41 (2019) 357–370, https://doi.org/10.1097/FTD.0000000000000583.

[17] K. Purschke, S. Heinl, O. Lerch, F. Erdmann, F. Veit, Development and validation of an automated liquid-liquid extraction GC/MS method for the determination of THC, 11-OH-THC, and free THC-carboxylic acid (THC-COOH) from blood serum, Anal. Bioanal. Chem. 408 (2016) 4379–4388, https://doi.org/10.1007/S00216-016-9537-5.

[18] J.A. Hubbard, B.E. Smith, P.M. Sobolesky, S. Kim, M.A. Hoffman, J. Stone, M. A. Huestis, D.J. Grelotti, I. Grant, T.D. Marcotte, R.L. Fitzgerald, Validation of a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to detect cannabinoids in whole blood and breath, Clin. Chem. Lab. Med. 58 (2020) 673–681, https://doi.org/10.1515/CCLM-2019-0600.

[19] E.W. Schwilke, E.L. Karschner, R.H. Lowe, A.M. Gordon, J.L. Cadet, R.I. Herning, M.A. Huestis, Intra- and intersubject whole blood/plasma cannabinoid ratios determined by 2-dimensional, electron impact GC-MS with cryofocusing, Clin. Chem. 55 (2009) 1188–1195, https://doi.org/10.1373/CLINCHEM.2008.114405.

[20] N. Korać, D. Vidić, D. Sutlović, Modified QuEChERS extraction and GC-MS analysis of selected cannabinoids from human urine, Glas. Hemičara i Tehnol. Bosne i Hercegovine Bull. Chem. Technol. Bosnia Herzeg. 54 (2020) 37–44, https://doi.org/10.35666/GHTBH.2020.54.07.

[21] M.P. Dybowski, A.L. Dawidowicz, R. Typek, M. Rombel, Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – troubles with reliable CBD quantitation when acidic precipitation agents are applied, Talanta 220 (2020), 121390, https://doi.org/10.1016/j.talanta.2020.121390.

[22] R. Lewis, S. Ward, R. Johnson, D.T. Burns, Distribution of the principal cannabinoids within bars of compressed cannabis resin, Anal. Chim. Acta 538 (2005) 399–405, https://doi.org/10.1016/J.ACA.2005.02.014.

[23] E. Han, Y. Park, E. Kim, S. In, W. Yang, S. Lee, H. Choi, S. Lee, H. Chung, J. myong Song, Simultaneous analysis of Δ9-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol in hair without different sample preparation and derivatization by gas chromatography–tandem mass spectrometry, J. Pharm. Biomed. Anal. 55 (2011) 1096–1103, https://doi.org/10.1016/J.JPBA.2011.03.020.

[24] A.L. Dawidowicz, A. Fijalkowska, Determination of propofol in blood by HPLC. Comparison of the extraction and precipitation methods, J. Chromatogr. Sci. 33 (1995) 377–382, https://doi.org/10.1093/CHROMSCI/33.7.377.

Publikacja D4

P. Hołowiński, R. Typek, A.L. Dawidowicz, **M. Rombel**, M.P. Dybowski

Formation of trifluoroacetic artefacts in gas chromatograph injector during Cannabidiol analysis

J. Chromatogr. A. 1671 (2022) 463020

https://doi.org/10.1016/J.CHROMA.2022.463020

IF 2-letni: 4,1; MEiN: 100 pkt

Journal of Chromatography A 1671 (2022) 463020

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Formation of trifluoroacetic artefacts in gas chromatograph injector during Cannabidiol analysis

Piotr Holowinski, Rafal Typek, Andrzej L. Dawidowicz*, Michal Rombel, Michal P. Dybowski

Department of Chromatography, Faculty of Chemistry, Institute of Chemical Sciences, Maria Curie Sklodowska University in Lublin, Lublin 20-031, Poland

ARTICLE INFO

Article history:
Received 26 January 2022
Revised 30 March 2022
Accepted 31 March 2022
Available online 4 April 2022

Keywords:
CBD transformation
Trifluoroacetic esters of THC
Plasma precipitation
GC–MS

ABSTRACT

The knowledge of compounds stability in the process of sample preparation for analysis and during analysis itself helps assess the accuracy and precision of estimating their concentration in tested samples. The present paper shows that a significant amount of CBD present in the blood/plasma sample analyzed by means of GC transforms in the hot GC injector not only to 9α-hydroxyhexahydrocannabinol, 8-hydroxy-iso-hexahydrocannabinol, delta-9-tetrahydrocannabinol, Δ8-tetrahydrocannabinol, and cannabinol but also to the trifluoroacetic esters of Δ9-THC and Δ8-THC, when trifuoroacetic acid is used as protein precipitation agent. The amount of those newly revealed CBD transformation products depends on the GC injector temperature and on the extrahent type when extracts of the supernatants centrifuged from human plasma samples are analyzed after their preliminary protein precipitation by trifuoroacetic acid.

Although trifuoroacetic acid as a protein precipitating agent has many disadvantages, it is quite often used for this purpose due to its very high protein precipitation efficiency. The results presented in the study demonstrate why the use of trifuoroacetic acid for plasma samples deproteinization should be avoided when CBD is determined by GC.

1. Introduction

Cannabidiol (CBD), 2-[(1R,6R)−3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]−5-pentylbenzene-1,3-diol is one of the ingredients of marijuana and hemp plants most frequently discussed in the literature [1–4]. This compound, devoid of psychotropic effect, unlike delta-9-tetrahydrocannabinol (Δ9-THC), has recently been extensively researched due to its biological properties suggesting therapeutic benefits. Although the potential activity of CBD is especially emphasized in the treatment of epileptic syndromes [5,6], the compound is also espoused as supporting the treatment of immune dysfunctions [7], diabetes [8,9], addictive behavior [10] and cancer [11,12]. Preclinical studies have also demonstrated its anti-nausea and analgesic effects [13,14]. High interest in CBD resulting from research and clinical observations, as well as a marked increase in the use of dietary supplements containing CBD in self-healing therapies [15], require the development of reliable and sensitive analytical procedures for its quantitative determination in blood/plasma samples.

Several analytical procedures have been developed for measuring CBD and other cannabinoids together with their metabolites in blood/plasma samples applying GC [16,17] and HPLC [18–20] equipment. Most of them involve classical or automated liquid-liquid extraction (LLE) or solid-phase extraction (SPE) as sample preparation method. QuEChERS is also recommended as sample clean-up technique for cannabinoids analysis [21,22]. As CBD is a highly hydrophobic molecule and strongly binds with plasma proteins [23,24], some reports recommend using for this purpose the analytical procedures involving protein precipitation [19,25]. As protein precipitation is a very simple and quick sample preparation method not requiring special equipment, it is willingly used in many analytical procedures of xenobiotics estimation, including cannabinoids, in blood/plasma samples [19,24].

Several protein precipitation agents are used in the analytical procedures of drug assay in blood/plasma, most often organic solvents (e.g. acetonitrile, methanol, acetone), acidic agents (e.g. H_2SO_4, CF_3COOH, $ZnSO_4$, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, CCl_3COOH, $HClO_4$, $CHCl_3$) and neutral salts ($MgSO_4$, Na_2SO_4, NaCl, $MgCl_2$, CH_3COONH_4, $HCOONH_4$) [24,26–32]. As demonstrated in [33,34], if an acidic precipitation agent is used, a significant amount of CBD in a sample analyzed by GC transforms in

* Corresponding author.
E-mail address: dawid@poczta.umcs.lublin.pl (A.L. Dawidowicz).

https://doi.org/10.1016/j.chroma.2022.463020

the hot GC injector to 9α-hydroxyhexahydrocannabinol (9α-OH-HHC), 8-hydroxy-iso-hexahydrocannabinol (8-OH-iso-HHC), delta-9-tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC), and cannabinol (CBN).

One of the longest-used protein-precipitating reagents is CF_3COOH (TFA). The major disadvantage of TFA, and other protein precipitating agents, is sometimes insufficient sample clean-up, which may hinder the chromatographic separation and quantification of the analytes. Nevertheless, TFA is still used due to its very high protein precipitation efficiency in relation to other agents. According to Andrews and Paterson [35], the anhydride of this acid (trifluoroacetic anhydride - TFAA) is able to react with CBD and Δ9-THC, forming a stable Δ9-THC-TFA ester. If so, it needs to be established whether TFA derivatives with Δ9-THC and eventually with other CBD transformation products (9α-OH-HHC, 8-OH-iso-HHC, Δ8-THC and CBN) are formed when TFA is applied as protein precipitation agent in the sample preparation for CBD analysis in plasma by GC ? The chemical structures of CBD and all its mentioned transformation products suggest the possibility of forming esters with TFA. Hypothetically, as many as 77 mono-, di-, tri- and tetra-TFA esters could be formed (their structures are shown in the supplementary materials). The answer to the above question is not only theoretically interesting but may also be practically valuable for the accuracy of CBD quantification in plasma samples by GC when TFA is used for protein precipitation. An additional argument to answer the above question is that TFA's has much weaker acylation abilities of OH groups in organic compounds than TFAA. Thus, the aim of the study is to find out whether GC allows for accurate quantification of CBD in blood/plasma samples if TFA is used for their deproteinization.

2. Materials and methods

2.1. Reagents and standards

Acetonitrile (ACN) (LC/MS grade), anhydrous magnesium sulfate (99.5% powder; $MgSO_4$) and sodium chloride were purchased from Merck (Warszawa, Poland). The standards (certified reference materials) of Δ9-THC (1.0 mg/mL in methanol - Cerilliant) and CBD (1.0 mg/mL in methanol - Cerilliant), CBD-D_3 (1.0 mg/mL in methanol - Cerilliant), THC-D_3 (1.0 mg/mL in methanol - Cerilliant), trifluoroacetic acid (TFA) (>99%) and trifluoroacetic anhydride (TFAA) were acquired from Sigma-Aldrich (Poznan, Poland). Dichloromethane (DCM), hexane, chloroform ($CHCl_3$) and ethyl acetate (EtOAc), all of analytical grade, were purchased from the Polish Chemical Plant POCh (Gliwice, Poland). DMSO-d_6 was bought from Armar AG (Döttingen, Switzerland). CBD crystal (>99%) was a gift from CannLAB (Kraków, Poland). Deionized water was purified by the Milli-Q system (Millipore Sigma, Bedford, MA, USA).

2.2. Preparation of CBD-TFA and THC-TFA esters

Preliminary studies have indicated that not only 9α-OH-HHC, 8-OH-iso-HHC, Δ9-THC, Δ8-THC and CBN [34] but also trifluoroacetic esters of CBD or THC are formed in the GC injector. Therefore, in separate experiments, CBD and THC were esterified using trifluoroacetic acid anhydride. The obtained TFA esters were tested by NMR and GC–MS. The GC-MS data were useful in identifying the compounds formed in the GC injector.

The procedure of syntethizing trifluoroacetic ester of CBD or THC was as follows. The trifluoroacyl derivatives of CBD and THC were prepared heating a mixtures composed of TFAA/DCM (20:80) (500 µL) and CBD or THC solution in DCM (20 mg/mL) (500 µL) at 65 °C for 60 min. The molar ratio of TFAA to CBD or THC was 0.72: 0.03. The liquid phase from individual reaction mixtures was subsequently evaporated under nitrogen stream. The dry residue was dissolved in an appropriate solvent (DMSO-d6 or acetonitrile) and subjected to further measurements. THC for the synthesis was obtained from CBD following the procedure described in [36].

2.3. Plasma protein precipitation procedure

TFA, the precipitation agent (25 µL), was added to 475 µL of human plasma containing CBD (10 µg/mL). The samples were vortex mixed, incubated for 1 h and centrifuged for 5 min at 18,600 x g. The separated supernatants were analyzed by GC-MS and LC-MS. Protein precipitation in the experiments was performed with the use of excess amount of TFA, as provided for the precipitation procedures.

2.4. Extraction of supernatants from plasma

The extraction process of supernatants from blood/plasma samples is used in some cases as an additional sample purification step and may involve different solvents. In order to determine the effect of the solvent type on CBD transformation in the GC injector, test samples were prepared in the following way. To supernatants centrifuged from human plasma samples spiked with CBD (10 µg/mL), after their preliminary protein precipitation by TFA (500 µL), ACN or DCM or EtOAc or hexane (500 µL) was added and vortex mixed (2 min). Next, the mixtures were centrifuged for 5 min at 18,600 x g and the separated organic phases were subjected to GC-MS analysis. When ACN was used as extracting solvent, NaCl/$MgSO_4$ (1/4–250 mg) was added to the mixture before its vortexing to reduce the miscibility of ACN and H_2O, and to allow phase separation of these liquids. It is worth mentioning that anhydrous $MgSO_4$ is a strong water binding agent, the heat emitted during water binding reaction favors the extraction of analytes from the sample matrix – see QuEChERS technique [37]

2.5. GC–MS measurements

Qualitative analyses of CBD, CBD-TFA esters and TFA esters of CBD transformation products were conducted using a gas chromatograph hyphenated with a triple quadruple tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan). GC–MS conditions were as follows: capillary column - Zebron ZB5-MSi (30 m x 0.25 mm i.d., 0.25 µm film thickness; Phenomenex, Torrance, CA, USA); carrier gas: helium (grade 5.0); flow rate: 1.0 ml/min; splitless/split injection mode (sampling time: 1.00 min); glass wool packed liner (AG0–4683, Phenomenex) – 3.4 mm ID x 95 mm L x 5 mm OD; injector temperature: 280; 295 and 310 °C; injection volume: 1 µL; temperature program - initial temperature 60 °C held for 3 min and then the temperature increase to 310 °C at a rate of 12 °C/min. The final temperature was held for 15 min. Mass spectrometer parameters: normalized electron energy of 70 eV; ion source temperature: 225 °C.

The full SCAN mode with range 40–750 m/z and SIM mode for *m/z*=410, 428, 506, 524, 542, 620, 638 and 716 were used. These *m/z* values correspond with molecular ions of individual esters from Fig. 1S.

In order to analyse extracts from the supernatants centrifuged from human plasma samples spiked with CBD after their preliminary protein precipitation by TFA, multiple reaction monitoring (MRM) mode was used. GC-MS/MS analysis was performed using characteristic MRM transitions at optimal collision energies (CE) for Δ8-THC-TFA and Δ9-THC-TFA. Three MRM transitions (*m/z* => *m/z*) of the highest intensity were selected for further experiments:410 => 327 (CE = 20 eV), 410 => 367 (CE = 15 eV) and 395 => 367 (CE = 12 eV) for Δ8-THC-TFA and for Δ9-THC-TFA.

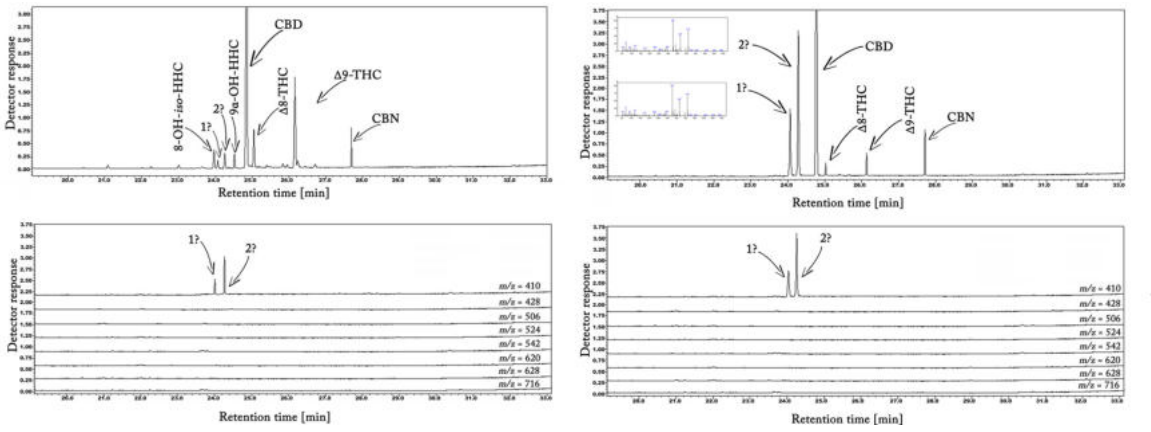


Fig. 1. GC-MS chromatograms (A, B in Scan and C, D in SIM mode) of the supernatants centrifuged from human plasma samples spiked with CBD (10 µg/mL) after their preliminary protein precipitation by TFA (A,C), and CBD solution (10 µg/mL) in acetonitrile containing TFA (B,D).

2.6. LC-MS measurements

An LC-MS system composed of an UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) and a linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) was applied for the chromatographic analyses of the examined supernatants. ESI source operating in the positive ionization mode at needle potential of 4.5 kV was employed. Nitrogen (>99.98%) was used as sheath gas (at 40 arbitrary units), auxiliary gas (at 10 arbitrary units) and sweep gas (at 10 arbitrary units). Capillary temperature was maintained at 320 °C. The resolution of MS was 60,000. Separations were performed on a Gemini C18 column (4.6 × 100 mm, 3 µm; Phenomenex) using gradient elution. Mobile phase A was 25 mM formic acid in water; mobile phase B was 25 mM formic acid in acetonitrile. The gradient program started at 30% B increasing to 90% for 40 min, and ended with isocratic elution (90% B) for 20 min. The total run time was 60 min at the mobile phase flow rate 0.4 mL/min.

Analysing the examined samples, the SIM function was used to better visualize the chromatographic separation and to remove the signals from insignificant mixture components like the plasma components and the precipitation agent. Pseudo molecular ions $[M+H]^+$ of m/z=411, 429, 507, 525, 543, 621, 639 and 717, corresponding with esters presented in Fig. 1S, were monitored.

2.7. NMR measurements

NMR measurements were performed at 298 K using a Ascend 600 MHz instrument (Bruker, Bremen, Germany). The DMSO-d_6 solutions of the obtained samples were examined using ^{1}H, ^{13}C, DEPT 135, ^{19}F, ^{1}H–^{1}H COSY, multiplicity-edited ^{1}H–^{13}C HSQC and selective 1D TOCSY techniques.

3. Results and discussion

To find out whether TFA ester is formed when using protein precipitation process as sample preparation procedure in estimating CBD presence in human plasma, (1) the supernatants centrifuged from its samples spiked with CBD (10 µg/mL) after their preliminary protein precipitation by TFA, and (2) CBD solutions (10 µg/mL) in acetonitrile containing TFA were examined using GC-MS working in SCAN and SIM modes. In order to facilitate the identification of CBD transformation products in the GC injector, plasma samples containing a high concentration of the analyte were used deliberately. In the course of chromatographic separation in SIM mode, *m/z*=410, 428, 506, 524, 542, 620, 638 and 716 ions corresponding to the molecular weight of individual esters (see Fig. 1S) were searched for. The results of the GC-MS analyses are shown in Fig. 1A–D. The obtained chromatograms indicate only the presence of *m/z*=410 compounds, which can be attributed with great probability to the mono-TFA esters of CBD and/or THC. In order to confirm this preliminary assumption, appropriate amounts of CBD and THC were esterified using TFAA in separate experiments (see 2.2 in Experimental). The structures and chromatographic data of the obtained TFA esters were determined using NMR and GC-MS. The results of the NMR measurements are presented in Figs. 2A–C and 2S–10S.

^{19}F spectrum (Fig. 3S) acquired for the reaction products of CBD with TFAA shows a strong signal in −74.00 ppm and multiple minor signals at similar positions, correlating well with the typical chemical shift range for trifluoroacyl groups [38], and thus confirming its presence in the obtained derivatives. The region of aromatic protons from ^{1}H spectrum (Figs. 2A and 2S) reveals the presence of two doublets in positions 6.76 and 6.63 ppm that can be assigned as correlating aromatic protons (see COSY and HSQC spectra – Figs. 6S and 7S in supplementary materials). These signals are significantly shifted toward higher chemical shifts comparing to the analogous resonances of CBD or Δ9-THC [39], which - together with the observed strong fluorine resonance – indicates the presence of a trifluoroacyl moiety in place of the OH phenolic group. The lack of significant signals in the range 9–10 ppm, in which protons of phenolic OH are observed for CBD and THC (see CBD 1H spectrum in Fig. 8S in supplementary material), additionally indicates the presence of the trifluoroacyl group attached to the aromatic ring. Moreover, the signal in position 5.75 ppm can be identified as resonance from the alkene proton of the cycloalkene ring of the CBD derivative. Using COSY and HSQC correlations observed for this ring and comparing 1D selective TOCSY spectrum obtained for 5.75 ppm resonance with similar 1D selective TOCSY spectrum for the analogues proton of Δ9-THC (see Fig. 9S in supplementary material), it can be seen that the considered trifluoroacyl ester contains a cycloalkenyl ring identical to that of Δ9-THC. All the above observations allow us to identify the main reaction product as a trifluoroacyl ester of Δ9-THC (see structure no. 62 in Fig. 1S). The examined sample also contains trifluoroacyl esters of other THC isomers and non-modified THC isomers, as can be inferred from the presence of multiple small doublets in the ranges 6.8–6.6 ppm and 6.2–6.0 ppm (see Fig. 2A,B), respectively. The second trifluoroacyl ester of THC in the examined sample is Δ8-THC-TFA (see structure no. 61 in Fig. 1S). It results from the presence of resonances in positions at 5.42 and 5.39 ppm (see Fig. 2C), which can be attributed to alkene protons of the cycloalkene rings of Δ8-

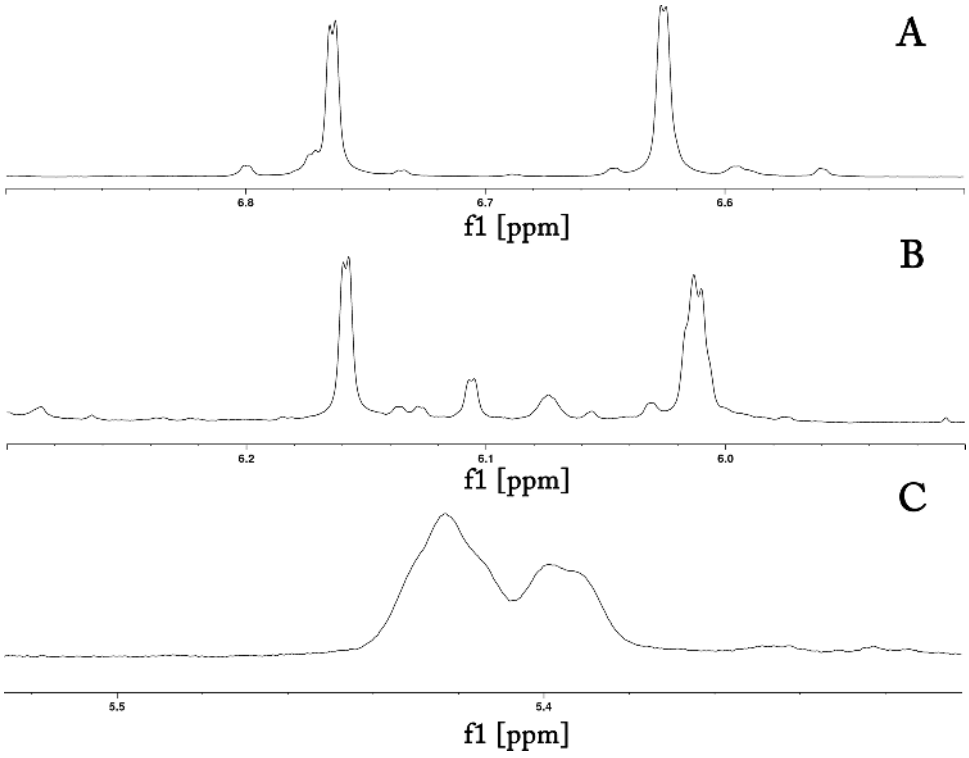


Fig. 2. 1H spectrum of CBD-TFA reaction products (DMSO6) in the ranges of 6.9–6.5 ppm (A), 6.3–5.9 ppm (B) and 5.5–5.3 ppm (C).

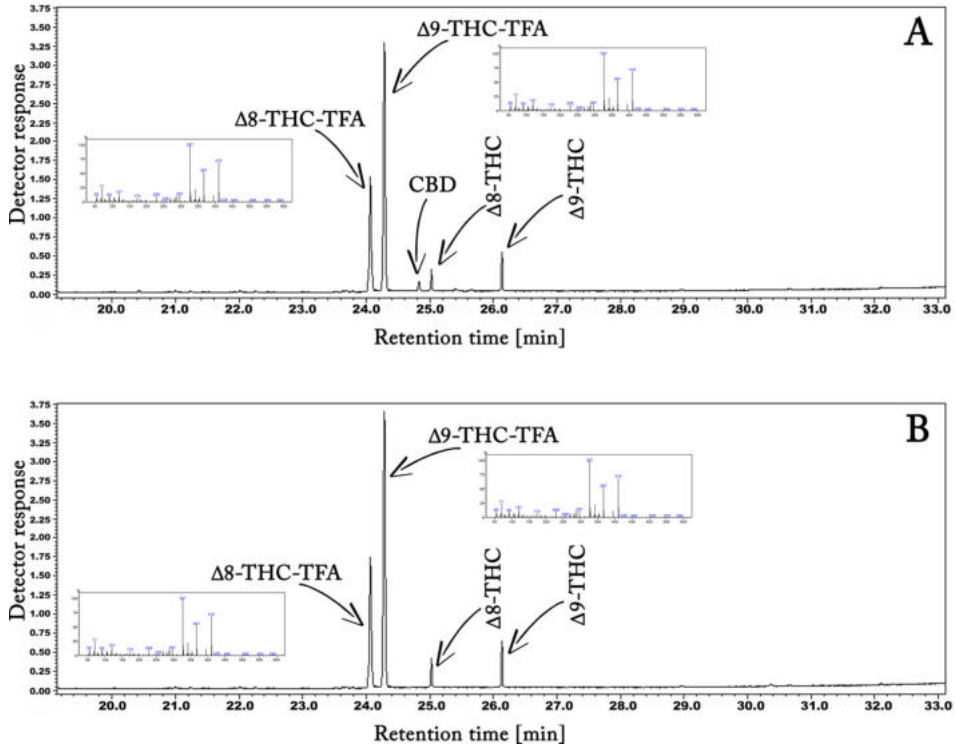


Fig. 3. GC-MS chromatograms (SIM mode) of Δ8-THC-TFA and Δ9-THC-TFA mixtures obtained after esterification of CBD (A) and Δ9-THC (B) by TFAA.

THC and its TFA ester. The observed signals are consistent with the data reported for Δ8-THC in $CDCl_3$ in [39]. Hence, the NMR measurements show that the esterification of CBD by TFAA leads to the formation of two TFA monoesters of THC, Δ9-THC-TFA and Δ8-THC-TFA, of molecular weight equal 410. It should be stressed that the same esters are formed during the esterification of Δ9-THC by TFAA. For confirmation see Fig. 10S in the supplementary materials. Δ9-THC-TFA structure and its NMR data are presented in Fig. 11S and Table 1S, respectively.

The GC-MS chromatograms of ACN solutions of Δ8-THC-TFA and Δ9-THC-TFA mixtures (10 μg/mL) obtained after esterification of CBD and Δ9-THC by TFAA are presented in Fig. 3. They show

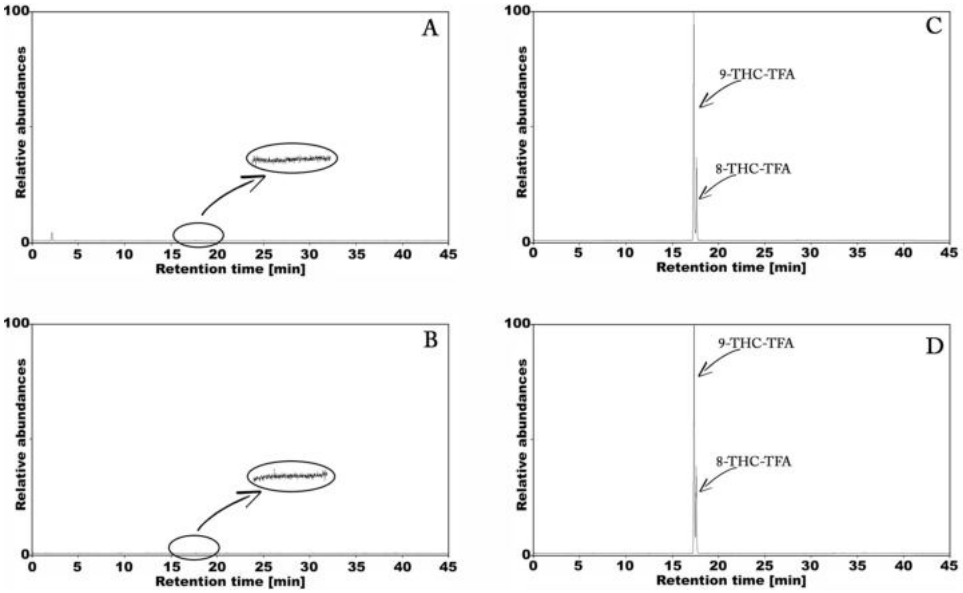


Fig. 4. LC-MS chromatograms (SIM mode in positive polarization) of: - the supernatant centrifuged from human plasma sample spiked with CBD (10 μg/mL) after their preliminary protein precipitation by TFA (A); - CBD solution (10 μg/mL) in acetonitrile containing TFA (B); - acetonitrile solutions of Δ8-THC-TFA and Δ9-THC-TFA mixtures (100 μg/mL) obtained after esterification of CBD by TFAA; - acetonitrile solutions of Δ8-THC-TFA and Δ9-THC-TFA mixtures (100 μg/mL) obtained after esterification of Δ9-THC by TFAA.

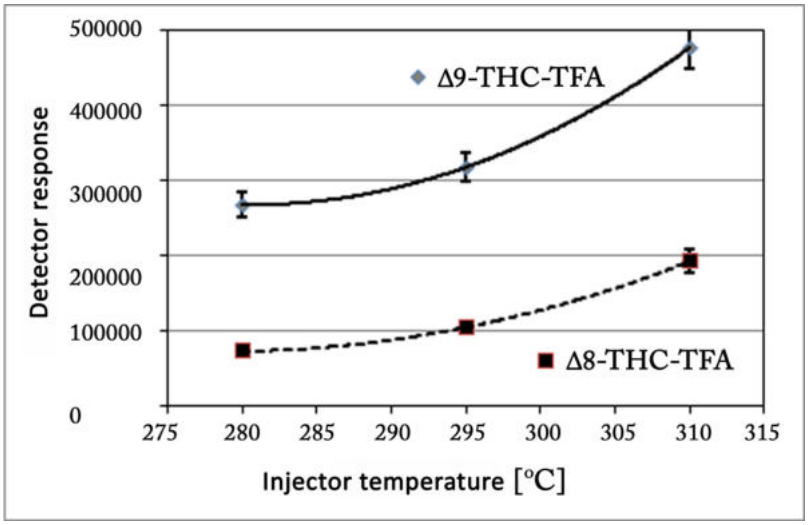


Fig. 5. The influence of GC injector temperature on the GC-MS signal magnitude of Δ9-THC-TFA (solid line with diamonds) and Δ8-THC-TFA (dashed line with squares).

that the retention data and MS spectra of these esters are the same as those registered for the compounds of m/z=410 when analysing supernatant centrifuged from human plasma samples spiked with CBD after their preliminary protein precipitation by TFA and/or CBD solution in ACN containing TFA (see Fig. 1). Hence, if TFA is used as protein precipitation agent, CBD contained in the sample analyzed by GC transforms not only to 9α-OH-HHC, 8-OH-iso-HHC, Δ9-THC, Δ8-THC and CBN but also to Δ9-THC-TFA and Δ8-THC-TFA. In the GC system they elute in the order from Δ8-THC-TFA to Δ9-THC-TFA, which results from the elution order and peak intensities of their precursors, i.e. Δ8-THC and Δ9-THC, respectively.

Another related question is when exactly the esterification process of Δ8-THC and Δ9-THC by TFA occurs: during protein precipitation or in the hot GC injector. Therefore it was decided to test the supernatants centrifuged from human plasma samples spiked with CBD (10 μg/mL) after their preliminary protein precipitation by TFA as well as properly prepared solutions of CBD and THC by LC-MS. Fig. 4 presents LC-MS chromatograms (SIM mode in positive polarization) of:

- the supernatant centrifuged from human plasma spiked with CBD (10 μg/mL) after preliminary protein precipitation by TFA (A),
- CBD solution (10 μg/mL) in acetonitrile containing TFA (B),
- acetonitrile solutions of Δ8-THC-TFA and Δ9-THC-TFA mixtures (100 μg/mL) obtained after esterification of CBD by TFAA (C),
- acetonitrile solutions of Δ8-THC-TFA and Δ9-THC-TFA mixtures (100 μg/mL) obtained after esterification of Δ9-THC by TFAA (D).

In the course of chromatographic separation, ions corresponding to the molecular weights of esters from Fig. 1S were searched for. The absence of Δ9-THC-TFA and Δ8-THC-TFA in the supernatant centrifuged from the human plasma sample spiked with CBD and in CBD solution containing TFA indicates that either these

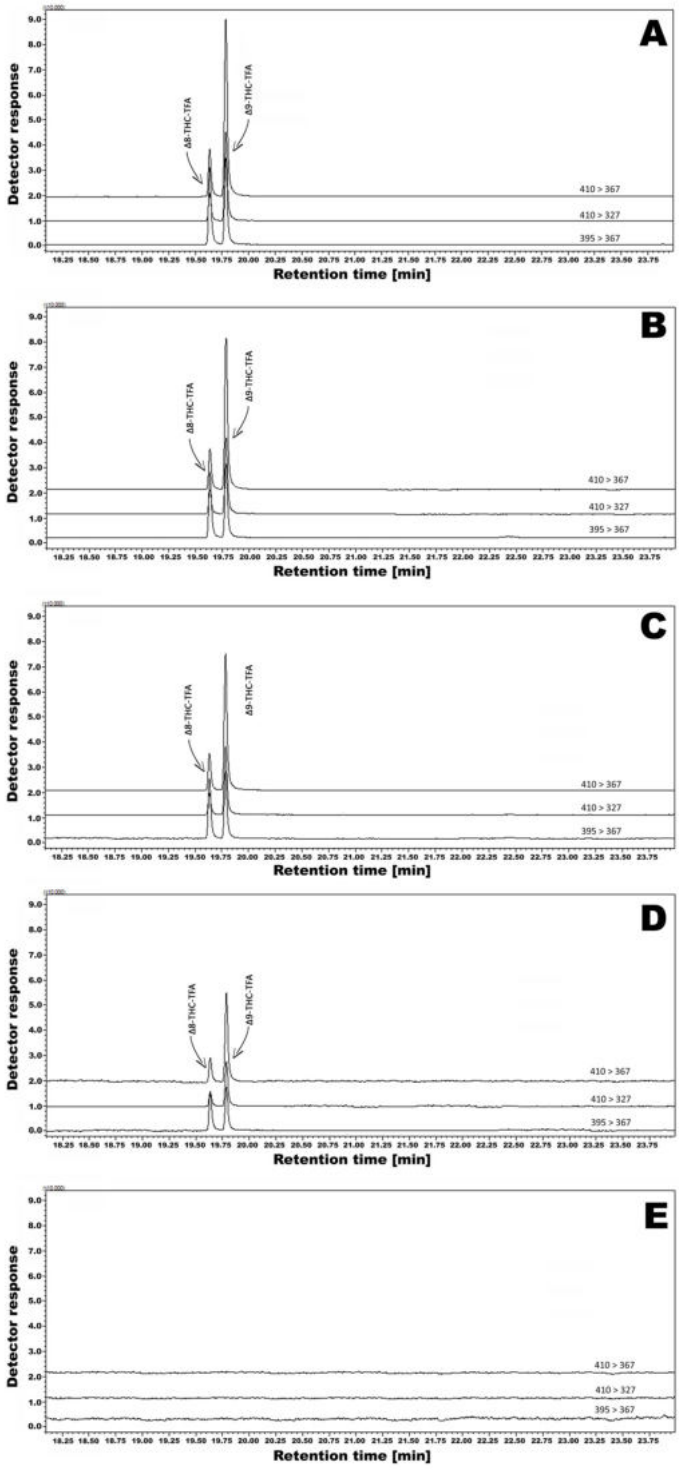


Fig. 6. GC-MS/MS chromatograms (in MRM mode) of extracts from the supernatants centrifuged from human plasma samples spiked with CBD (100 ng/mL) after their preliminary protein precipitation by TFA, which were obtained using ACN (A), $CHCl_3$ (B) DCM (C), EtOAc (D) and hexane (E).

esters do not form during protein precipitation by TFA, or they do, but their formation kinetics is very slow. These results and those presented in Fig. 1 indicate that Δ9-THC-TFA and Δ8-THC-TFA are formed in the hot GC injector when TFA is used as protein precipitation agent. An increase in the GC injector temperature favors their formation, as seen in the diagram in Fig. 5 showing the change of the GC-MS signal magnitude of Δ9-THC-TFA and Δ8-THC-TFA as a function of the GC injector's temperature.

The type of solvent in which a chemical reaction occurs, including esterification, is also a factor influencing the reaction kinetics. It is worth noticing that the extraction process of supernatants from blood/plasma samples is used in some cases as an additional sample purification step [40,41]. GC-MS chromatograms

(using MRM function) of the extracts from the supernatants centrifuged from human plasma samples spiked with CBD after their preliminary protein precipitation by TFA, obtained using hexane, EtOAc, DCM, $CHCl_3$ and ACN are shown in Fig. 6. They indicate that the amount of Δ8-THC-TFA and Δ9-THC-TFA formed in the GC injector depends on the polarity of the extracting solvent. The peaks of Δ8-THC-TFA and Δ9-THC-TFA do not appear on the GC chromatogram when hexane, non-polar solvent, is used as extracting solvent. The TFA derivatives do form in the presence of other extracting solvents, but in varying amounts. The results of the last experiment might allow to find out the relationship between the degree of CBD transformation in the GC injector and the polarity of the extracting solvent by relating them to the polarity of individual solvents at temperature of the GC injector (i.e. in 310 °C). Unfortunately, all commonly known solvent polarity scales were developed under normal conditions. In all probability, the observed differences in the amount of the formed TFA derivatives are connected with different amounts of TFA co-extracting with CBD to a given solvent from blood/plasma sample after protein precipitation. Various enthalpy of the processes occurring in the GC injector and different polarity and density of sample vapor in the GC injector due to the presence of different solvents may also play a part, yet the first explanation seems most probable.

It is also worth noting Fig. 6B showing the formation of Δ8-THC-TFA and Δ9-THC-TFA when $CHCl_3$ as the extractant in purification step is used. Its content does not quite agree with Holler et al. [42], who showed that $CHCl_3$ prevents the transformation of CBD during its acylation with TFAA. It should be remembered, however, that $CHCl_3$ at the temperature of GC injector partially decomposes to HCl, which in turn acidifies the injector atmosphere and catalyzes CBD transformation. Hence, the result in Fig. 6B is not surprising.

4. Conclusions

Methods for the analysis of cannabinoids in biological matrices are continually being developed, specifically to achieve the accuracy and precision in estimating their concentration in tested samples. As reported in [33,34], if protein precipitation by acidic agents is applied as sample preparation method in blood/plasma analysis, a significant part of CBD contained in the sample analyzed by GC transforms to 9α-OH-HHC, 8-OH-iso-HHC, Δ9-THC, Δ8-THC and CBN. The present study takes this knowledge further by demonstrating the formation of two additional CBD derivatives in the GC injector, Δ8-THC-TFA and Δ9-THC-TFA, if TFA is used for protein precipitation. Although TFA, unlike TFAA, has a much lower acylation capacity of OH groups in organic compounds and does not cause cannabinoids esterification during protein precipitation performed at ambient temperature, it is able to form Δ8-THC-TFA and Δ9-THC-TFA esters in GC injector conditions. The amount of Δ8-THC-TFA and Δ9-THC-TFA esters strongly depends on the GC injector temperature and the solvent type in the injected sample.

The knowledge of cannabinoids stability in the process of sample preparation for analysis and during analysis itself helps assess the accuracy and precision of estimating their concentration in tested samples. The obtained results demonstrate why the use of trifuoroacetic acid for plasma samples deproteinization should be avoided when CBD is determined by GC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463020.

CRediT authorship contribution statement

Piotr Holowinski: Writing – original draft, Investigation, Methodology, Data curation. **Rafal Typek:** Writing – original draft, Investigation, Methodology, Data curation. **Andrzej L. Dawidowicz:** Conceptualization, Writing – original draft, Investigation. **Michal Rombel:** Writing – original draft, Investigation, Data curation. **Michal P. Dybowski:** Writing – original draft, Writing – review & editing, Investigation, Methodology, Data curation, Visualization.

References

[1] M.M. Delgado-Povedano, C. Sánchez-Carnerero Callado, F. Priego-Capote, C. Ferreiro-Vera, Untargeted characterization of extracts from Cannabis sativa L. cultivars by gas and liquid chromatography coupled to mass spectrometry in high resolution mode, Talanta 208 (2019) 120384, doi:10.1016/j.talanta.2019.120384.
[2] Y.H. Hsu, M.C. Fang, S.C. Huang, Y.M. Kao, S.H. Tseng, D.Y. Wang, Determination of cannabinoids in hemp oil based cosmetic products by LC-tandem MS, J. Food Drug Anal. 29 (2021) 502–509, doi:10.38212/2224-6614.3370.
[3] G. Micalizzi, F. Vento, F. Alibrando, D. Donnarumma, P. Dugo, L. Mondello, Cannabis Sativa L.: a comprehensive review on the analytical methodologies for cannabinoids and terpenes characterization, J. Chromatogr. A 1637 (2021) 461864, doi:10.1016/J.CHROMA.2020.461864.
[4] A. Nemeškalová, K. Hájková, L. Mikulů, D. Sýkora, M. Kuchař, Combination of UV and MS/MS detection for the LC analysis of Cannabidiol-rich products, Talanta 219 (2020) 121250, doi:10.1016/J.TALANTA.2020.121250.
[5] O. Devinsky, M.R. Cilio, H. Cross, J. Fernandez-Ruiz, J. French, C. Hill, R. Katz, V. Di Marzo, D. Jutras-Aswad, W.G. Notcutt, J. Martinez-Orgado, P.J. Robson, B.G. Rohrback, E. Thiele, B. Whalley, D. Friedman, Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders, Epilepsia 55 (2014) 791–802, doi:10.1111/epi.12631.
[6] V. Cardenia, T. Gallina, S. Scappini, R.C. Rubino, M.T. Rodriguez-Estrada, Development and validation of a Fast gas chromatography/mass spectrometry method for the determination of cannabinoids in Cannabis sativa L, J. Food Drug Anal. 26 (2020) 9, doi:10.1016/j.jfda.2018.06.001.
[7] A.L. Osborne, N. Solowij, K. Weston-Green, A systematic review of the effect of Cannabidiol on cognitive function: relevance to schizophrenia, Neurosci. Biobehav. Rev. 72 (2017) 310–324, doi:10.1016/j.neubiorev.2016.11.012.
[8] A.B. El-Remessy, Y. Khalifa, S. Ola, A.S. Ibrahim, G.I. Liou, Cannabidiol protects retinal neurons by preserving glutamine synthetase activity in diabetes, Mol. Vis. 16 (2010) 1487–1495.
[9] V. Brighenti, M. Licata, T. Pedrazzi, D. Maran, D. Bertelli, F. Pellati, S. Benvenuti, Development of a new method for the analysis of cannabinoids in honey by means of high-performance liquid chromatography coupled with electrospray ionisation-tandem mass spectrometry detection, J. Chromatogr. A 1597 (2019) 179–186, doi:10.1016/J.CHROMA.2019.03.034.
[10] M. Prud'Homme, R. Cata, D. Jutras-Aswad, Cannabidiol as an intervention for addictive behaviors: a systematic review of the evidence, Subst. Abus. Res. Treat. 9 (2015) 33–38, doi:10.4137/SART.S25081.
[11] M.M. Caffarel, C. Andradas, E. Pérez-Gómez, M. Guzmán, C. Sánchez, Cannabinoids: a new hope for breast cancer therapy? Cancer Treat. Rev. 38 (2012) 911–918, doi:10.1016/j.ctrv.2012.06.005.
[12] R.G. Pertwee, Cannabidiol as a potential medicine, in: Cannabinoids as Therapeutics, Birkhäuser-Verlag, 2005, pp. 47–65, doi:10.1007/3-7643-7358-x_3.
[13] R. Gottardo, D. Sorio, M. Ballotari, F. Tagliaro, First application of atmospheric-pressure chemical ionization gas chromatography tandem mass spectrometry to the determination of cannabinoids in serum, J. Chromatogr. A 1591 (2019) 147–154, doi:10.1016/J.CHROMA.2019.01.041.
[14] E.M. Rock, D. Bolognini, C.L. Limebeer, M.G. Cascio, S. Anavi-Goffer, P.J. Fletcher, R. Mechoulam, R.G. Pertwee, L.A. Parker, Cannabidiol, a nonpsychotropic component of cannabis, attenuates vomiting and nausea-like behaviour via indirect agonism of 5-HT 1A somatodendritic autoreceptors in the dorsal raphe nucleus, Br. J. Pharmacol. 165 (2012) 2620–2634, doi:10.1111/j.1476-5381.2011.01621.x.
[15] R.A. Vlad, L. Farczádi, C.M. Toma, S. Imre, P. Antonoaea, E.M. Rédai, D.L. Muntean, A. Ciurba, Cannabidiol content evaluation in commercial dietary supplements and stability in oil vehicle, Stud. Univ. Babes Bolyai Chem. 66 (2021) 299–308, doi:10.24193/SUBBCHEM.2021.2.26.
[16] D.M. Andrenyak, D.E. Moody, M.H. Slawson, D.S. O'Leary, M. Haney, Determination of Δ-9-tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC and Cannabidiol in human plasma using gas chromatography–tandem mass spectrometry, J. Anal. Toxicol. 41 (2017) 277, doi:10.1093/JAT/BKW136.
[17] R. Andrews, S. Paterson, A validated method for the analysis of cannabinoids in post-mortem blood using liquid-liquid extraction and two-dimensional gas chromatography-mass spectrometry, Forensic Sci. Int. 222 (2012) 111–117, doi:10.1016/j.forsciint.2012.05.007.

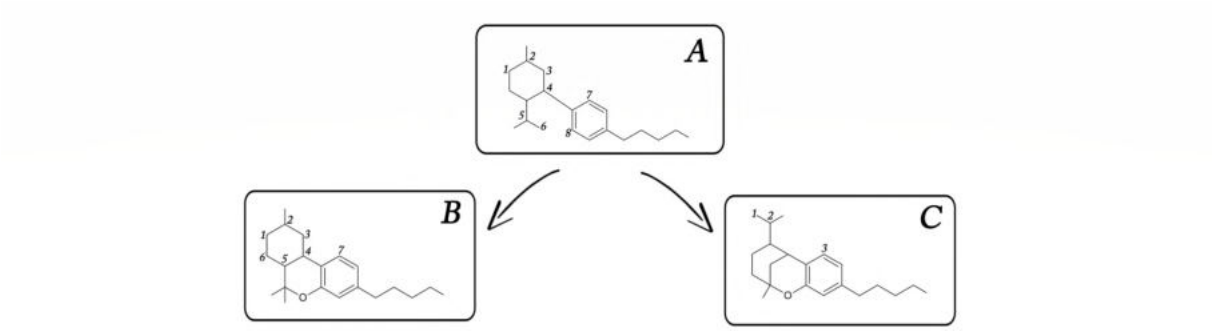
[18] K.D. Cliburn, M.A. Huestis, J.R. Wagner, P.M. Kemp, Identification and quantification of cannabinoids in postmortem fluids and tissues by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1652 (2021) 462345, doi:10.1016/J.CHROMA.2021.462345.
[19] M.J. Roslawski, R.P. Remmel, A. Karanam, I.E. Leppik, S.E. Marino, A.K. Birnbaum, Simultaneous quantification of 13 cannabinoids and metabolites in human plasma by liquid chromatography tandem mass spectrometry in adult epilepsy patients, Ther. Drug Monit. 41 (2019) 357–370, doi:10.1097/FTD.0000000000000583.
[20] S. Pichini, S. Malaca, M. Gottardi, A.P. Pérez-Acevedo, E. Papaseit, C. Perez-Maña, M. Farré, R. Pacifici, A. Tagliabracci, G. Mannocchi, F.P. Busardò, UHPLC-MS/MS analysis of Cannabidiol metabolites in serum and urine samples. Application to an individual treated with medical cannabis, Talanta 223 (2021) 121772, doi:10.1016/j.talanta.2020.121772.
[21] J.H. Lee, A.Y. Min, J.H. Han, Y.J. Yang, H. Kim, D. Shin, Development and validation of LC-MS/MS method with QuEChERS clean-up for detecting cannabinoids in foods and dietary supplements, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 37 (2020) 1413–1424, doi:10.1080/19440049.2020.1769200.
[22] A.L. Dawidowicz, M.P. Dybowski, M. Rombel, R. Typek, Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: oleamide-induced transient matrix effect, J. Pharm. Biomed. Anal. 204 (2021) 114265, doi:10.1016/J.JPBA.2021.114265.
[23] M.W. Elmes, M. Kaczocha, W.T. Berger, K.N. Leung, B.P. Ralph, L. Wang, J.M. Sweeney, J.T. Miyauchi, S.E. Tsirka, I. Ojima, D.G. Deutsch, Fatty acid-binding proteins (FABPs) are intracellular carriers for Δ9-tetrahydrocannabinol (THC) and Cannabidiol (CBD), J. Biol. Chem. 290 (2015) 8711–8721, doi:10.1074/jbc.M114.618447.
[24] J. Klawitter, C. Sempio, S. Mörlein, E. De Bloois, J. Klepacki, T. Henthorn, M.A. Leehey, E.J. Hoffenberg, K. Knupp, G.S. Wang, C. Hopfer, G. Kinney, R. Bowler, N. Foreman, J. Galinkin, U. Christians, J. Klawitter, An atmospheric pressure chemical ionization MS/MS assay using online extraction for the analysis of 11 cannabinoids and metabolites in human plasma and urine, Ther. Drug Monit. 39 (2017) 556–564, doi:10.1097/FTD.0000000000000427.
[25] W.H. Abd-Elsalam, M.A. Alsherbiny, J.Y. Kung, D.W. Pate, R. Löbenberg, LC-MS/MS quantitation of phytocannabinoids and their metabolites in biological matrices, Talanta 204 (2019) 846–867, doi:10.1016/j.talanta.2019.06.053.
[26] L. Jiang, L. He, M. Fountoulakis, Comparison of protein precipitation methods for sample preparation prior to proteomic analysis, J. Chromatogr. A 1023 (2004) 317–320, doi:10.1016/j.chroma.2003.10.029.
[27] S.H. Chiang, C.Y. Chang, Antioxidant properties of caseins and whey proteins from colostrums, J. Food Drug Anal. 13 (2020) 6, doi:10.38212/2224-6614.2550.
[28] X. Chen, C. Liang, L. Cui, J. Le, Z. Qian, R. Zhang, Z. Hong, Y. Chai, A rapid LC-MS/MS method for simultaneous determination of quetiapine and duloxetine in rat plasma and its application to pharmacokinetic interaction study, J. Food Drug Anal. 27 (2020) 21, doi:10.1016/j.jfda.2018.07.003.
[29] K.S. Shiau, T.L. Chen, Initial protein concentration effects on precipitation by salt, Biotechnol. Bioeng. 53 (1997) 202–206 doi:10.1002/(SICI)1097-0290(19970120)53:2<202::AID−BIT10>3.0.CO;2-M.
[30] M. Niknafs, R. Kaviani, A. Gharekhani, A. Jouyban, A. Shayanfar, Salting-out liquid–liquid microextraction to the determination of mycophenolic acid in plasma samples, Chem. Pap. 74 (2020) 1663–1668, doi:10.1007/S11696-019-01018-Y.
[31] T. Li, L. Zhang, L. Tong, Q. Liao, High-throughput salting-out-assisted homogeneous liquid-liquid extraction with acetonitrile for determination of baicalin in rat plasma with high-performance liquid chromatography, Biomed. Chromatogr. 28 (2014) 648–653, doi:10.1002/BMC.3083.
[32] M. Li, H. Wang, X. Huan, N. Cao, H. Guan, H. Zhang, X. Cheng, C. Wang, Simultaneous LC-MS/MS bioanalysis of alkaloids, terpenoids, and flavonoids in rat plasma through salting-out-assisted liquid-liquid extraction after oral administration of extract from tetradium ruticarpum and glycyrrhiza uralensis: a sample preparation strategy to broaden analyte coverage of herbal medicines, Anal. Bioanal. Chem. 413 (2021) 5871–5884, doi:10.1007/S00216-021-03568-1.
[33] K. Watanabe, Y. Itokawa, S. Yamaori, T. Funahashi, T. Kimura, T. Kaji, N. Usami, I. Yamamoto, Conversion of Cannabidiol to Δ9-tetrahydrocannabinol and related cannabinoids in artificial gastric juice, and their pharmacological effects in mice, Forensic Toxicol. 25 (2007) 16–21, doi:10.1007/s11419-007-0021-y.
[34] M.P. Dybowski, A.L. Dawidowicz, R. Typek, M. Rombel, Conversion of Cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – Troubles with reliable CBD quantitation when acidic precipitation agents are applied, Talanta 220 (2020) 121390, doi:10.1016/j.talanta.2020.121390.
[35] R. Andrews, S. Paterson, Production of identical retention times and mass spectra for Δ9-tetrahydrocannabinol and Cannabidiol following derivatization with trifluoracetic anhydride with 1,1,1,3,3,3-hexafluoroisopropanol, J. Anal. Toxicol. 36 (2012) 61–65, doi:10.1093/jat/bkr017.
[36] G.R.B. Webster, L.P. Sarna, R. Mechoulam, Conversion of CBD to Δ8-THC and Δ9-THC, US 7,399,872 B2, 2008.
[37] M.P. Dybowski, A.L. Dawidowicz, Application of the QuEChERS procedure for analysis of Δ9-tetrahydrocannabinol and its metabolites in authentic whole blood samples by GC–MS/MS, Forensic Toxicol. 36 (2018) 415–423, doi:10.1007/s11419-018-0419-8.
[38] R.M. Silverstein, G.C. Bassler, T. Morrill, Chapter three: infrared spectrometry, in: D. Sawicki, J. Stiefel (Eds.), Spectrometric Identification of Organic Compounds, 5th ed., John Wiley & Sons, Inc., 1991, pp. 91–164.
[39] Y.H. Choi, A. Hazekamp, A.M.G. Peltenburg-Looman, M. Frédérich, C. Erkelens, A.W.M. Lefeber, R. Verpoorte, NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of cannabis sativa, Phytochem. Anal. 15 (2004) 345–354, doi:10.1002/pca.787.
[40] S.J. Bruce, I. Tavazzi, V. Parisod, S. Rezzi, S. Kochhar, P.A. Guy, Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry, Anal. Chem. 81 (2009) 3285–3296, doi:10.1021/AC8024569/SUPPL_FILE/AC8024569_SI_001.PDF.
[41] Y.J. Xue, J.B. Akinsanya, J. Liu, S.E. Unger, A simplified protein precipitation/mixed-mode cation-exchange solid-phase extraction, followed by high-speed liquid chromatography/mass spectrometry, for the determination of a basic drug in human plasma, Rapid Commun. Mass Spectrom. 20 (2006) 2660–2668, doi:10.1002/RCM.2645.
[42] J.M. Holler, M.L. Smith, S.N. Paul, M.R. Past, B.D. Paul, Isomerization of delta-9-THC to delta-8-THC when tested as trifluoroacetyl-, pentafluoropropionyl-, or heptafluorobutyryl- derivatives, J. Mass Spectrom. 43 (2008) 674–679, doi:10.1002/JMS.1375.

Supplementary materials

Formation of trifluoroacetic artefacts in gas chromatograph injector during Cannabidiol analysis

Piotr Hołowiński, Rafał Typek, Andrzej L. Dawidowicz, Michał Rombel, Michał P. Dybowski

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031, Lublin, Poland



A

Structure No.	R1	R2	R3	R4	R5	R6	R7	R8
		– C1 = C2 –		– C2 – C3 –		– C3 – C4 –		– C5 = C6 –
1	– H	—		– H,H	– H	—	– H,H	– TFA	– OH
2	– H	—		– H,H	– H	—	– H,H	– TFA	– TFA
		– C1 – C2 –		– C2 = C3 –		– C3 – C4 –		– C5 = C6 –
3	– H,H	—		– H	– H	—	– H,H	– TFA	– OH
4	– H,H	—		– H	– H	—	– H,H	– TFA	– TFA
		– C1 – C2 –		– C2 – C3 –		– C3 = C4 –		– C5 = C6 –
5	– H,H	– H		– H	—	—	– H,H	– TFA	– OH
6	– H,H	– H		– H	—	—	– H,H	– TFA	– TFA
		– C1 = C2 –		– C2 – C3 –		– C3 – C4 –		– C5 – C6 –
7	– H	—		– H,H	– H	– TFA	– H,H	– OH	– OH
8	– H	—		– H,H	– H	– TFA	– H,H	– TFA	– OH
9	– H	—		– H,H	– H	– TFA	– H,H	– TFA	– TFA
10	– H	—		– H,H	– H	– H	– H,TFA	– OH	– OH
11	– H	—		– H,H	– H	– H	– H,TFA	– TFA	– OH
12	– H	—		– H,H	– H	– H	– H,TFA	– TFA	– TFA
		– C1 – C2 –		– C2 = C3 –		– C3 – C4 –		– C5 – C6 –
13	– H,H	—		– H	– H	– TFA	– H,H	– OH	– OH
14	– H,H	—		– H	– H	– TFA	– H,H	– TFA	– OH
15	– H,H	—		– H	– H	– TFA	– H,H	– TFA	– TFA
16	– H,H	—		– H	– H	– H	– H,TFA	– OH	– OH
17	– H,H	—		– H	– H	– H	– H,TFA	– TFA	– OH
18	– H,H	—		– H	– H	– H	– H,TFA	– TFA	– TFA
		– C1 – C2 –		– C2 = C3 –		– C3 – C4 –		– C5 – C6 –
19	– H,H	– H		– H	—	– TFA	– H,H	– OH	– OH
20	– H,H	– H		– H	—	– TFA	– H,H	– TFA	– OH
21	– H,H	– H		– H	—	– TFA	– H,H	– TFA	– TFA
22	– H,H	– H		– H	—	– H	– H,TFA	– OH	– OH
23	– H,H	– H		– H	—	– H	– H,TFA	– TFA	– OH
24	– H,H	– H		– H	—	– H	– H,TFA	– TFA	– TFA
		– C1 – C2 –		– C2 – C3 –		– C3 – C4 –		– C5 = C6 –
25	– H,TFA	– H		– H,H	– H	—	– H,H	– OH	– OH
26	– H,TFA	– H		– H,H	– H	—	– H,H	– TFA	– OH
27	– H,TFA	– H		– H,H	– H	—	– H,H	– TFA	– TFA
28	– H,H	– TFA		– H,H	– H	—	– H,H	– OH	– OH
29	– H,H	– TFA		– H,H	– H	—	– H,H	– TFA	– OH
30	– H,H	– TFA		– H,H	– H	—	– H,H	– TFA	– TFA
31	– H,H	– H		– H,TFA	– H	—	– H,H	– OH	– OH
32	– H,H	– H		– H,TFA	– H	—	– H,H	– TFA	– OH
33	– H,H	– H		– H,TFA	– H	—	– H,H	– TFA	– TFA
34	– H,H	– H		– H,H	– TFA	—	– H,H	– OH	– OH
35	– H,H	– H		– H,H	– TFA	—	– H,H	– TFA	– OH
36	– H,H	– H		– H,H	– TFA	—	– H,H	– TFA	– TFA
		– C1 – C2 –		– C2 – C3 –		– C3 – C4 –		– C5 – C6 –
37	– H,TFA	– H		– H,H	– H	– TFA	– H,H,H	– OH	– OH
38	– H,TFA	– H		– H,H	– H	– TFA	– H,H,H	– TFA	– OH
39	– H,TFA	– H		– H,H	– H	– TFA	– H,H,H	– TFA	– TFA
40	– H,TFA	– H		– H,H	– H	– H	– H,H,TFA	– OH	– OH
41	– H,TFA	– H		– H,H	– H	– H	– H,H,TFA	– TFA	– OH
42	– H,TFA	– H		– H,H	– H	– H	– H,H,TFA	– TFA	– TFA
43	– H,H	– TFA		– H,H	– H	– TFA	– H,H,H	– OH	– OH
44	– H,H	– TFA		– H,H	– H	– TFA	– H,H,H	– TFA	– OH
45	– H,H	– TFA		– H,H	– H	– TFA	– H,H,H	– TFA	– TFA
46	– H,H	– TFA		– H,H	– H	– H	– H,H,TFA	– OH	– OH
47	– H,H	– TFA		– H,H	– H	– H	– H,H,TFA	– TFA	– OH
48	– H,H	– TFA		– H,H	– H	– H	– H,H,TFA	– TFA	– TFA
49	– H,H	– H		– H,TFA	– H	– TFA	– H,H,H	– OH	– OH
50	– H,H	– H		– H,TFA	– H	– TFA	– H,H,H	– TFA	– OH
51	– H,H	– H		– H,TFA	– H	– TFA	– H,H,H	– TFA	– TFA
52	– H,H	– H		– H,TFA	– H	– H	– H,H,TFA	– OH	– OH
53	– H,H	– H		– H,TFA	– H	– H	– H,H,TFA	– TFA	– OH
54	– H,H	– H		– H,TFA	– H	– H	– H,H,TFA	– TFA	– TFA
55	– H,H	– H		– H,H	– TFA	– TFA	– H,H,H	– OH	– OH
56	– H,H	– H		– H,H	– TFA	– TFA	– H,H,H	– TFA	– OH
57	– H,H	– H		– H,H	– TFA	– TFA	– H,H,H	– TFA	– TFA
58	– H,H	– H		– H,H	– TFA	– H	– H,H,TFA	– OH	– OH
59	– H,H	– H		– H,H	– TFA	– H	– H,H,TFA	– TFA	– OH
60	– H,H	– H		– H,H	– TFA	– H	– H,H,TFA	– TFA	– TFA

B

Structure No.	R1	R2	R3	R4	R5	R6	R7
	– C1 = C2 –		– C2 – C3 –		– C3 – C4 –	– C4 – C5 –	– C5 – C6 –	
61	– H	—		– H,H	– H	– H	– H,H	– TFA
	– C1 – C2 –		– C2 = C3 –		– C3 – C4 –	– C4 – C5 –	– C5 – C6 –	
62	– H,H	—		– H	– H	– H	– H,H	– TFA
	– C1 – C2 –		– C2 – C3 –		– C3 = C4 –	– C4 – C5 –	– C5 – C6 –	
63	– H,H	– H		– H	—	– H	– H,H	– TFA
	– C1 – C2 –		– C2 – C3 –		– C3 – C4 –	– C4 – C5 –	– C5 – C6 –	
64	– H,TFA	– H		– H,H	– H	– H	– H,H	– OH
65	– H,TFA	– H		– H,H	– H	– H	– H,H	– TFA
66	– H,H	– TFA		– H,H	– H	– H	– H,H	– OH
67	– H,H	– TFA		– H,H	– H	– H	– H,H	– TFA
68	– H,H	– H		– H,TFA	– H	– H	– H,H	– OH
69	– H,H	– H		– H,TFA	– H	– H	– H,H	– TFA
70	– H,H	– H		– H,H	– TFA	– H	– H,H	– OH
71	– H,H	– H		– H,H	– TFA	– H	– H,H	– TFA
	– C1 = C2 –		– C2 – C3 –		– C3 = C4 –	– C4 – C5 –	– C5 = C6 –	
72	– H	—		– H	—	—	– H	– TFA

C

Structure No.	R1	R2	R3
	– C1 = C2 –		
73	– H,H	—	– TFA
	– C1 – C2 –		
74	– H,H,TFA	– H	– OH
75	– H,H,TFA	– H	– TFA
76	– H,H,H	– TFA	– OH
77	– H,H,H	– TFA	– TFA

Figure 1S. TFA esters of CBD and CBD transformation products that can be formed when protein precipitation process by TFAA is applied to the procedure of CBD estimation in blood/plasma samples.

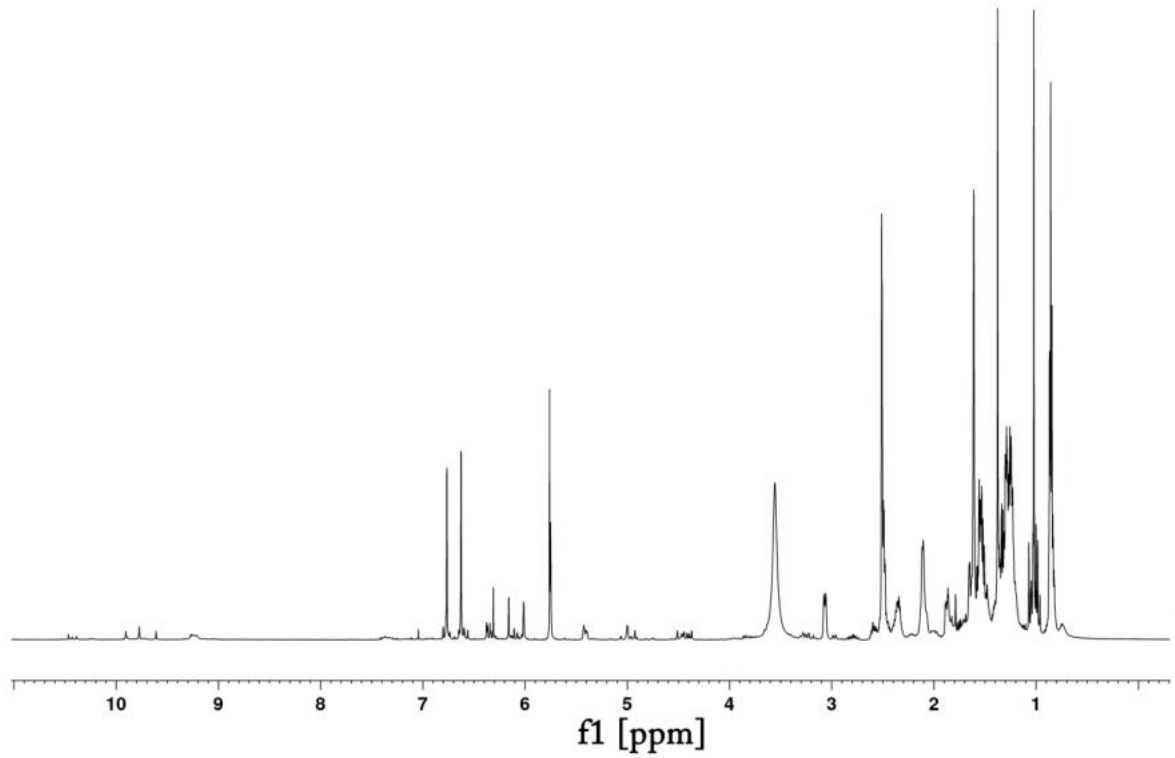


Figure 2S. ^{1}H spectrum of reaction products of CBD with TFAA ($DMSO_6$).

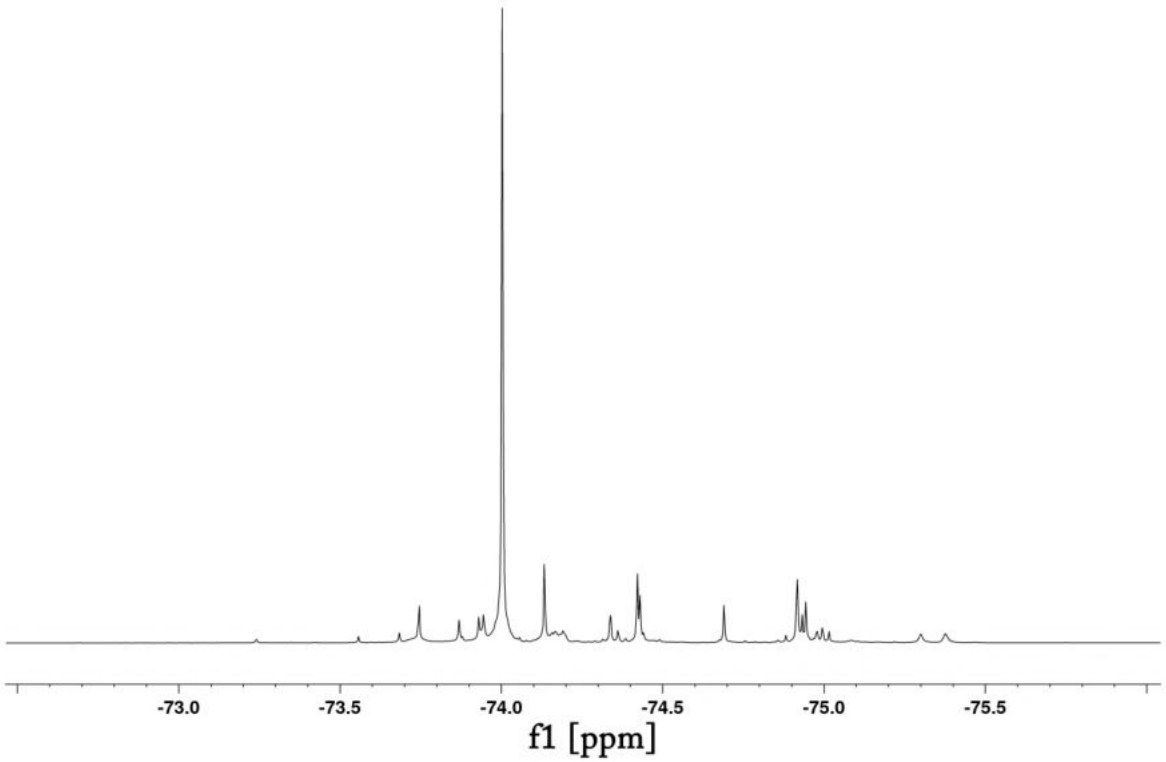


Figure 3S. ^{19}F spectrum of reaction products of CBD with TFAA ($DMSO_6$).

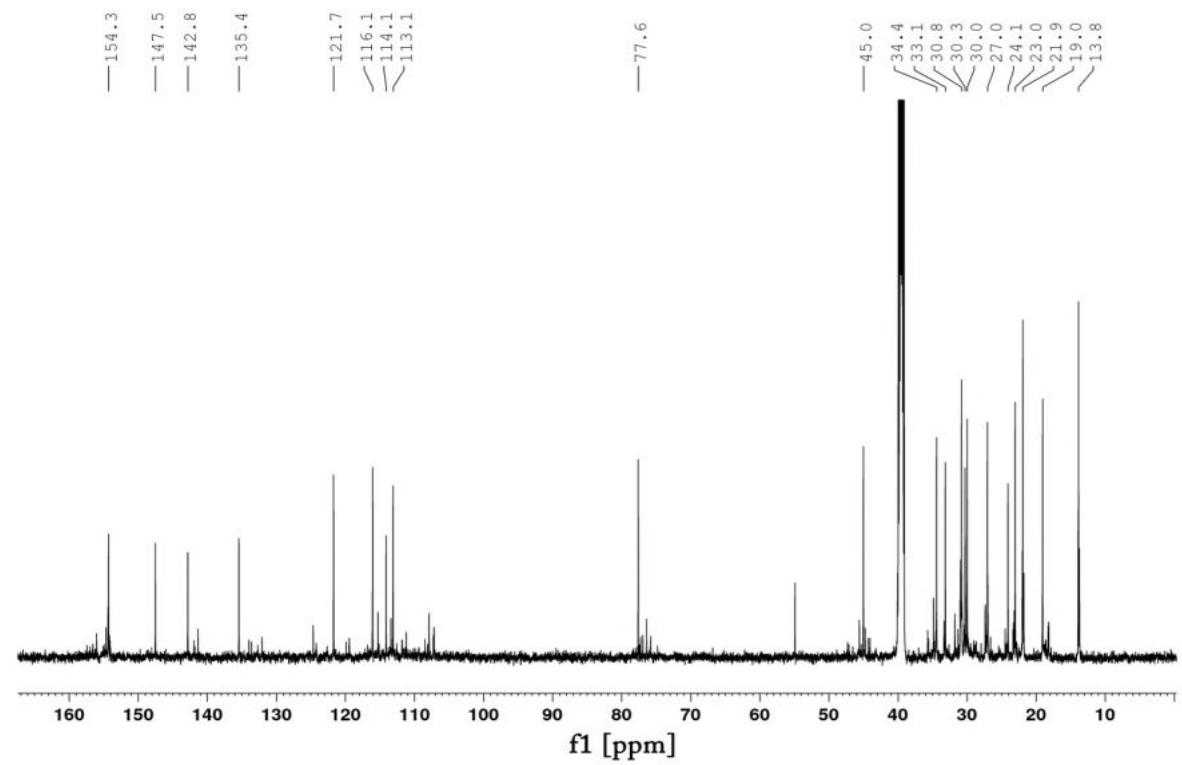


Figure 4S. ^{13}C spectrum of reaction products of CBD with TFAA ($DMSO_6$).

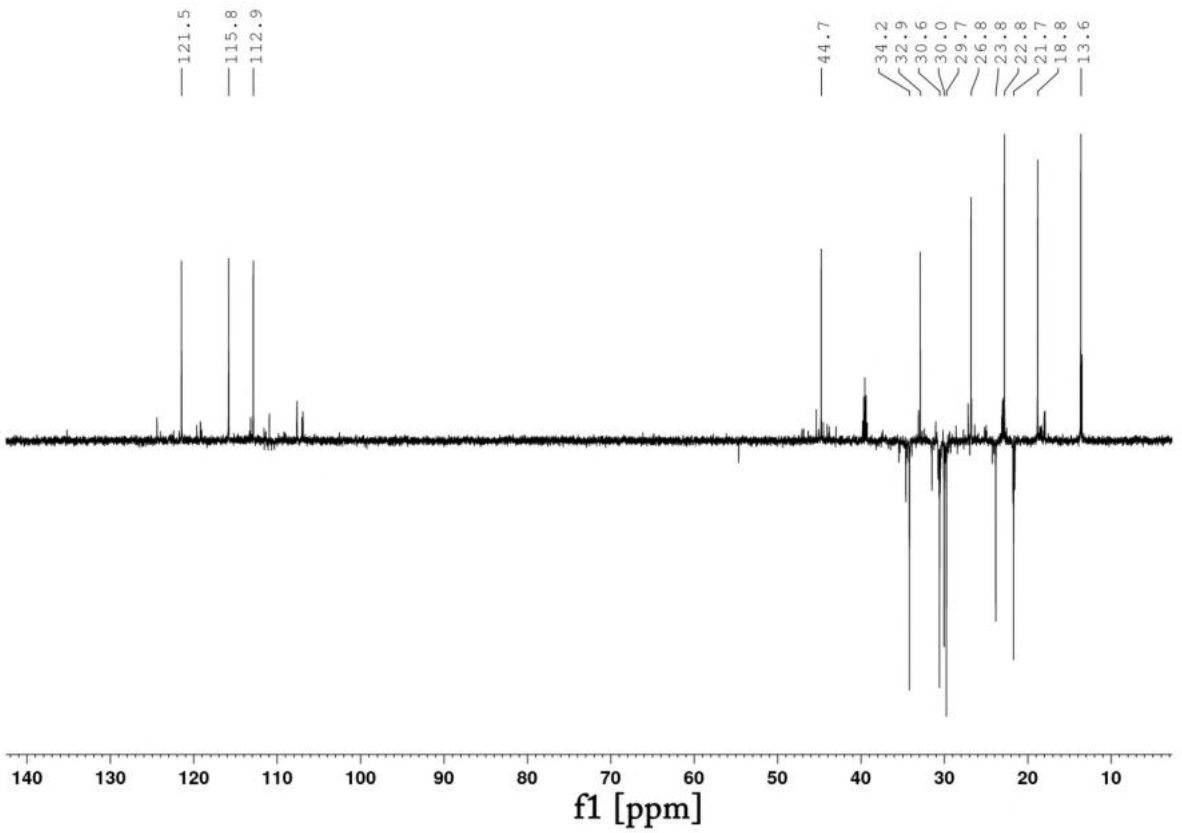


Figure 5S. DEPT 135 spectrum of reaction products of CBD with TFAA ($DMSO_6$).

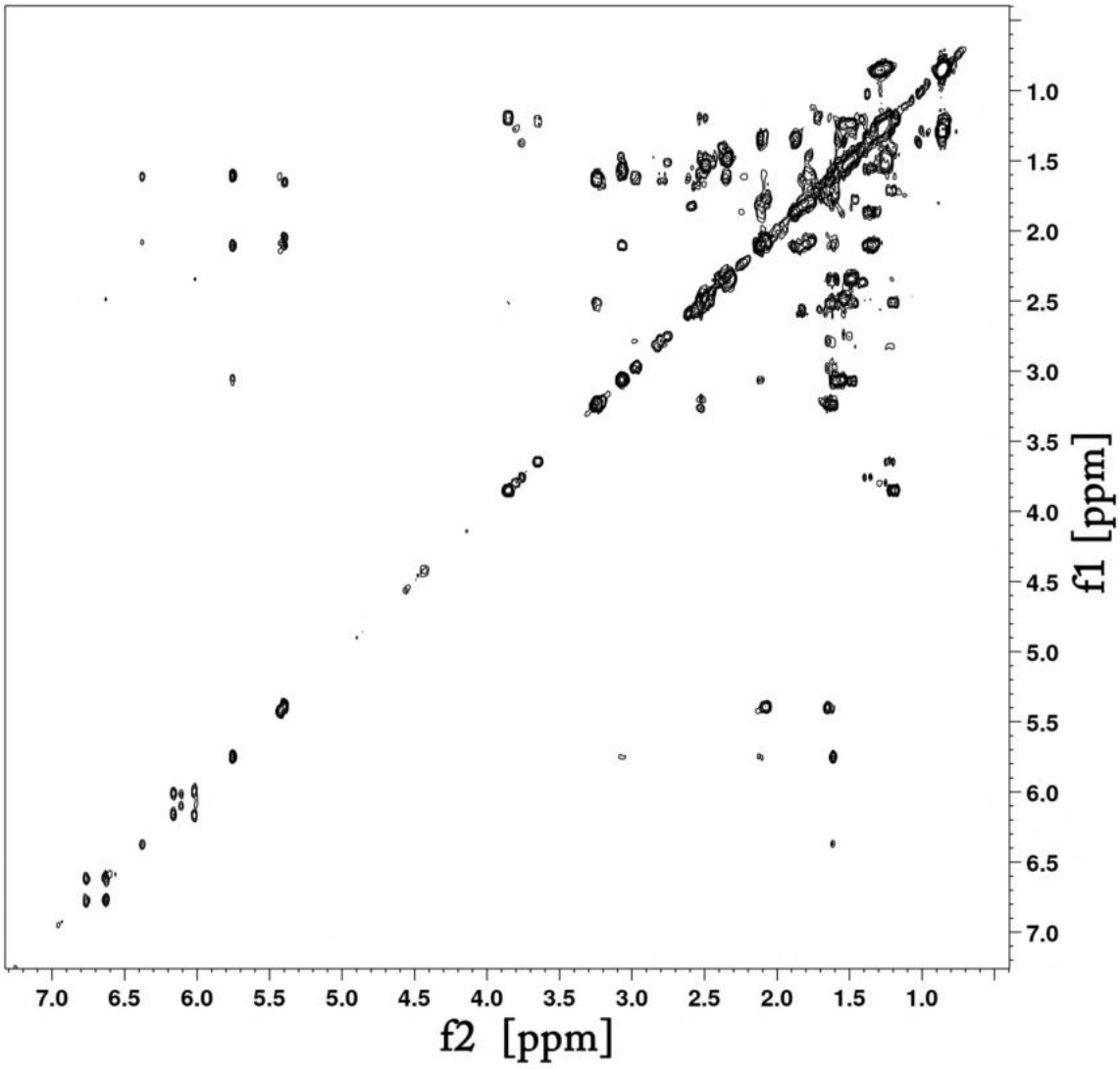


Figure 6S. ^{1}H-^{1}H DQF COSY spectrum of reaction products of CBD with TFAA ($DMSO_6$).

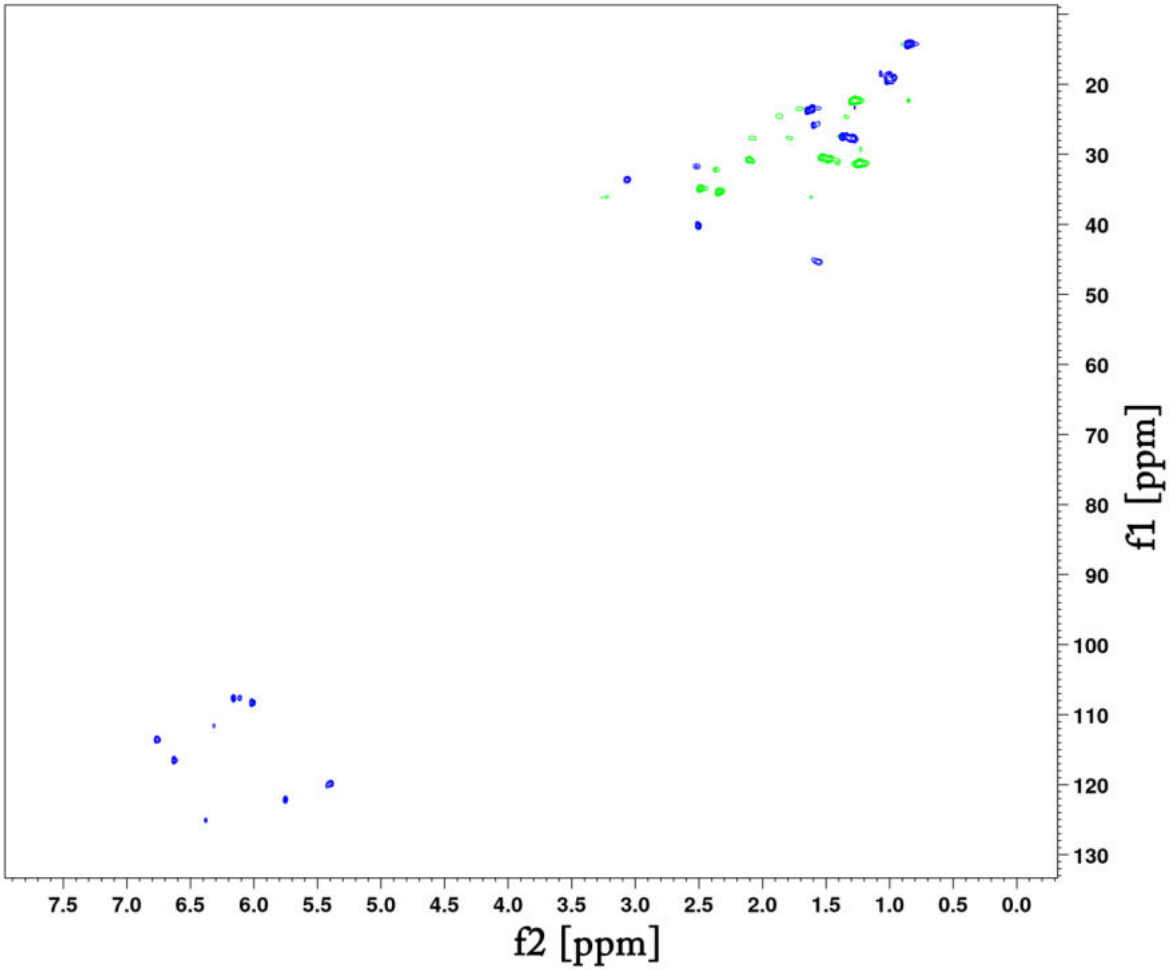


Figure 7S. ^{1}H-^{13}C multiplicity edited HSQC spectrum of reaction products of CBD with TFAA ($DMSO_6$) with CH_2 cross peaks shown as negative signals (green) and CH_3 and CH cross peaks shown as positive signals (blue).

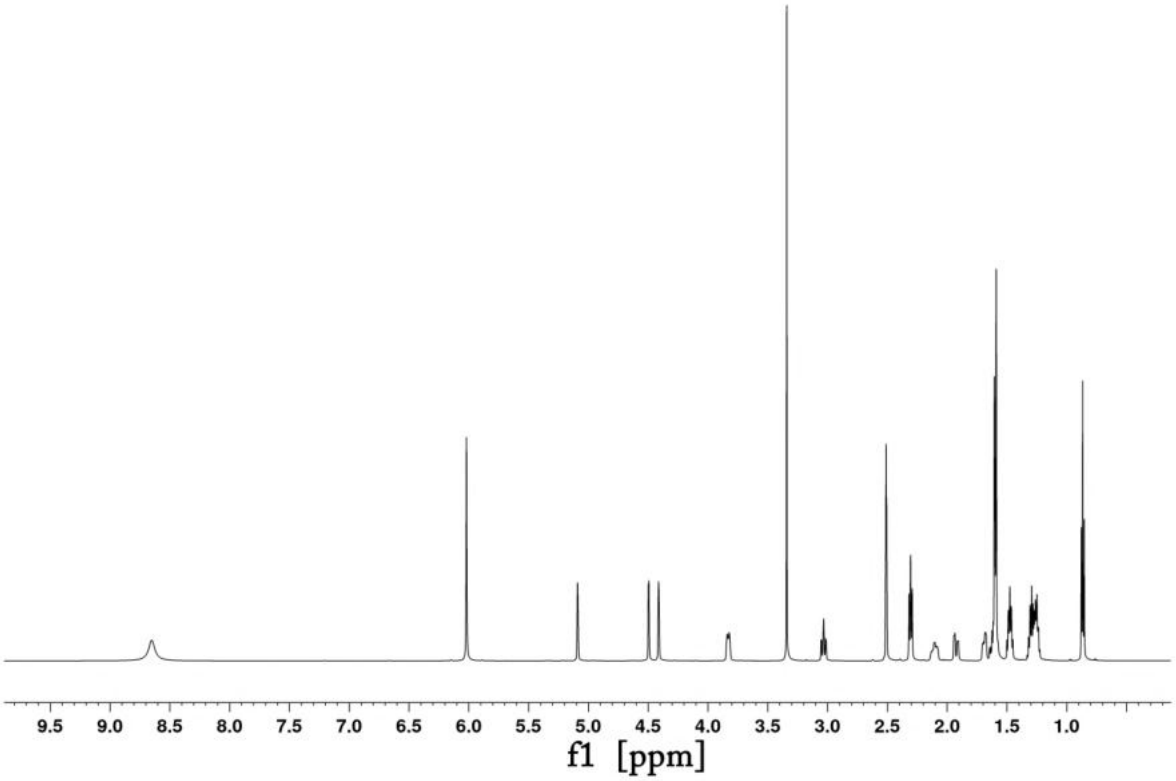


Figure 8S. ^{1}H spectrum of CBD ($DMSO_6$).

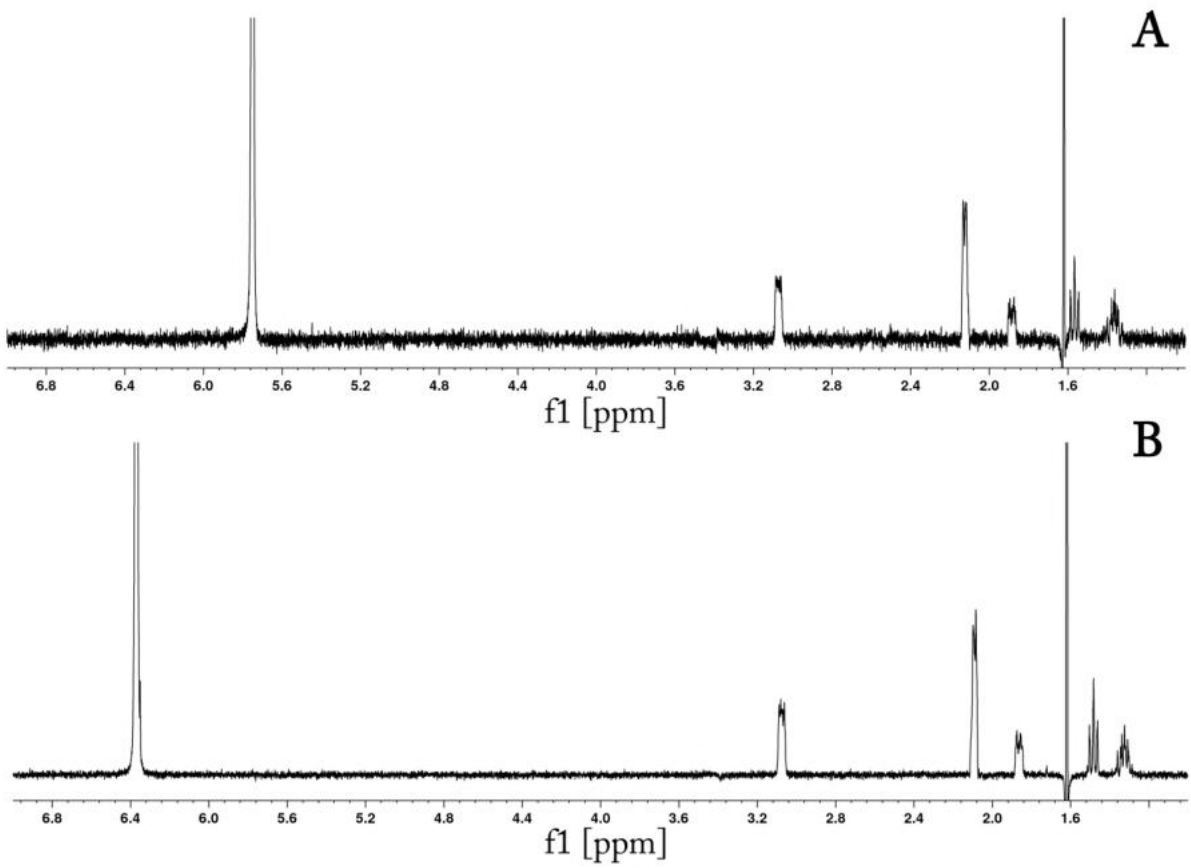


Figure 9S. ^{1}H selective TOCSY spectra obtained by irradiation of Δ9-THC-TFA ester in 5.75 ppm (A) and by irradiation of Δ9-THC in 6.37 ppm (B).

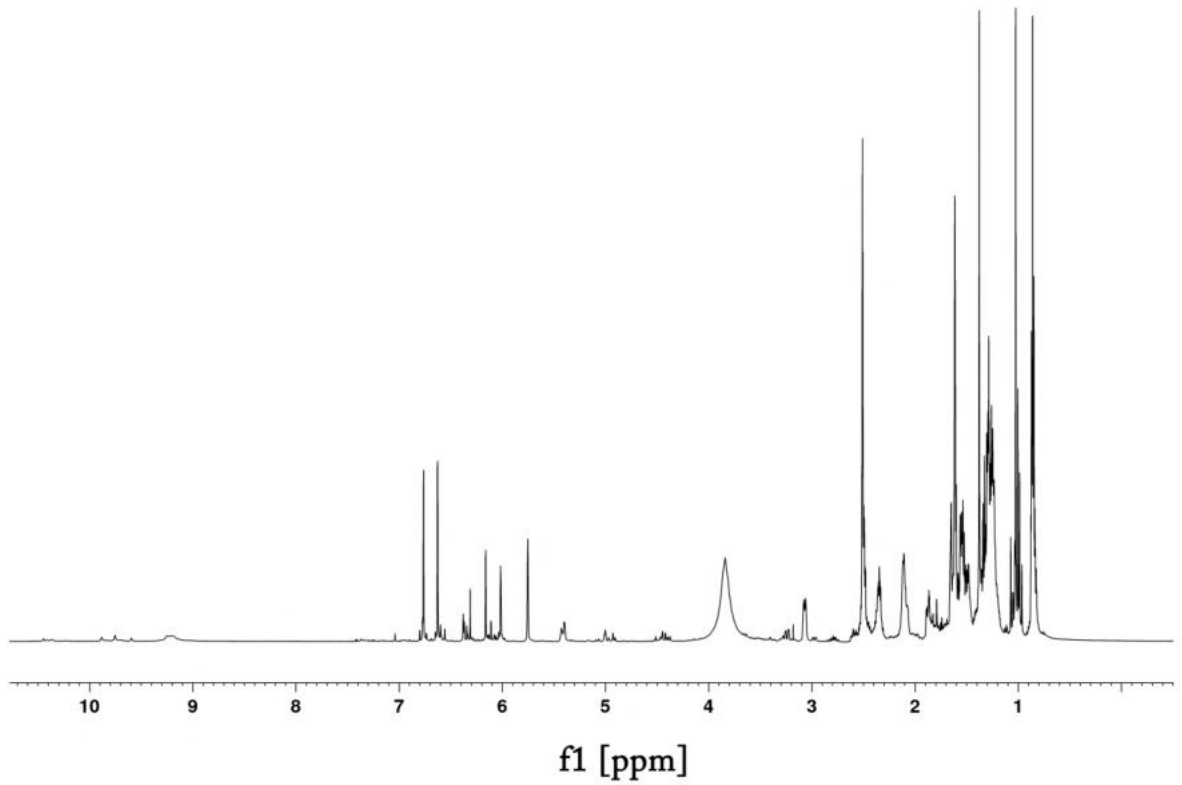


Figure 10S. ^{1}H spectrum of reaction products of Δ9-THC with TFAA ($DMSO_6$).

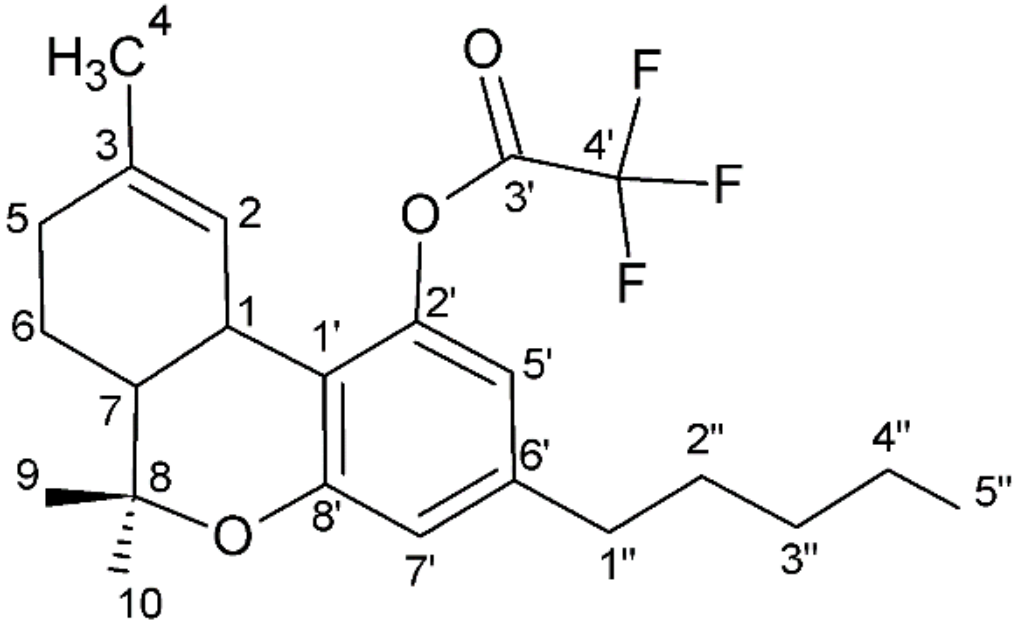


Figure 11S. Enumeration of nuclei in Δ9-THC-TFA ester.

Table 1S. ^{1}H and ^{13}C NMR data of the Δ9-THC-TFA ester in $DMSO_6$.

No.	δ_C	δ_H (m, J/Hz)[b]
1	33.1, CH	3.06 (br d, 10.7)
2	121.7, CH	5.75 (t, 1.4)
3	135.4, C	–
4	23.0, CH_3	1.61 (br s)
5	30.8, CH_2	2.11[c] (m)
6	24.1, CH_2	1.87[d] (m) 1.34[e] (m)
7	45.0, CH	1.56[f] (m)
8	77.6, C	–
9	27.0, CH_3	1.37 (s)
10	19.0, CH_3	1.02 (s)
1′	114.1, C	–
2′	147.5, C	–
3′	–[a], C	–
4′	114.8, CF_3 (J_{C-F}=285 Hz)	–
5′	113.1, CH	6.76 (d, 1.4)
6′	142.8, C	–
7′	116.1, CH	6.63 (d, 1.3)
8′	154.3, C	–
1″	34.4, CH_2	2.49[g] (m)
2″	30.0, CH_2	1.54[f] (m)
3″	30.3, CH_2	1.24[e] (m)
4″	21.9, CH_2	1.28[e] (m)
5″	13.8, CH_3	0.86 (t, 7.1)

[a] 3′ carbon chemical shift could not be clearly determined due to the lack of compound long-term stability.

[b-g] Overlapped signals are reported without designated multiplicity.

Publikacja D5

R. Typek, P. Holowinski, A.L. Dawidowicz, M.P. Dybowski, **M. Rombel**

Chromatographic analysis of CBD and THC after their acylation with blockade of compound transformation

Talanta 251 (2023) 123777

https://doi.org/10.1016/J.TALANTA.2022.123777

IF 2-letni: 6,1; MEiN: 100 pkt

Talanta 251 (2023) 123777

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Chromatographic analysis of CBD and THC after their acylation with blockade of compound transformation

Rafal Typek, Piotr Holowinski, Andrzej L. Dawidowicz *, Michal P. Dybowski, Michal Rombel

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031, Lublin, Poland

ARTICLE INFO

Keywords:
CBD
THC
CBD and THC transformation
CBD and THC acylation
Sample derivatization

ABSTRACT

Methods: for the analysis of cannabinoids in bio-matrices are continually improved to achieve best possible sensitivity in their detection and accurate quantification. It has been well documented that CBD cyclizes to Δ9-THC and Δ9-THC isomerizes to Δ8-THC under acidic conditions by means of a Lewis-acid-catalyzed process, causing difficulty in accurate quantification of Δ9-THC in the presence of CBD, of CBD itself and of Δ9-THC itself when these compounds have to be derivatized by acylation. The present paper shows that CBD cyclization and Δ9-THC isomerization can be blocked by tertiary amines or azines, which capture protons appearing in the derivatizing mixture during acylation. The efficiency of the described acylation of CBD depends on the time and temperature of the derivatizing process, whereas the degree of CBD acylation, i.e. the synthesis of mono- or di-acylate CBD derivative, depends on the mutual ratio of the cannabinoid, the acylating agent and the proton binding compound. The way of mono- and di-acyl CBD derivatives formation described in the paper has not been reported yet. The paper contains a comprehensive analytical characterization of two types of CBD acyl derivatives, CBD-TFA and CBD-Ac, obtained by NMR, GC–MS and LC–MS.

1. Introduction

Of the nearly 120 cannabinoids identified in marijuana and hemp plants, the most frequently analyzed recently in various types of samples, especially bio-matrices, are THC and CBD. The first compound due to its psychoactivity. THC is prohibited in many countries and its abuse in any form is considered a crime [1,2]. The second one, CBD, due to its bioactive, but not psychotropic properties. It is suggested in medicinal treatments of many diseases, and in particular in antiepileptic therapy [3–5]. For these reasons methods for analysis of CBD and THC in bio-matrices are continually being developed to achieve a proper sensitivity required for their detection and accuracy in their quantification. The most frequent way of increasing the chromatographic analysis sensitivity of examined compounds is their preliminary derivatization. However, the derivatization process may prevent quantification of easily transforming analytes. A well-described case is just the analysis of CBD and Δ9-THC, which - during derivatization with acid anhydrides [e.g. with trifluoroacetic anhydride (TFAA)]- are easily converted to Δ9-THC and Δ8-THC, and to Δ8-THC [6–11], respectively. The amounts of these transformation products are dependent on the reaction conditions (i.e. acidic strength of by-products, reaction time, and temperature) [12–14]. The acylation process of CBD and THC results in identical retention times and mass spectra of their derivatives, which makes impossible to quantify their precursors [8,15,16]. Even if the object of analysis is Δ9-THC only, consideration must be given to the possibility of CBD presence in the sample when acid anhydride is used as derivatizing agent. Difficulties in accurate quantification of THC in the presence of CBD or of CBD itself when derivatized with acidic anhydride is due to the different rates of CBD cyclization to Δ9-THC and Δ9-THC isomerization to Δ8-THC, and the acylation reaction of CBD and/or THC. The cyclization and isomerization processes, running under acidic conditions according to the Lewis-acid-catalyzed process [17,18], are much faster. The present work describes and discusses the procedure of CBD and THC acylation with their transformation blockage. The described method of the transformation blockage of these compounds during their derivatization by acylation allows for accurate and reliable quantification of CBD in the presence of THC, THC in the presence of CBD and CBD/THC itself.

* Corresponding author.
E-mail address: dawid@poczta.umcs.lublin.pl (A.L. Dawidowicz).

https://doi.org/10.1016/j.talanta.2022.123777
Received 6 May 2022; Received in revised form 19 July 2022; Accepted 23 July 2022
Available online 10 August 2022

2. Experimental

2.1. Materials

Acetonitryle (ACN) (LC/MS grade), anhydrous magnesium sulfate (99.5% powder; MgSO4) and sodium chloride were purchased from Merck (Warszawa, Poland). The standards (certified reference materials) of Δ9-THC (1.0 mg/mL in methanol - Cerilliant) and CBD (1.0 mg/mL in methanol - Cerilliant), trifluoroacetic anhydride (TFAA), acetic anhydride (Ac_2O), acyl chloride (AcCl), trimethylamine (TEA), N-ethyldiisopropylamine (EDiPA), N,N,N′,N'- tetramethylethylenediamine (TMEDA), morpholine (Mor), N-methylmorpholine (NMM), pyridine (Pyr), 4-dimethylaminopyridine (DMAP), nicotine (Nico), azobenzene (AB) were acquired from Sigma-Aldrich (Poznan, Poland). Dichloromethane (DCM) and toluen (analytical grade) were purchased from the Avantor Performance Materials Poland (Gliwice, Poland). Deuterated chloroform ($CDCl_3$) DMSO-d_6 was bought from Armar AG (Döttingen, Switzerland). CBD crystal (>99%) was a gift from CannLAB (Kraków, Poland). Deionized water was purified by a Milli-Q system (Millipore Sigma, Bedford, MA, USA).

The blood samples were collected by a registered nurse from volunteers, after obtaining their informed consent, using a single closed system containing an S-Monovette coagulation activator, according to the manufacturer instructions (Sarstedt AG, Nümbrecht, Germany), and thoroughly mixed in order to maintain their homogeneity.

2.2. Derivatization of CBD, Δ9-THC or CBD/d9-THC mixture by TFAA in the absence of proton capturing agent (classical acylation way)

The acylation of CBD or Δ9-THC, or CBD/Δ9-THC mixture was performed heating their solutions in dichloromethane (50 μL) with TFAA (20 μL) at 65 °C for 60 min. The concentration of cannabinoids in their dichloromethane solutions was 1 mg/mL in the case of CBD or Δ9-THC. In the case of CBD/Δ9-THC mixture, the concentration of each cannabinoid was 0.5 mg/mL. Next, the low volatile components of the reaction mixture were evaporated under nitrogen stream and the obtained dry residue was dissolved in ACN (1 mL) and subjected to GC-MS analysis. The high CBD and/or THC concentrations, unexpected in real blood samples for forensic analysis, were used merely for demonstration purposes.

2.3. Derivatization of CBD and Δ9-THC by TFAA in the presence of TEA as proton capturing agent – the influence of reaction time and temperature on the conversion degree of CBD to CBD-di-TFA and Δ9-THC to d9-THC-TFA

The mixture composed of:

- 500 μL of toluene solution of CBD (0.1 mg/mL) or Δ9-THC (0.1 mg/mL),
- 250 μL of toluene solution of TFAA (5.0 mg/mL) and
- 250 μL of toluene solution of TEA (2.4 mg/mL)

was heated at 25, 65 and 105 °C for 5, 10, 20, 30, 40, 50, 60 and 70 min. Finally, the low volatile components of the mixture were evaporated under nitrogen stream and the obtained dry residue was dissolved in ACN (1 mL) and subjected to GC-MS analysis.

To avoid sediment precipitation in the reaction mixture containing proton capturing agent, toluene instead of dichloromethane was used in these experiments. Moreover, the used toluene solutions of CBD/THC and acylating agent were more diluted but amounts of these components in reaction mixtures were the same as in the described before classical acylation procedure.

2.4. Derivatization of CBD or Δ9-THC, or CBD/d9-THC mixture by TFAA or Ac_2O or AcCl in the presence of various proton capturing agents

To the mixture composed of:

- 500 μL of toluene solution of CBD (0.1 mg/mL) or Δ9-THC (0.1 mg/mL) or CBD/Δ9-THC mixture (0.05/0.05 mg/mL) and
- 250 μL of toluene solution of TFAA (5.0 mg/mL) or Ac_2O (2.4 mg/mL) or AcCl (1.8 mg/mL),

the toluene solution of a proton capturing agent (250 μL) was added. The type of capturing agent (amine/azine) and its concentration is given in Table 1. The obtained mixture was heated at 65 °C for 60 min. Finally, the low volatile components of the mixture were evaporated under nitrogen stream and the obtained dry residue was dissolved in ACN (1 mL) and subjected to GC-MS analysis.

2.5. Preparation of di-acyl CBD derivative (CBD-di-TFA and CBD-di-Ac) in amounts sufficient for NMR analysis

Trifluoroacyl and acyl derivatives of CBD (CBD-di-TFA and CBD-di-Ac) were prepared by carefully mixing 250 μL of toluene solution of TEA (TEA concentration 25 mg/mL) with 500 μL of toluene solution of CBD (CBD concentration 2 mg/mL). Next, 250 μL of toluene solution of TFAA (TFAA concentration 55 mg/mL) or Ac_2O (Ac_2O concentration 25 mg/mL) was added and the resulting mixture was heated at 65 °C for 60 min. Finally, excessive amounts of acylating agent and toluene were evaporated under nitrogen stream and the dry residue was dissolved in 1 mL of $CDCl_3$ for NMR analysis, or in 10 mL of ACN for GC-MS and LC-MS analysis.

2.6. Preparation of mono-acyl CBD derivative (CBD-mono-TFA and CBD-mono-Ac) in amounts sufficient for NMR analysis

The preparation process of mon-acyl CBD derivatives, both CBD-TFA and CBD-Ac, was performed in the same way as that for di-acyl CBD derivatives. The only difference was the concentration of the applied proton capturing agent, TEA, which amounted to 50 mg/mL.

2.7. Preparation of blood samples for GC-MS analysis

Two steps can be distinguished in the applied procedure of blood sample preparation for GC-MS analysis:

- isolation of examined cannabinoid from plasma using QuEChERS and
- acylation of dry residue obtained from QuEChERS aliquot.

$MgSO_4$ (200 mg) and NaCl (50 mg) were added to a blood sample (700 μL) spiked properly with CBD or Δ9-THC or mixture of CBD/Δ9-THC – concentrations of individual cannabinoids are given below. After vortexing for 1 min, acetonitrile (700 μL) was introduced and the whole suspension was vortexed again and then centrifuged at 12,000 rpm for 3 min. The isolated aliquot (600 μL) was evaporated under nitrogen stream.

The dry residue obtained from QuEChERS aliquot was dissolved in 300 μL of TEA solution in toluene (TEA concentration 5 mg/mL). To this mixture, 300 μL of toluene solution of TFAA (5 mg/mL) was added and the resulting composition was heated at 65 °C for 40 min. Finally, the

Table 1
Concentration of individual proton-capturing agents used in acylation process.

TEA	EDiPA	TMEDA	Mor	NMM	Pyr	DMAP	Nico	AB
Concentration [mg/mL]								
2.4	**3.1**	**1.4**	**2.0**	**2.4**	**1.8**	**1.8**	**1.9**	**2.2**

excessive amounts of acylating agent and toluene were evaporated under nitrogen stream. The obtained dry residue was dissolved in ACN (600 μL) and subjected to GC-MS analysis.

The examined blood samples contained the following cannabinoid concentrations:

- Δ9-THC - 20 ng/mL;
- CBD - 20 ng/mL;
- CBD - 5 ng/mL and Δ9-THC - 15 ng/mL;
- CBD - 10 ng/mL and Δ9-THC - 10 ng/mL;
- CBD - 15 ng/mL and Δ9-THC - 5 ng/mL.

The above CBD/THC concentrations are in the range of those determined in real blood samples of cannabis/hemp consumers.

2.8. GC–MS measurements

Qualitative analyses of CBD, CBD-TFA and CBD-Ac esters were conducted using a gas chromatograph hyphenated with a triple quadruple tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan). GC–MS conditions were as follows:

- capillary column: Zebron ZB5-MSi from Phenomenex, Torrance, CA, USA (30 m × 0.25 mm i. d., 0.25 μm film thickness)
- carrier gas: helium (grade 5.0);
- flow rate: 1.0 mL/min;
- injector temperature: 310 °C;
- injection volume: 1 μL;
- temperature program: initial temperature 60 °C held for 3 min, and then the temperature increase to 310 °C at a rate of 12 °C/min. The final temperature was held for 15 min;
- mass spectrometer operated with normalized electron energy of 70 eV;
- ion source temperature: 225 °C.

The full SCAN mode with range 40–650 *m/z* was used.

2.9. LC-MS measurements

A LC-MS system composed of an UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) and a linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) was applied for the chromatographic analyses of the examined supernatants. An ESI ionization source operating in the positive polarization mode at needle potential of 4.5 kV was employed. Nitrogen (>99.98%) was used as sheath gas (at 40 arbitrary units), auxiliary gas (at 10 arbitrary units) and sweep gas (at 10 arbitrary units). Capillary temperature equalled 320 °C.

The resolution of MS was 60,000. Separations were performed on a Gemini C18 column (4.6 × 100 mm, 3 μm; Phenomenex, USA) using gradient elution. Mobile phase A was 25 mM formic acid in water; mobile phase B was 25 mM formic acid in acetonitrile. The gradient program started at 30% B increasing to 90% for 40 min, and ended with isocratic elution (90% B) for 20 min. The total run time was 60 min at the mobile phase flow rate 0.4 mL/min.

The function of secondary (MS^2) ion fragmentation was applied for spectra of the CBD esters under investigation.

2.10. NMR measurements

NMR measurements were performed at 298 K using a Bruker Ascend 600 MHz instrument. The $CDCl_3$ or DMSO-d_6 solutions of the obtained samples were examined using 1H, 13C, 19F, 1H–1H COSY, multiplicity edited 1H–13C HSQC and selective 1D TOCSY techniques.

3. Result and discussion

Fig. 1 presents GC-MS chromatograms (in SCAN mode) of samples containing Δ9-THC (A), CBD (B) and Δ9-THC/CBD mixture (C) after their preliminary TFAA derivatization performed in the classical acylation way (see experimental section) during which the reaction environments become acidic. As can be seen in each case, there are two main peaks in the chromatograms, which are identified as trifluoroacetic esters of Δ8-THC and Δ9-THC (Δ8-THC-TFA and Δ9-THC-TFA – peak 1 and 2, respectively). Such effect of CBD and Δ9-THC derivatization process by TFAA is very well known from the literature [8,9] and results from the acidification of reaction environments during esterification, which favours the closure of the open moiety present on the CBD structure to a closed pyran ring, thus producing Δ9-THC and the isomerization of Δ9-THC to Δ8-THC. These transformation processes preclude the quantification of THC in the presence of CBD or CBD itself after their prior acylation.

3.1. Derivatization of CBD, Δ9-THC or CBD/d9-THC mixture by TFAA in the presence of TEA playing the role of proton capturing agent

Our experiments show that the CBD cyclization and THC isomerization can be blocked by tertiary amines capturing protons *in statu nascendi*, which appear in the reaction medium during the esterification process. GC-MS chromatograms of samples containing Δ9-THC, CBD and Δ9-THC/CBD mixture after their preliminary TFAA derivatization performed in the presence of TEA as a proton-binding agent are presented in Fig. 2 A-C. To better illustrate the differences in the chromatographic picture of the sample containing THC and CBD after its derivatization with and without TEA addition in the derivatizing mixture, Fig. 2 shows again the chromatogram of the sample containing both cannabinoids after its derivatization by TFAA at the absence of TEA (see Fig. 2 D). All chromatograms are presented in SCAN mode. The analysis of (A-C) chromatograms in Fig. 2 shows that neither CBD cyclization to Δ9-THC nor Δ9-THC isomerization to Δ8-THC occur when TEA is present in the derivatizing mixture. In this case, TFAA reacts with Δ9-THC forming only Δ9-THC-TFA. This derivative is well known and described in literature [8,19]. The single peak in chromatogram (B) and peak 1 in chromatogram (C), both in Fig. 2, can be attributed with high probability to the trifluoroacetic CBD derivative (CBD-TFA). Because, to the authors' knowledge, there is no literature data allowing to confirm the above identification, it was decided to synthetize this CBD-TFA derivative in greater amount, to examine its structure using NMR and LC-MS/MS, and to compare its GC-MS data with those for the single peak in Fig. 2B and peak 1 in Fig. 2C.

3.2. NMR, LC-MS and GC-MS data of CBD-di-TFA

^1H and ^{19}F NMR spectra of the synthetized CBD-TFA derivative dissolved in $CDCl_3$ are shown in Fig. 3A and B. The proton spectrum shows the presence of CBD-like spin systems in terms of the number of resonances and chemical shifts of nuclei. Significant differences are observed only for aromatic ring resonances. Broad signals of two aromatic protons are seen in the position 6.9 ppm. These protons are notably deshielded comparing to the analogous protons of CBD in $CDCl_3$, for which the positions are 6.28 ppm and 6.16 ppm (see Table 1S in supplementary material). The last observation and the lack of phenolic OH groups signals suggest that both OH groups were acetylated with trifluoroacetyl groups. The latters resonate at −74.3 ppm and −74.6 ppm (see ^{19}F NMR spectrum in Fig. 3B). The NMR data obtained for the "CBD-di-TFA derivative" are summarized in Table 2S (see supplementary material).

The exemplary LC–MS chromatogram of the CBD-di-TFA methanolic solution is shown in Fig. 1S (see supplementary material), whereas HR-MS data corresponding to this compound are gathered in Table 3S (see supplementary material). The obtained HR-MS data indicate that

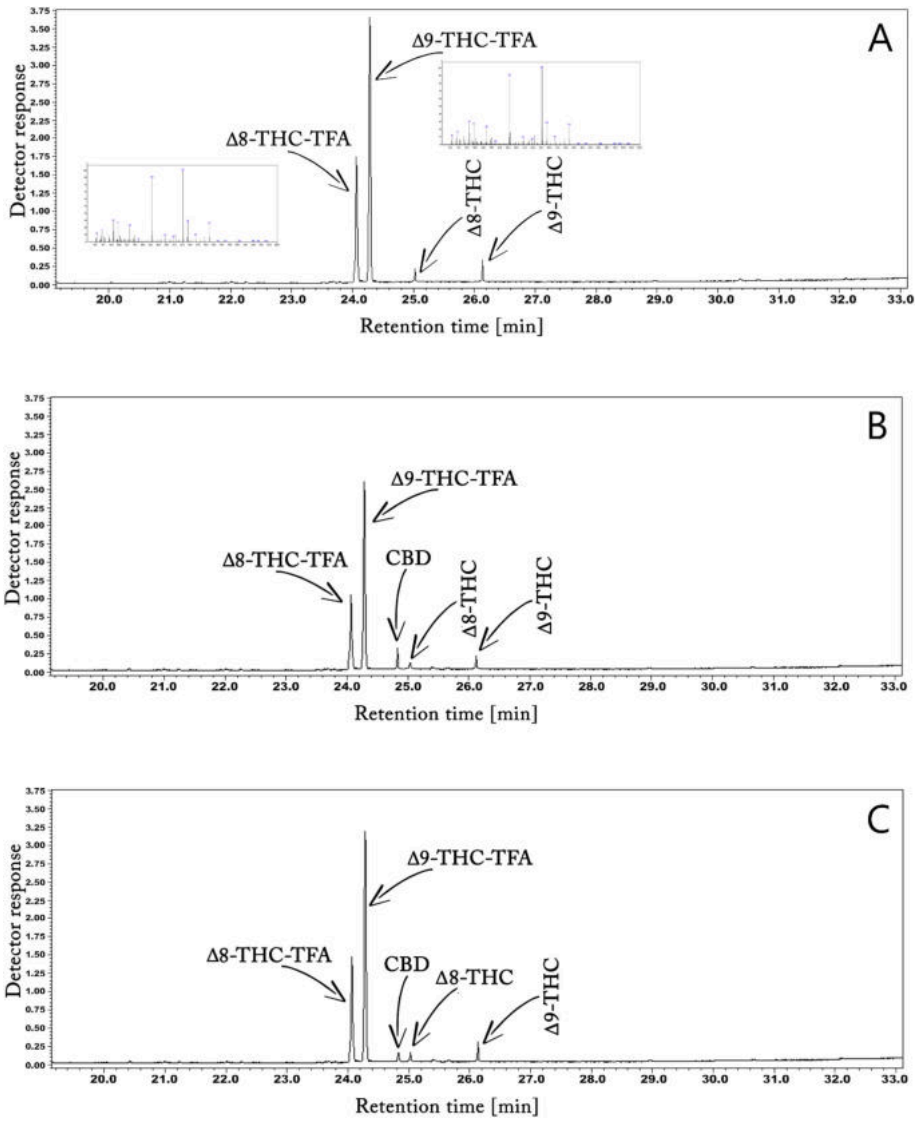


Fig. 1. GC-MS chromatogram (in SCAN mode) of sample containing Δ9-THC (A), or CBD (B), or Δ9-THC/CBD mixture (C) after its TFAA derivatization performed in a classical way, i.e. without addition of a proton-binding agent.

elemental composition of CBD-di-TFA $[M + H]^+$ ion is $C_{25}H_{29}F_6O_4$, and its molecular weight equals 507.19709 Da. The very low difference between the theoretical and the experimental mass (Δppm) for CBD-di-TFA ions prove the correctness of NMR analysis. The *m/z* values and intensities of individual product ions obtained by direct injection of CBD-di-TFA solution into the ion source of the ion-trap mass spectrometer are also collected in Table 3S. As can be seen, the eight ions appear on CBD-di-TFA spectrum of *m/z* equal:123.1; 193.2; 313.1; 325.1; 341.2; 394.2; 410.2 and 439.1.

The NMR and HR-MS results presented above are the first published report concerning CBD-di-TFA.

The GC-MS chromatogram for the synthetized CBD-di-TFA derivative is presented in Fig. 2S (see supplementary material). Its retention value and MS spectrum are the same as the corresponding data for the compound represented by the single peak in Fig. 2B and by peak 1 in Fig. 2C. Hence, the two peaks are identified as CBD-di-TFA, which is formed during CBD derivatization, when CBD cyclization to THC is blocked by TEA. As results from Fig. 2, the ratios of signal magnitudes in chromatograms (C) and (D). are almost the same. It indicates that THC quantification in the presence of CBD is possible when CBD cyclization to THC and Δ9-THC isomerization to Δ8-THC are blocked.

3.3. Derivatization of CBD, Δ9-THC or CBD/d9-THC mixture by Ac_2O in the presence of TEA playing the role of proton capturing agent

Acetic acid anhydride (Ac_2O) is another acylation agent used for derivatization of chromatographed compounds even more frequently than TFAA. Fig. 4 shows the GC-MS chromatograms (in SCAN mode) of samples containing Δ9-THC (A), CBD (B) and Δ9-THC/CBD mixture (C) after their preliminary Ac_2O derivatization performed with the use of TEA as proton-binding agent. In this case, CBD cyclization to Δ9-THC and Δ9-THC isomerization to Δ8-THC is also not observed, just like in the case of TFAA with TEA. A single peak in Fig. 4B and peak 1 in Fig. 4C belong to the di-acetic CBD derivative (CBD-di-Ac). This identification results from the comparison of its GC-MS data with those for CBD-di-Ac, obtained separately in greater amount, and from the NMR and LC-MS/MS data for this separately prepared compound – see discussion below.

3.4. NMR, LC-MS and GC-MS data of CBD-di-Ac

The ^{1}H NMR spectrum of the separately synthetized "acetyl CBD derivative" is shown in Fig. 5. Compared to the ^{1}H NMR data for CBD in DMSO (see Table 4S in supplementary material), it reveals signals of two additional groups in range 2.30–2.09 ppm, which can be identified as

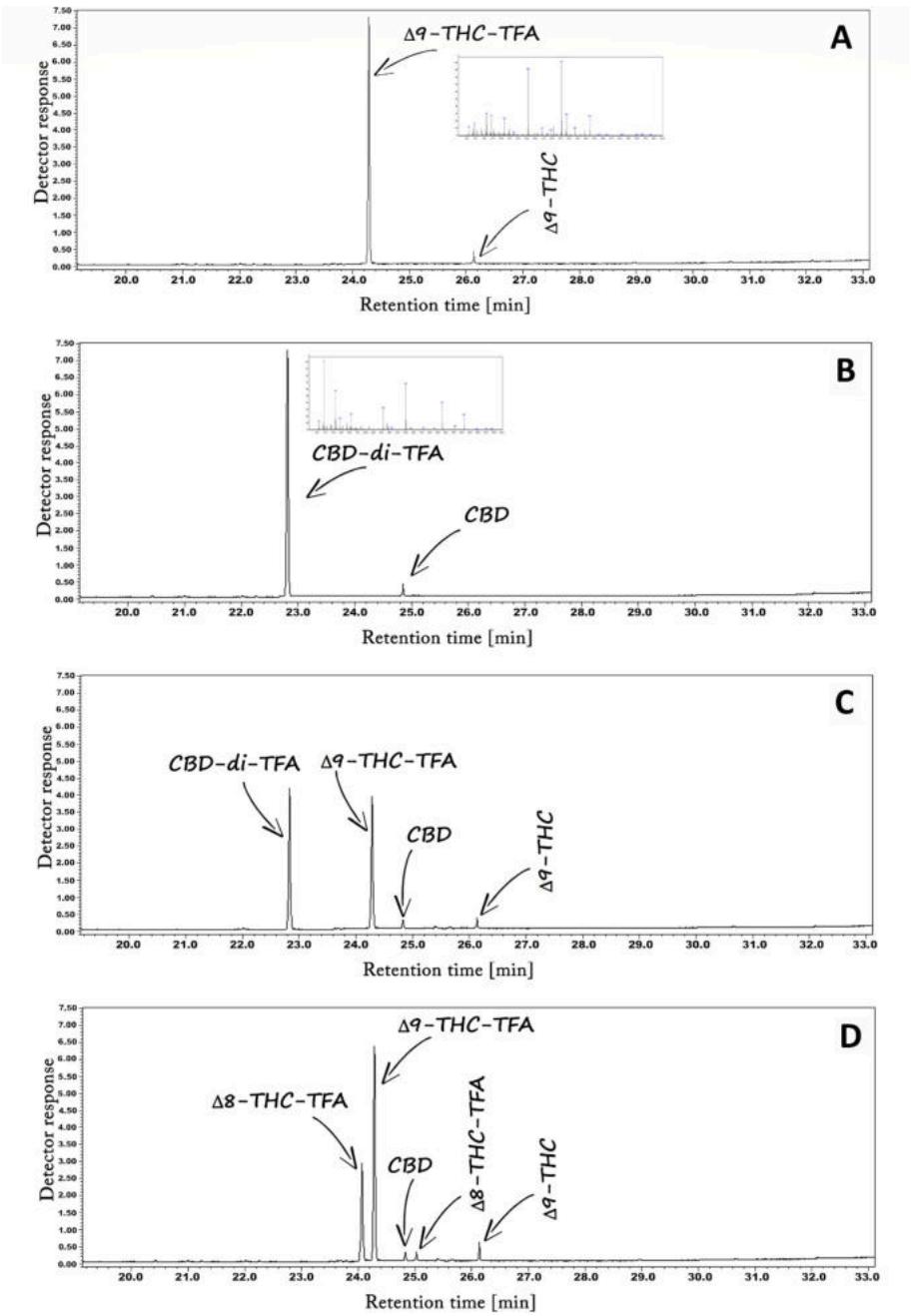


Fig. 2. GC-MS chromatogram (in SCAN mode) of sample containing Δ9-THC (A), or CBD (B), or Δ9-THC/CBD mixture (C) after its TFAA derivatization performed in the presence of TEA playing the role of a proton-binding agent. Chromatogram (D) of Δ9-THC/CBD mixture after its TFAA derivatization performed in a classical way, i.e. without addition of a proton-binding agent - inserted for easier data comparison of the changes in the chromatographic picture.

two methyl groups. It clearly indicates the attachment of two acetyl moieties to CBD. The 6.73 ppm signal from two aromatic protons observed in Fig. 5 provides evidence for the acetylation of both phenol OH groups. The signal from two aromatic protons for CBD dissolved in DMSO occurs at 6.02 ppm. The position difference of aromatic resonances results from the influence of the attached acetyl groups. ^{1}H and ^{13}C NMR data obtained for the "acetyl CBD derivative" are summarized in Table 5S (see supplementary material).

The LC–MS (see Fig. 3S in supplementary material) and HR-MS (see Table 6S in supplementary material) results indicate the following molecular weight and elemental composition of CBD-di-Ac: 399.25343 Da and $C_{25}H_{35}O_4$, respectively. The very low difference between the theoretical and the experimental mass (Δppm) for CBD-di-Ac ions proves the correctness of NMR analysis. The *m/z* values and intensities of individual product ions obtained by direct injection of CBD-di-Ac solution into the ion source of the ion-trap mass spectrometer are also collected in Table 6S. As can be seen, the six ions appear on CBD-di-Ac spectrum of *m/z* equal: 123.1; 193.2; 297.2; 313.1; 339.2 and 355.2.

This is the first report presenting the NMR and HR-MS results for CBD-di-Ac similarly as for CBD-di-TFA.

The GC-MS chromatogram for the synthetized CBD-di-Ac derivative is presented in Fig. 4S (see supplementary material). The retention value and the MS spectrum of this derivative are the same as the corresponding data for the compound represented by the single peak in Fig. 4B and by peak 1 in Fig. 4C. Hence, the presented data confirm that CBD cyclization and Δ9-THC isomerization can be blocked by TEA also when the derivatization of these cannabinoids by Ac_2O is performed.

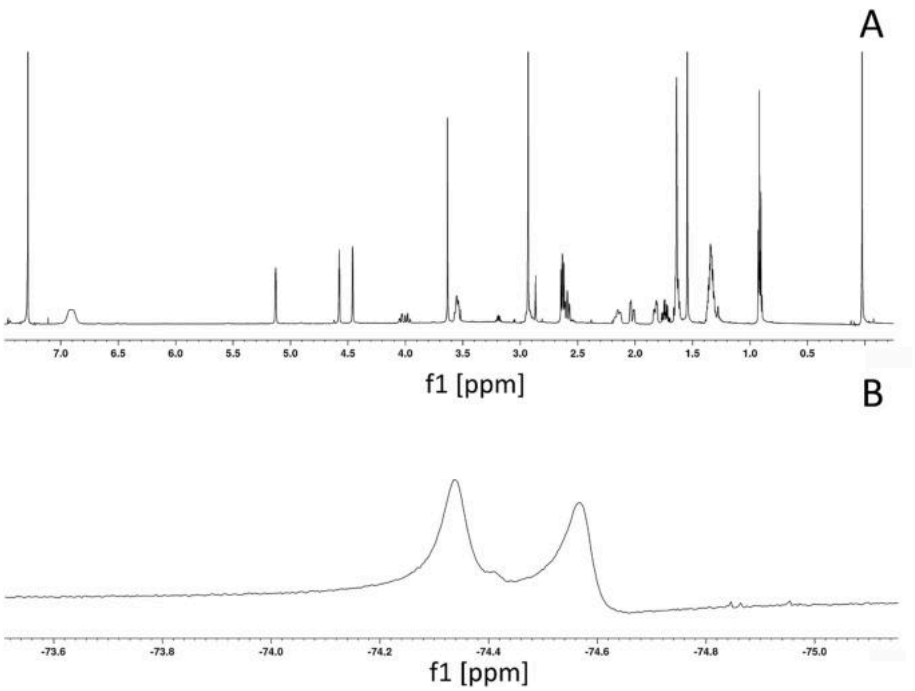


Fig. 3. 1H (A) and 19F (B) NMR spectra of the synthetized CBD-TFA derivative.

3.5. CBD derivatization by TFAA, Ac_2O or AcCl in the presence of different proton capturing agents

The results presented so far have been obtained using TEA as an agent capturing protons released during CBD and Δ9-THC acylation by acid anhydrides. It should be noted that the blockade of CBD cyclization and Δ9-THC isomerization can also be achieved by using other compounds highly capable of proton coordination. The effect of the use of different amines and azines for blocking the possible CBD cyclization during acylation of this compound by TFAA or Ac_2O or AcCl is summarized in Table 2. The (+) and (±) signs indicate that a total CBD amount in sample (+) or only its portion (±) is acylated during sample derivatization, but THC and its acyl derivative do not appear in the post-reaction mixture. The (−) sign indicates that the process of CBD acylation does not occur - the acylation process of primary and secondary amine is quicker than that of CBD. The arrow (↓) sign informs about the appearance of solid precipitate in the post-reaction mixture. As appears from the table, CBD cyclization is blocked when tertiary amines and azines are used as proton-capturing agents. Primary and secondary amines are easily acylated and therefore no blockage of CBD cyclization is observed in their presence - see the results concerning morpholine, a heterocyclic compound featuring secondary amine groups. When AcCl is used for CBD acylation, a solid precipitate can appear in the reaction mixture. It is a hydrochloride of the amine applied as a proton-capturing agent in CBD acylation. There is no precipitate in the case of AB, but it is an azine not amine compound.

3.6. The influence of reaction time and temperature on CBD-di-TFA and d9-THC-TFA formation

The coordinate bond between nitrogen and hydrogen ion is thermolabile. Hence, the effect of blocking of CBD cyclization and Δ9-THC isomerization by amines during the acylation process of these cannabinoids can depend on temperature. On the other hand, temperature determines the rate of chemical reaction, including acylation. Hence, the net temperature effect is worth considering. Fig. 6 shows the influence of acylation time of CBD (Fig. 6 A) and Δ9-THC (Fig. 6 B) on the degree of its their conversion to CBD-di-TFA and Δ9-THC-TFA in the presence of TEA, estimated at three different temperatures: 25, 65 and 105 °C. Increase in the applied temperature range promotes CBD and Δ9-THC acylation. Total CBD and Δ9-THC acylation by TFAA in the presence of TEA can be achieved even within 10 min when the process is performed at 105 °C.

3.7. NMR, LC-MS and GC-MS data of CBD-mono-TFA and CBD-mono-Ac

The acylation process for analytical purposes is usually carried out using an excessive amount of acylating reagent which, in the case of CBD, leads to the modification of two OH groups of this molecule. It should be noted, however, that the monoacyl derivative of CBD may appear in the reaction mixture if insufficient acylating agent amount or excessive proton capturing agent amount is used. The following section briefly discusses the analytical properties of CBD-mono-TFA and CBD-mono-Ac which are helpful in identifying these CBD monoacyl derivatives, if they accidently formed in the reaction mixture.

NMR and LC-MS/MS data for CBD-mono-TFA and CBD-mono-Ac are presented in Tables 7S and 8S (see supplementary material), whereas Fig. 5S (see supplementary material) shows their MS spectra from GC-MS. As results from Table 7S A, summarizing ^{1}H, ^{13}C and ^{19}F NMR data for CBD-mono-TFA in $CDCl_3$, two distinct signals of aromatic protons are observed in positions 6.49 ppm and 6.64 ppm in the ^{1}H spectrum. These signals, as in the case of CBD-di-TFA, are shifted to positions higher than for CBD, although the deshielding effect in this compound is less pronounced, indicating mono acylation with the trifluoacetyl group. The identification correlates well with the presence of the 6.07 ppm signal that can be assigned to a single proton of the phenolic OH group. The fluorine resonance of the attached trifluoroacetyl group can be observed in position −74.4 ppm in the ^{19}F spectrum (see Table 7S A in supplementary material).

The ^{1}H and ^{13}C NMR data for CBD-mono-Ac in DMSO are collected in Table 7S B. The ^{1}H spectrum of "CBD-acyl derivative" reveals the presence of one methyl group in position 2.11 ppm, which can be attributed to the acetyl group. Moreover, the presence of two doublets of

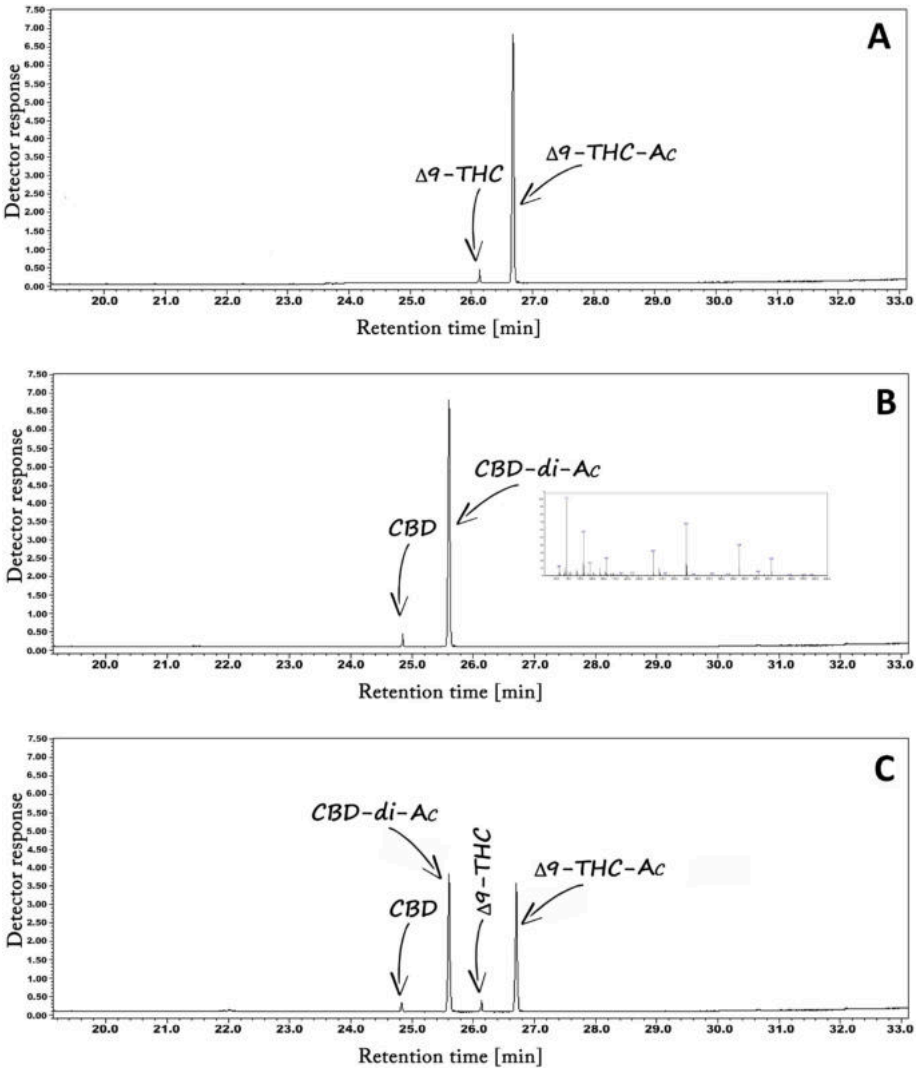


Fig. 4. GC-MS chromatogram (in SCAN mode) of sample containing Δ9-THC (A), or CBD (B), or Δ9-THC/CBD mixture (C) after its Ac_2O derivatization performed in the presence of TEA.

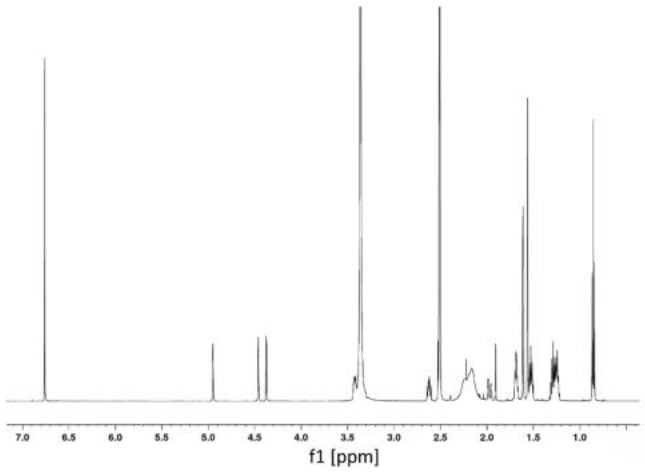


Fig. 5. 1H spectrum of reaction products of CBD with TFAA (DMSO6).

aromatic protons at positions 6.45 ppm and 6.23 ppm is observed in this spectrum. These chemical shifts are intermediate between the value observed for the analogous nuclei of "diacetyl-CBD" and CBD in DMSO. The broad singlet in 9.33 ppm relating to only one proton of the phenolic OH group is also observed for the considered aromatic ring. These signals justify the identification of the obtained derivative as "mono-acetylated CBD".

As results from Table 8S A, the molecular weight and elemental composition of CBD-mono-TFA are as follows: 411.21477 Da and $C_{23}H_{30}F_3O_3$, respectively, whereas *m/z* of its most intensive fragment ions are: 313.2, 325.1 and 341.2. Analogous data for CBD-mono-Ac are as follows: 357.24292, $C_{25}H_{35}O_4$, and 313.2 (see Table 8S B in supplementary material).

The MS spectra from GC-MS measurements for CBD-mono-TFA and CBD-mono-Ac are shown in Fig. 5S A and 5S B. A comparison of the data in Fig. 5S A and Fig. 2 or 1S, and the data in Fig. 5S B and Fig. 4 or 4S shows the MS spectra for the respective mono- and di-acyl CBD derivatives are very similar. There is one ion less in the EI spectrum of the mono-acyl derivatives.

According to the authors' best knowledge, the above data are the first reported analytical characteristics concerning mono-acylated CBD.

3.8. Chromatographic analysis of CBD and THC in blood samples - the cannabinoids acylation with blockade of their transformation

The utility of the described procedure is shown in Fig. 7 illustrating

Table 2
The effect of amines and azines usage for CBD cyclization blockage during the cannabinoid acylation by TFAA or Ac_2O or AcCl.

Acylation agent	Protective agent								
	TEA	EDiPA	TMEDA	Mor	NMM	Pyr	DMAP	Nico	AB
TFAA	+	+	+	–	+	+	+	+	+/–
Ac2O	+	+	+	–	+	+	+	+	+
AcCl	+↓	+↓	+↓	-↓	+↓	+↓	+↓	+↓	+

+ formation of CBD derivatives only.
- deactivation of acylation agent.
+/– partial deactivation of acylation agent.
↓ precipitate formation during derivatization process.

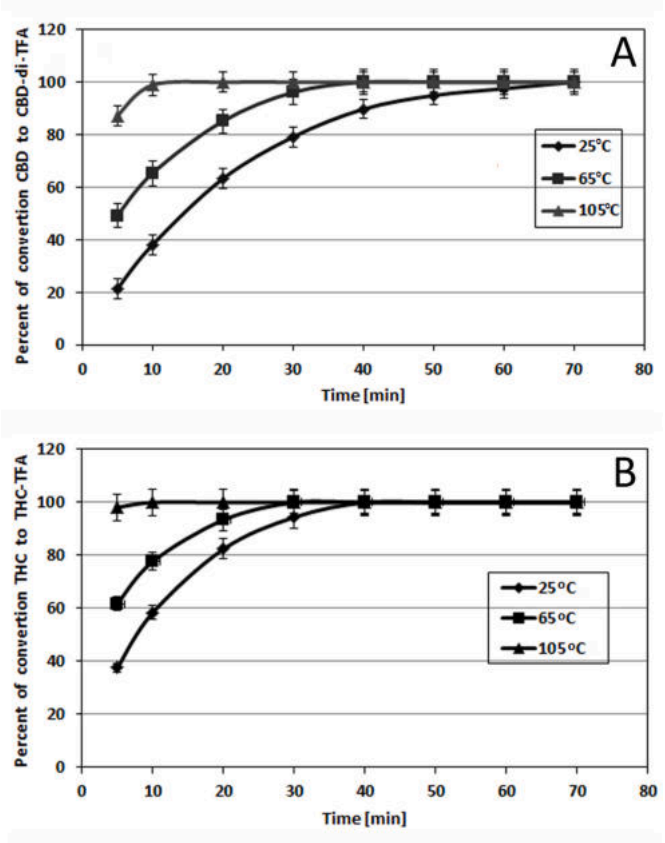


Fig. 6. The influence of acylation time of CBD (A) and Δ9-THC (B) by TFAA in the presence of TEA on the derivatization degree of these cannabinoids. Temperature of acylation process: 25 (dashed line with triangles), 65 (solid line with squares) and 105 °C (dotted line with diamonds).

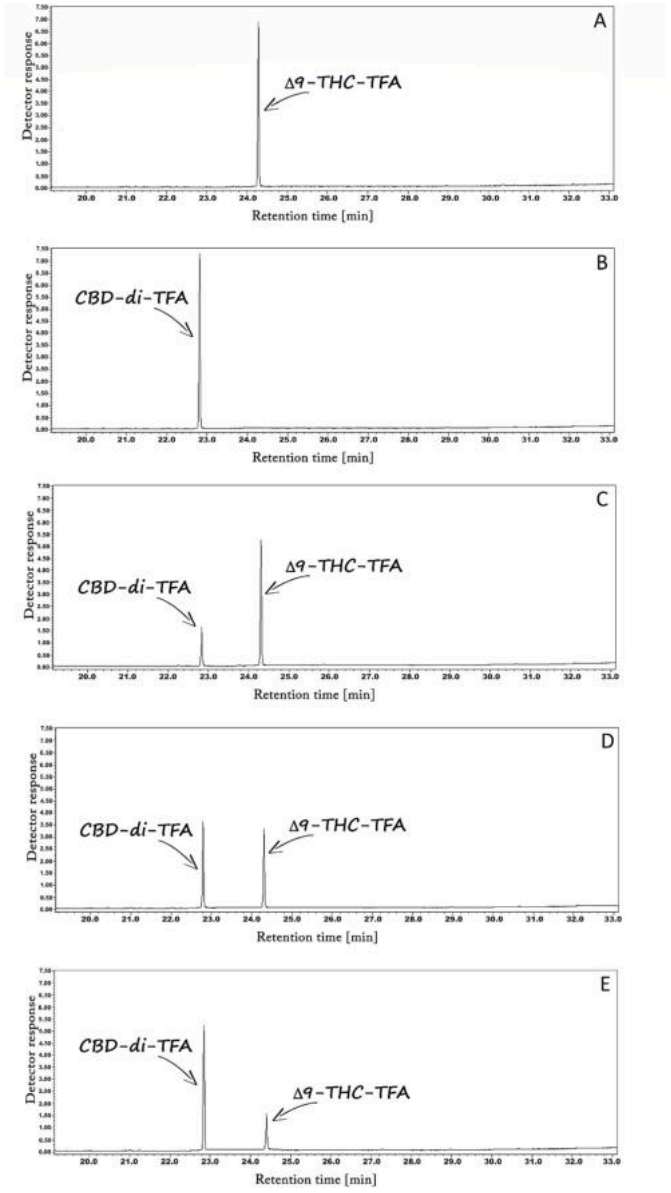


Fig. 7. GC-MS chromatogram (in SIM mode) of blood sample containing- Δ9-THC (A), or - CBD (B), or - mixture of Δ9-THC/CBD of molar ratio equal 3/1 (C) or - mixture of Δ9-THC/CBD of molar ratio equal 1/1 (D) or - mixture of Δ9-THC/CBD of molar ratio equal 1/3 (E) after its appropriate preparation procedure involving TFAA derivatization performed at the presence of TEA – for cannabinoid concentration see experimental part.

the results of GC-MS analyses of Δ9-THC and CBD in blood samples after their appropriate preparation involving TFAA derivatization performed in the presence of TEA. As can be seen, only trifluoroacetic esters of CBD and/or Δ9-THC (CBD-di-TFA and Δ9-THC-di-TFA) are registered on the chromatogram, although the initial cannabinoids and their other derivatives were also searched for in GC column outlet during the analyses (see caption to Fig. 7). Hence, the presented results prove that the quantification of THC in the presence of CBD is possible not only in an artificial sample but also in a natural one, when the processes of CBD cyclization to THC and Δ9-THC isomerization to Δ8-THC are blocked.

4. Conclusions

Methods for the analysis of cannabinoids in bio-matrices are continually improved to achieve best possible sensitivity for their detection and accuracy in their quantification. The most frequent way of

sensitivity increase of examined compounds analysis is their preliminary derivatization. Although silylation of compounds, including cannabinoids, is used for this purpose most often, there are many examples indicating that their acylation with acid anhydride or acid chloride is more advisable.

Difficulty in accurate quantification of Δ9-THC in the presence of CBD or of CBD itself or Δ9-THC itself when derivatization of these compounds by acylation is necessary, is due to a higher speed of CBD cyclization to Δ9-THC and/or Δ9-THC isomerization to Δ8-THC in relation to the speed of CBD and/or THC acylation. The presented results demonstrate that CBD cyclization and THC isomerization can be blocked by capturing protons *in statu nascendi* which appear in the reaction medium during the acylation process, using tertiary amines or azines. The described acylation procedure is applicable using both anhydrides and acid chlorides. The efficiency of CBD acylation depends on the time and temperature of the process, and the degree of CBD acylation depends on the mutual ratio of the cannabinoid, the acylating agent and the proton-binding compound.

Authors' contributions

Andrzej L. Dawidowicz: Conceptualization, Writing – original draft, Investigation, Writing- Reviewing and Editing, Methodology. Michal P. Dybowski: Writing- Original draft preparation, Writing- Reviewing and Editing, Investigation, Methodology, Data curation, Visualization. Piotr Holowinski: Writing- Original draft preparation, Investigation, Methodology, Data curation. Rafal Typek: Writing- Original draft preparation, Investigation, Methodology, Data curation. Michal Rombel: Writing- Original draft preparation, Investigation, Data curation

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123777.

References

[1] European Monitoring Centre for Drugs and Drug Addiction, Cannabis Legislation in Europe: an Overview, 2018.
[2] C. Duchateau, J.M. Kauffmann, M. Canfyn, C. Stévigny, K. De Braekeleer, E. Deconinck, Discrimination of legal and illegal Cannabis spp. according to European legislation using near infrared spectroscopy and chemometrics, Drug Test. Anal. 12 (2020) 1309–1319, https://doi.org/10.1002/DTA.2865.
[3] O. Devinsky, M.R. Cilio, H. Cross, J. Fernandez-Ruiz, J. French, C. Hill, R. Katz, V. Di Marzo, D. Jutras-Aswad, W.G. Notcutt, J. Martinez-Orgado, P.J. Robson, B. G. Rohrback, E. Thiele, B. Whalley, D. Friedman, Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders, Epilepsia 55 (2014) 791–802, https://doi.org/10.1111/epi.12631.
[4] K.M. Nelson, J. Bisson, G. Singh, J.G. Graham, S.N. Chen, J.B. Friesen, J.L. Dahlin, M. Niemitz, M.A. Walters, G.F. Pauli, The essential medicinal chemistry of cannabidiol (CBD), J. Med. Chem. 63 (2020) 12137–12155, https://doi.org/10.1021/acs.jmedchem.0c00724.
[5] C. Scuderi, D. De Filippis, T. Iuvone, A. Blasio, A. Steardo, G. Esposito, Cannabidiol in medicine: a review of its therapeutic potential in CNS disorders, Phyther. Res. 23 (2009) 597–602, https://doi.org/10.1002/ptr.2625.
[6] R. Mechoulam, L. Hanuš, Cannabidiol: an overview of some chemical and pharmacological aspects. Part I: chemical aspects, Chem, Phys. Lipids 121 (2002) 35–43, https://doi.org/10.1016/S0009-3084(02)00144-5.
[7] G.R.B. Webster, L.P. Sarna, R. Mechoulam, Conversion of CBD to d8-THC and d9-THC, U. S. Jpn. Outlook 7 (872 B2) (2008) 399.
[8] R. Andrews, S. Paterson, Production of identical retention times and mass spectra for Δ9-tetrahydrocannabinol and cannabidiol following derivatization with trifluoracetic anhydride with 1,1,1,3,3,3-hexafluoroisopropanol, J. Anal. Toxicol. 36 (2012) 61–65, https://doi.org/10.1093/jat/bkr017.
[9] J.M. Holler, M.L. Smith, S.N. Paul, M.R. Past, B.D. Paul, Isomerization of delta-9-THC to delta-8-THC when tested as trifluoroacetyl-, pentafluoropropionyl-, or heptafluorobutyryl- derivatives, J. Mass Spectrom. 43 (2008) 674–679, https://doi.org/10.1002/JMS.1375.
[10] M.P. Dybowski, A.L. Dawidowicz, R. Typek, M. Rombel, Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – troubles with reliable CBD quantitation when acidic precipitation agents are applied, Talanta 220 (2020), 121390, https://doi.org/10.1016/j.talanta.2020.121390.
[11] K. Watanabe, Y. Itokawa, S. Yamaori, T. Funahashi, T. Kimura, T. Kaji, N. Usami, I. Yamamoto, Conversion of cannabidiol to Δ9-tetrahydrocannabinol and related cannabinoids in artificial gastric juice, and their pharmacological effects in mice, Forensic Toxicol. 25 (2007) 16–21, https://doi.org/10.1007/s11419-007-0021-y.
[12] G.R. Handrick, R.K. Razdan, D.B. Uliss, H.C. Dalzell, E. Boger, Hashish. Synthesis of (±)-.Δ1- and d6-3,4-cis-cannabidiols and their isomerization by acid catalysis, J. Org. Chem. 42 (1977) 2563–2568, https://doi.org/10.1021/jo00435a007.
[13] Y. Gaoni, R. Mechoulam, Hashish—VII : the isomerization of cannabidiol to tetrahydrocannabinols, Tetrahedron 22 (1966) 1481–1488, https://doi.org/10.1016/S0040-4020(01)99446-3.
[14] R. Adams, C.K. Cain, W.D. Mchee, R.B. Wearn, Structure of cannabidiol. XII. Isomerization to tetrahydrocannabinols, J. Am. Chem. Soc. 63 (1941) 2209–2213, https://doi.org/10.1021/JA01853A052.
[15] T. Nadulski, F. Sporkert, M. Schnelle, A.M. Stadelmann, P. Roser, T. Schefter, F. Pragst, Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract, J. Anal. Toxicol. 29 (2005) 782–789, https://doi.org/10.1093/jat/29.8.782.
[16] M.J. Baptista, P.V. Monsanto, E.G. Pinho Marques, A. Bermejo, S. Ávila, A. M. Castanheira, C. Margalho, M. Barroso, D.N. Vieira, Hair analysis for delta(9)-THC, delta(9)-THC-COOH, CBN and CBD, by GC/MS-EI. Comparison with GC/MS-NCI for delta(9)-THC-COOH, Forensic Sci. Int. 128 (2002) 66–78, https://doi.org/10.1016/S0379-0738(02)00154-8.
[17] W. Quarles, G. Ellman, R. Jones, Toxicology of marijuana: conditions for conversion of cannabidiol to THC upon smoking, Clin. Toxicol. 6 (1973) 211–216, https://doi.org/10.3109/15563657308990520.
[18] P. Marzullo, F. Foschi, D.A. Coppini, F. Fanchini, L. Magnani, S. Rusconi, M. Luzzani, D. Passarella, Cannabidiol as the substrate in acid-catalyzed intramolecular cyclization, J. Nat. Prod. 83 (2020) 2894–2901, https://doi.org/10.1021/ACS.JNATPROD.0C00436/SUPPL_FILE/NP0C00436_SI_001.PDF.
[19] P. Holowinski, R. Typek, A.L. Dawidowicz, M. Rombel, M.P. Dybowski, Formation of trifluoroacetic artefacts in gas chromatograph injector during Cannabidiol analysis, J. Chromatogr. A 1671 (2022), 463020, https://doi.org/10.1016/J.CHROMA.2022.463020.

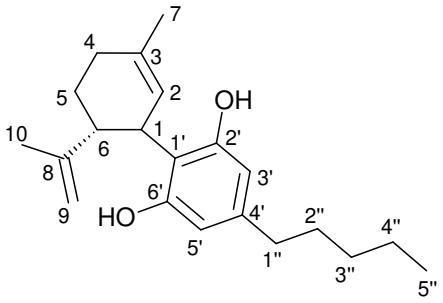
Supplementary materials

Chromatographic analysis of CBD and THC after their acylation with blockade of compound transformation

Rafał Typek, Piotr Hołowiński, Andrzej L. Dawidowicz, Michał P. Dybowski, Michał Rombel

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031, Lublin, Poland

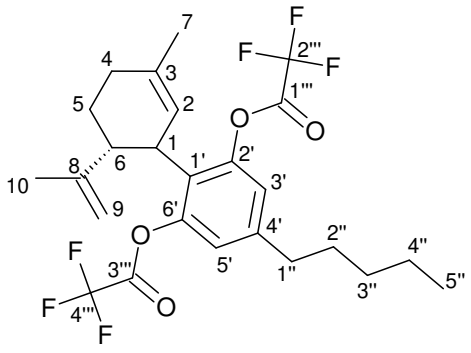
Table 1S. ^{1}H and ^{13}C NMR data of the CBD in $CDCl_3$.



No.	δ_C, type	δ_H (J in Hz)
1	37.3, CH	3.85, m
2	124.1, CH	5.57, s
3	140.0, C	-
4	30.4, CH_2	2.23, m 2.10, m
5	28.4, CH_2	1.82, m[a]
6	46.2, CH	2.39, m
7	23.7,CH_3	1.79, brs[a]
8	149.4, C	-
9	110.8, CH_2	4.66, quint (1.5) 4.56, brs
10	20.5,CH_3	1.66, s
1′	113.8, C	-
2′	153.8, C	-
3′	109.8, CH	6.28, brs
4′	143.1, C	-
5′	108.0, CH	6.16, brs
6′	156.0, C	-
2′-OH	-	4.68, brs
6′-OH	-	5.99, brs
1″	35.5, CH_2	2.43, t (7.7)
2″	30.6, CH_2	1.56, quint (7.5)
3″	31.5, CH_2	1.27, m[b]
4″	22.5, CH_2	1.31, m[b]
5″	14.0, CH_3	0.88, t (7.0)

[a, b] Overlapping signals.

Table 2S. ^{1}H, ^{13}C and ^{19}F NMR of CBD-di-TFA in $CDCl_3$.



No.	δ_C, type	δ_H (*J* in Hz) or δ_F
1	38.7, CH	3.52, m
2	121.9, CH	5.10, s
3	135.8, C	-
4	30.1, CH_2	2.13, m
		2.00, dd (17.7, 4.9)
5	28.5, CH_2	1.80, m
		1.71, qd (12.5, 5.4)
6	45.5, CH	2.57, ddd (12.5, 10.5, 2.4)
7	23.0, CH_3	1.62, s[b]
8	147.2, C	-
9	115.5, CH_2	4.55, t (1.7)
		4.44, s
10	19.5, CH_3	1.52, s
1′	126.4, C	-
2′	148.3, C	-
3′	121.5 or 120.1[a], CH	6.90, m[c]
4′	143.6, C	-
5′	121.5 or 120.1[a], CH	6.90, m[c]
6′	148.3, C	-
1″	35.1, CH_2	2.61, t (7.8)
2″	30.3, CH_2	1.61, m[b]
3″	31.3, CH_2	1.31, m[d]
4″	22.4, CH_2	1.32, m[d]
5″	14.0, CH_3	0.89, t (6.9)
1‴	155.6, C, brs	-
2‴	114.6, CF_3	-74.34 or -74.56[e], brs
	J_{C-F}= 286.0 Hz	
3‴	155.6, C, brs	-
4‴	114.6, CF_3,	-74.34 or -74.56[e], brs
	J_{C-F}= 286.0 Hz	

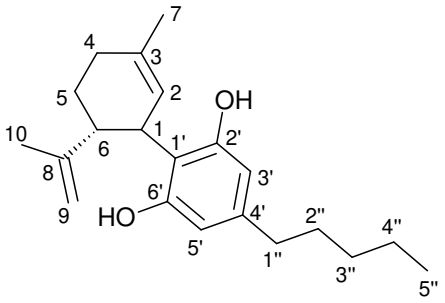
[a, e] Considered signals cannot be unequivocally assigned.
[b-d] Overlapping signals.

Table 3S. MS^2 and HR-MS data of CBD-di-TFA.

HRMS				
Theoretical mass (Da)	Experimental mass (Da)	Δ mDa	Δ ppm	Formula
507.19700	507.19709	0.09	0.18	$C_{25}H_{29}F_6O_4$

MS/MS			
MS^1	MS^2		
Parent ion	Base peak	Secondary peak	
m/z	m/z	m/z	Intensity (%)
507.2	410.2	123.1	3.2
		193.2	7.5
		313.1	12.4
		325.1	47.8
		341.2	27.3
		394.2	5.1
		439.1	23.2

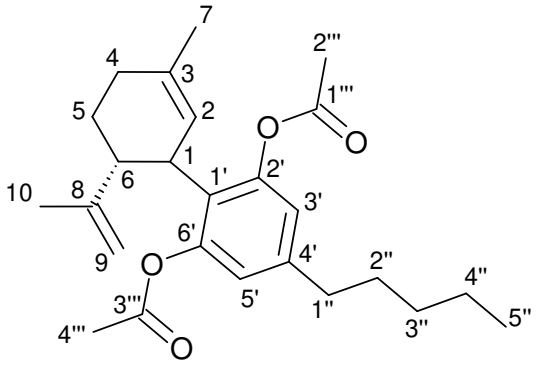
Table 4S. ^{1}H and ^{13}C NMR data for CBD in DMSO-d_6.



No.	δ_C, type	δ_H (*J* in Hz)
1	36.0, CH	3.83, m
2	127.3, CH	5.09, s
3	130.5, C	-
4	30.8, CH_2	2.10, td (11.3, 5.3)
		1.92, dd (17.2, 4.3)
5	30.0, CH_2	1.69, m
		1.63, m[a]
6	44.1, CH	3.03, ddd (12.5, 10.4, 2.3)
7	23.8,CH_3	1.60, brs[a]
8	149.6, C	-
9	110.1, CH_2	4.50, d (2.3)
		4.41, m
10	19.7,CH_3	1.59, s
1′	114.6, C	-
2′	156.7, C	-
3′	107.0, CH	6.02, s
4′	140.6, C	-
5′	107.0, CH	6.02, s
6′	156.7, C	-
2′-OH	-	8.65, brs
6′-OH	-	8.65, brs
1″	35.4, CH_2	2.30, t (7.6)
2″	30.8, CH_2	1.47, quint (7.5)
3″	31.5, CH_2	1.25, m[b]
4″	22.5, CH_2	1.29, m[b]
5″	14.4, CH_3	0.86, t (7.1)

[a, b] Overlapping signals.

Table 5S. ^{1}H and ^{13}C NMR data of CBD-di-Ac in DMSO-d_6.



No.	δ_C, type	δ_H (*J* in Hz)
1	37.9, CH	3.42, m
2	124.5, CH	4.95, brs
3	133.0, C	-
4	30.2, CH_2	2.17, m[b]
		1.97, brd (17.6)
5	28.5, CH_2	1.68, m
6	45.7, CH	2.62, m
7	23.7, CH_3	1.61, brs
8	141.9, C	-
9	111.5, CH_2	4.46, m
		4.37, brd (1.9)
10	19.4, CH_3	1.56, s
1′	126.4, C	-
2′	147.9, C	-
3′	119.9 or 121.8[a], CH	6.76, s
4′	141.9, C	-
5′	119.9 or 121.8[a], CH	6.76, s
6′	149.9, C	-
1″	34.6, CH_2	2.51, m
2″	30.4, CH_2	1.53, quint (7.6)
3″	31.2, CH_2	1.24, m[c]
4″	22.4, CH_2	1.29, m[c]
5″	14.4, CH_3	0.85, t (7.1)
1‴	169.5, C	-
2‴	21.1, CH_3	2.24 or 2.17 [d], brs[b]
3‴	169.5, C	-
4‴	21.1, CH_3	2.24 or 2.17 [d], brs[b]

[a, d] Considered signals cannot be unequivocally assigned.

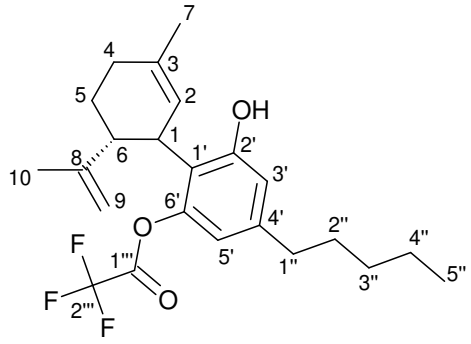
[b, c] Overlapping signals.

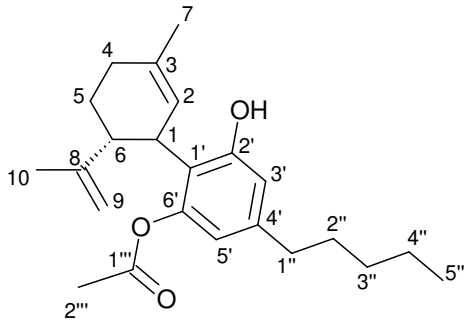
Table 6S. MS^2 and HR-MS data of CBD-di-Ac.

HRMS				
Theoretical mass (Da)	Experimental mass (Da)	Δ mDa	Δ ppm	Formula
399.25354	399.25343	-0.11	0.28	$C_{25}H_{35}O_4$

MS/MS			
MS^1	MS^2		
Parent ion	Base peak	Secondary peak	
m/z	m/z	m/z	Intensity (%)
399.3	355.2	123.1	3.5
		193.2	7.7
		297.2	4.2
		313.1	16.5
		339.2	21.4

Table 7S. ^{1}H, ^{13}C and ^{19}F NMR data for CBD-mono-TFA in $CDCl_3$ (Table 7S A) and ^{1}H and ^{13}C NMR data for CBD-mono-Ac in DMSO-d_6 (Table 7S B).





	A		B	
No.	δ_C, type	δ_H (*J* in Hz) or δ_F	δ_C, type	δ_H (*J* in Hz)
1	38.0, CH	3.48, brs	35.9, CH	3.84, brs
2	122.6, CH	5.52, s	125.9, CH	5.00, brs
3	141.2, C	-	131.8, C	-
4	30.3, CH_2	2.22, m 2.11, m	30.6, CH_2	2.15, m[c] 1.96, m
5	27.9, CH_2	1.83, m[a] 1.74, qd (12.4, 5.4)	29.2, CH_2	1.67, m 1.64, m
6	45.5, CH	2.43, m	46.1, CH	2.64, brs
7	23.6, CH_3	1.79, brs[a]	23.8, CH_3	1.61, brs[d]
8	146.7, C	-	148.6, C	-
9	111.9, CH_2	4.60, quint (1.5) 4.39, brs	110.8, CH_2	4.43, brs 4.41, brs
10	19.9, CH_3	1.55, s	19.5, CH_3	1.59, brs[d]
1′	118.5, C	-	120.5, C	-
2′	156.0, C	-	156.5, C	-
3′	112.7, CH	6.48, s	114.6, CH	6.23, d (1.5)
4′	143.5, C	-	141.2, C	-
5′	116.0, CH	6.64, s	112.8, CH	6.45, d (1.4)
6′	147.7, C	-	150.4, C	-
2′-OH	-	6.08, brs	-	9.4, brs
1″	35.3, CH_2	2.52, t (7.6)	35.0, CH_2	2.40, t (7.7)
2″	30.3, CH_2	1.58, quint (7.6)	30.6, CH_2	1.50, quint (7.5)
3″	31.4, CH_2	1.28, m[b]	31.3, CH_2	1.24, m[e]
4″	22.4, CH_2	1.31, m[b]	22.4, CH_2	1.29, m[e]
5″	14.0, CH_3	0.88, t (7.0)	14.4, CH_3	0.86, t (7.1)
1‴	155.6, C, brs	-	169.2, C	-
2‴	114.7, CF_3, J_{C-F}= 286.0 Hz	-74.41, brs	21.1, CH_3	2.11, brs[c]

[a-e] Overlapping signals.

Table 8S. MS^2 and HR-MS data of CBD- mono-TFA (Table 8S A) and CBD-mono-Ac (Table 8S B).

A

HRMS				
Theoretical mass (Da)	Experimental mass (Da)	Δ mDa	Δ ppm	Formula
411.21470	411.21477	0.07	0.17	$C_{23}H_{30}F_3O_3$

MS/MS			
MS^1	MS^2		
Parent ion	Base peak	Secondary peak	
m/z	m/z	m/z	Intensity (%)
411.2	313.2	123.1	2.7
		193.2	6.4
		325.1	34.2
		341.2	21.3
		410.2	5.5

B

HRMS				
Theoretical mass (Da)	Experimental mass (Da)	Δ mDa	Δ ppm	Formula
357.24297	357.24292	-0.05	0.14	$C_{25}H_{35}O_4$

MS/MS			
MS^1	MS^2		
Parent ion	Base peak	Secondary peak	
m/z	m/z	m/z	Intensity (%)
357.2	313.2	123.1	5.7
		193.2	7.5
		297.2	6.2
		356.2	9.7

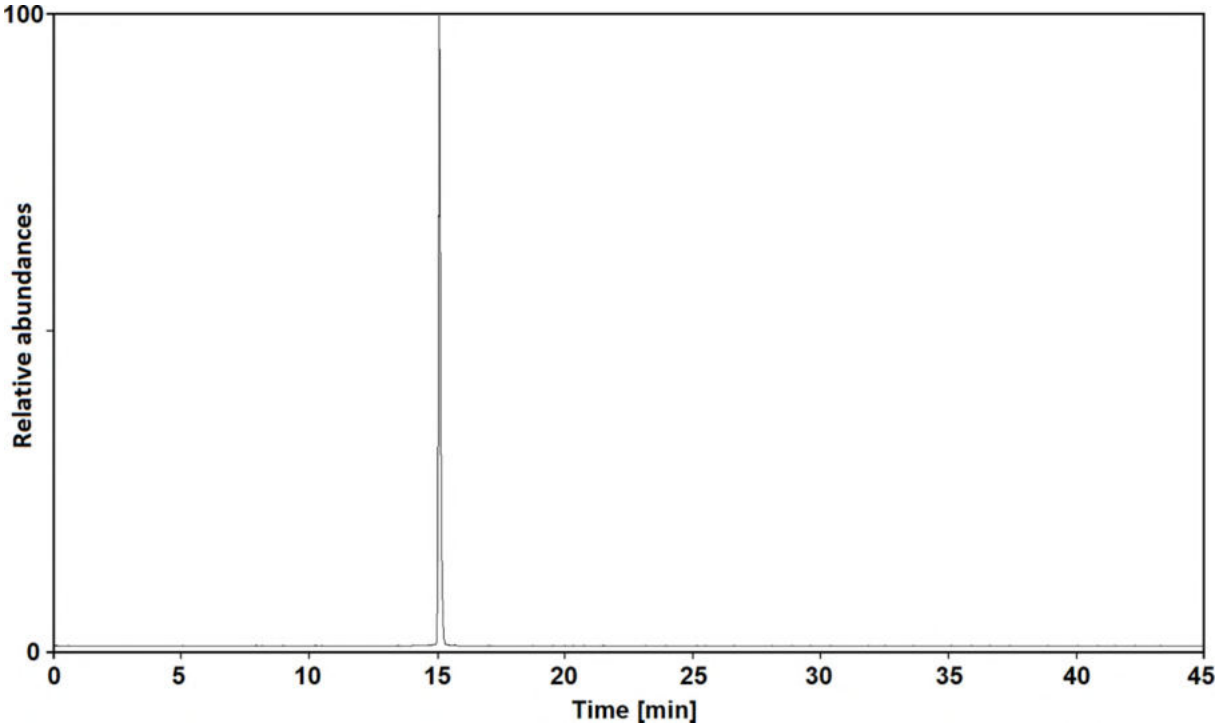


Fig. 1S. LC–MS chromatogram of CBD-di-TFA methanolic solution.

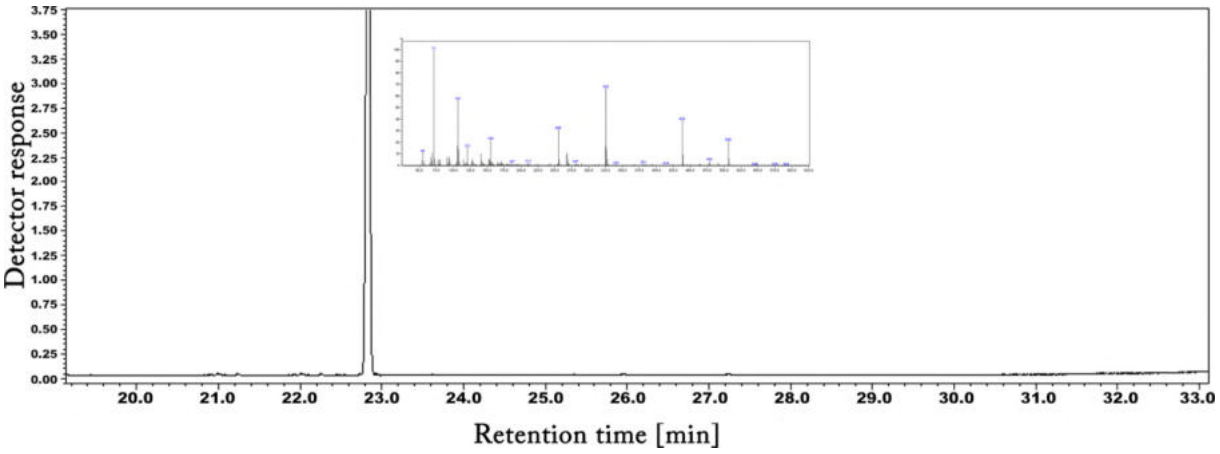


Fig. 2S. GC-MS chromatogram and MS data for the synthetized CBD-di-TFA derivative.

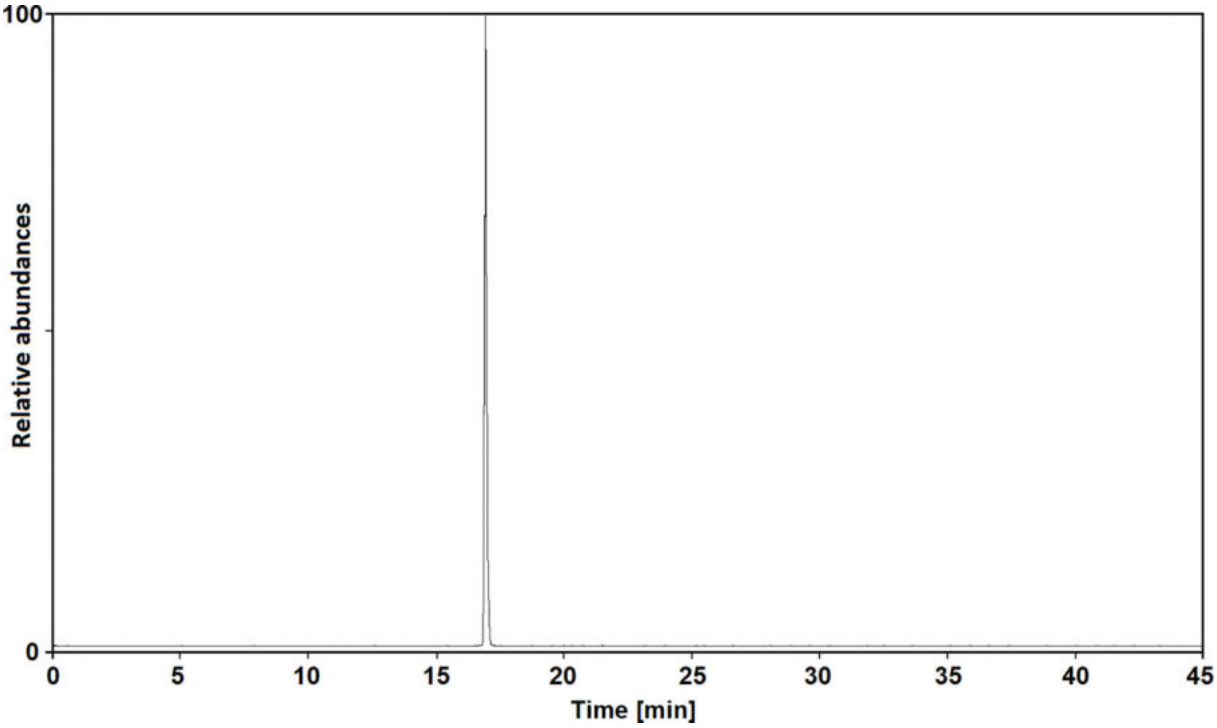


Fig. 3S. LC–MS chromatogram of CBD-di-Ac methanolic solution.

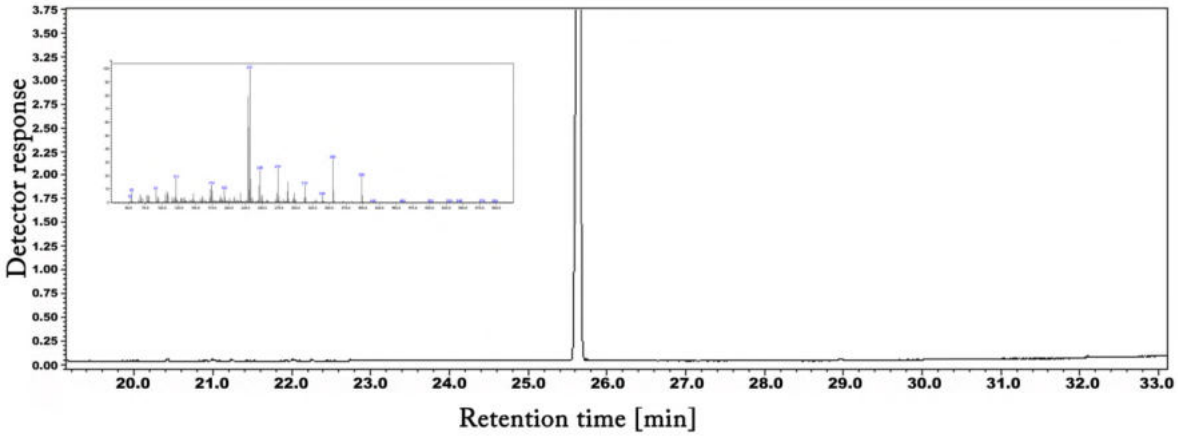


Fig. 4S. GC-MS chromatogram for the synthetized CBD-di-Ac derivative.

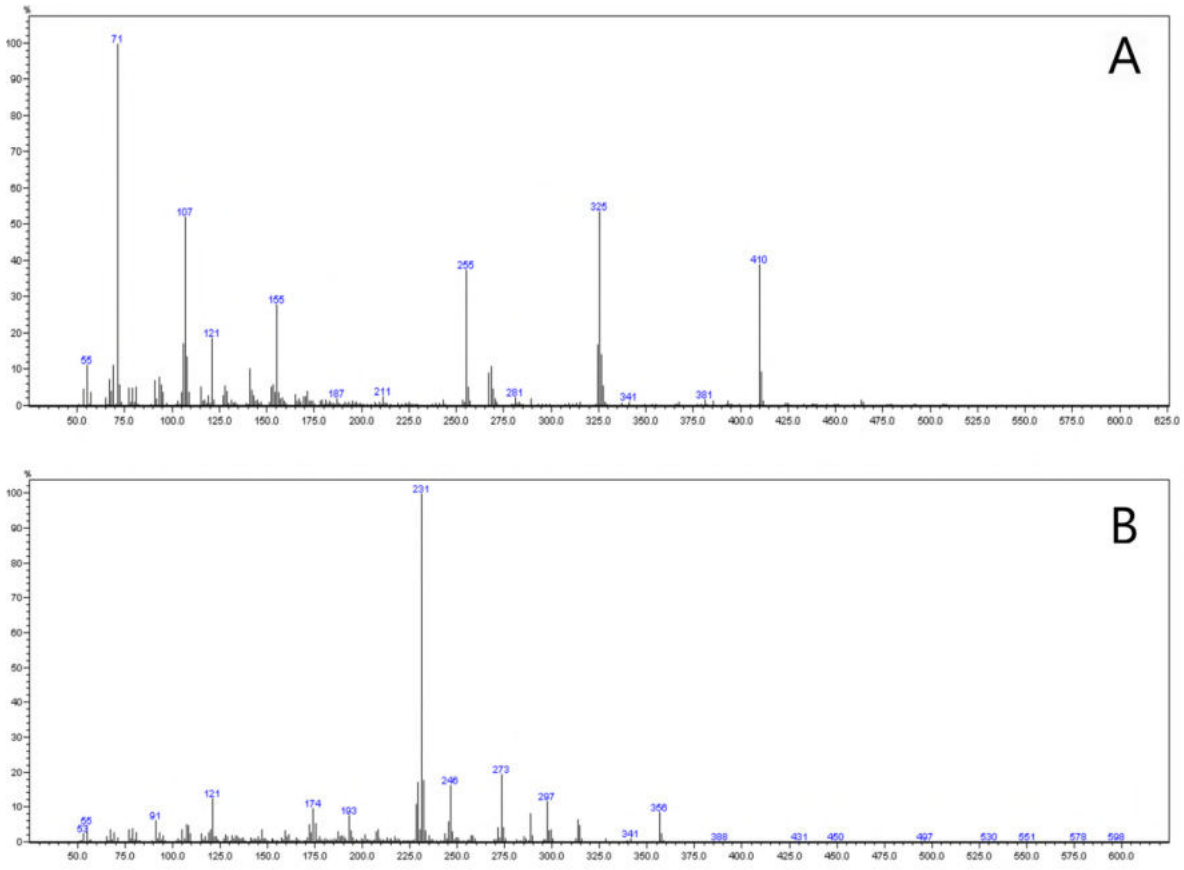


Fig. 5S. MS spectra from GC-MS for CBD-mono-TFA (A) and CBD-mono-Ac (B).

Publikacja D6

A.L. Dawidowicz, M.P. Dybowski, R. Typek, **M. Rombel**, P. Hołowiński

Unexpected formation of dichloroacetic and trichloroacetic artefacts in gas chromatograph injector during Cannabidiol analysis

J. Pharm. Biomed. Anal. 230 (2023) 115388

https://doi.org/10.1016/J.JPBA.2023.115388

IF 2-letni: 3,4; MEiN: 100 pkt

Journal of Pharmaceutical and Biomedical Analysis 230 (2023) 115388

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Short communication

Unexpected formation of dichloroacetic and trichloroacetic artefacts in gas chromatograph injector during Cannabidiol analysis

Andrzej L. Dawidowicz *, Michal P. Dybowski *, Rafal Typek, Michal Rombel, Piotr Holowinski

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie Sklodowska University in Lublin, Lublin 20-031, Poland

ARTICLE INFO

Keywords:
CBD transformation
Trichloroacetic esters of THC
Dichloroacetic esters of THC
Plasma precipitation
GC–MS

ABSTRACT

The knowledge about the stability of compounds and possible ways of their transformation in the process of sample preparation for analysis and during analysis itself is very helpful in the assessment of possible errors which can appear when an accurate and precise estimation of compound concentration in tested samples is attempted. The present paper shows that a significant amount of CBD present in the blood/plasma sample analyzed by means of GC transforms in the hot GC injector not only to 9α-hydroxyhexahydrocannabinol, 8-hydroxy-iso-hexahydrocannabinol, Δ9-tetrahydrocannabinol, Δ8-tetrahydrocannabinol, and cannabinol but also to the trichloroacetic esters of Δ9-THC and Δ8-THC and, unexpectedly, to their dichloroacetic esters when trichloroacetic acid is used as protein precipitation agent. The increase of GC injector temperature favors the formation of dichloroacetic esters of Δ9-THC and Δ8-THC in relation to their trichloroacetic ones. The appearance of dichloroacetic esters of Δ9-THC and Δ8-THC among CBD transformation products is probably the result of the thermal decomposition of their trichloroacetic esters. The transformation of trichloroacetic derivatives of organic compounds into their dichloroacetic derivatives in GC injector has not been reported yet. The instability of trichloroacetic derivatives of Δ8-/Δ9-THC during their GC analysis is probably accounts for the lack of their GC-MS spectra in the databases. NMR, GC-MS and LC-MS spectra of the newly discovered derivatives constitute an important element of the work. The obtained results demonstrate why the use of trichloroacetic acid for plasma samples deproteinization should be avoided when CBD and/or THC are determined by GC.

1. Introduction

Cannabidiol (CBD), 2-[(1 R,6 R)− 3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]− 5-pentylbenzene-1,3-diol is one of the ingredients of marijuana and hemp plants most frequently discussed in the literature [1–4]. This non-psychoactive cannabinoid, equally popular today as the psychoactive Δ9-tetrahydrocannabinol (Δ9-THC), is of interest for its bioactive properties suggesting therapeutic use. Current knowledge about the pharmacological actions of CBD is smaller than that of Δ9-THC, yet there is already no doubt that its pharmacological actions differ markedly from those of the latter compound [5,6]. Research has shown [7–12] that CBD may be applicable for:

(i) the management of epilepsy and certain other central motor disorders;
(ii) the treatment of anxiety, psychotic illnesses, and neurotoxicity, for instance associated with stroke;
(iii) inflammation treatment;
(iv) the attenuation of unwanted side-effects of Δ9-THC used as a medicine.

Other potential therapeutic uses for CBD include vomiting, glaucoma, sleep and appetite disorders, and cancer [13–17].

The growing interest in CBD resulting from research and clinical observations, as well as a marked increase in the use of dietary supplements containing CBD, especially CBD oils, in self-healing therapies [18] necessitate the development of reliable and sensitive analytical procedures for its quantitative determination in blood/plasma samples. Although the analytical procedures for the determination of CBD in blood/plasma samples by chromatographic methods recommend the use of LLE, SPE or QuEChERS as sample preparation methods, it should be noted that many analysts still adhere to the older method of sample preparation, i.e. protein precipitation. Protein precipitation as blood/plasma sample preparation method is especially recommended in analytical procedures of xenobiotics strongly binding to plasma proteins [19–22] one of them being hydrophobic CBD [23,24].

* Corresponding authors.
E-mail addresses: andrzej.dawidowicz@mail.umcs.pl (A.L. Dawidowicz), michal.dybowski@mail.umcs.pl (M.P. Dybowski).

https://doi.org/10.1016/j.jpba.2023.115388
Received 23 February 2023; Received in revised form 4 April 2023; Accepted 7 April 2023
Available online 8 April 2023

As shown in [25,26], a significant amount of CBD present in the blood/plasma sample analyzed by means of GC undergoes transformation in the hot GC injector when acidic protein precipitation agents are used for deproteination of the samples before analysis. CBD transformation products are particularly numerous in the GC injector when trifluoroacetic acid (TFA) is used as a deproteinizer. In case of TFA, CBD transforms not only to 9α-hydroxyhexahydrocannabinol (9α-OH-HHC), 8-hydroxy-iso-hexahydrocannabinol (8-OH-iso-HHC), Δ9-tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC), and cannabinol (CBN), which generally form when any acidic deproteining agent is used, but also to the trifluoroacetic esters of Δ9-THC and Δ8-THC.

A compound more commonly used to deproteinize blood/plasma samples than TFA is its analog, trichloroacetic acid (TCA). Due to its chemical reactivity, equally high as that of TFA, it is worth exploring whether TCA derivatives with Δ9-THC, Δ8-THC, and also with other CBD transformation products (9α-OH-HHC, 8-OH-iso-HHC and CBN) are formed when TCA is applied as a protein precipitation agent in sample preparation for CBD analysis in plasma by GC.

The study shows not only why the estimation CBD concentration in blood/plasma samples by GC is not possible if TCA is used for their deproteinization, but also presents unexpected transformation of trichloroacetic esters of Δ9-THC and Δ8-THC to their dichloroacetic derivatives in GC injector.

2. Materials and methods

2.1. Reagents and standards

Acetonitrile (ACN) (LC-MS grade) was purchased from Merck (Warszawa, Poland). The standards (certified reference materials) of Δ8-THC and Δ9-THC (1.0 mg/mL in methanol - Cerilliant) and CBD (1.0 mg/mL in methanol - Cerilliant), trichloroacetic acid (TCA) (>99%), formic acid, deuterated chloroform ($CDCl_3$), trichloroacetic anhydride (TCAA), and dichloroacetic anhydride (DCAA) were acquired from Sigma-Aldrich (Poznan, Poland). Dichloromethane (DCM), n-hexan and sodium bicarbonate were purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). CBD crystal (>99%) was a gift from CannLAB (Kraków, Poland). Deionized water was purified by the Milli-Q system (Millipore Sigma, Bedford, MA, USA).

The blood samples were collected by a registered nurse from volunteers, after obtaining their informed consent, using a single closed system containing an S-Monovette coagulation activator, according to the manufacturer instructions (Sarstedt AG, Nümbrecht, Germany), and thoroughly mixed in order to maintain their homogeneity. Blood donors did not take preparations containing cannabinoids. After collection, blood samples were stored at + 4 °C and used within 48 h.

2.2. Preparation of THC-TCA and THC-DCA esters

Preliminary studies have indicated that not only trichloroacetic esters of Δ8-THC and Δ9-THC but also dichloroacetic esters of Δ8-THC and Δ9-THC are formed in the GC injector during CBD analysis in blood/plasma samples if TCA is used for their deproteinization. In order to confirm the above, these esters were synthetized using CBD in separate experiments and tested by NMR, LC-MS and GC-MS.

As known from [27,28], CBD in an anhydrous acidic environment cyclizes into Δ9-THC, which than transforms into Δ8-THC. CBD acylation with acetic acylation reagents, like TCAA or DCAA, leads to acetic derivatives of Δ8-THC and Δ9-THC. The synthesis procedure of Δ8-, Δ9-THC-TCA and Δ8-, Δ9-THC-DCA mixtures was as follows:

2.2.1. Preparation of THC-TCA esters

The trichloroacyl derivatives of Δ8-THC and Δ9-THC were prepared by heating the mixture composed of TCAA solution in DCM (25%) (500 μL) and CBD solution in DCM (20 mg/mL) (500 μL) at 65 °C for 60 min. The molar ratio of TCAA to CBD was 0.68: 0.03. To remove the excess amount of the derivatizing agent and byproducts formed during the reaction, the obtained mixture was subsequently extracted with saturated sodium bicarbonate water solution (3 ×1 mL). Finally, the organic phase was divided into two portions, each of which was evaporated to dryness under nitrogen stream. The obtained dry residues were dissolved in an appropriate solvent ($CDCl_3$ or acetonitrile) and subjected to further analysis. The above procedure is optimal and results from studying the influence of time, temperature and analyte concentrations on the formation of THC-TCA esters in its DCM solution.

2.2.2. Preparation of THC-DCA esters

The dichloroacetic esters of Δ8-THC and Δ9-THC were prepared analogically to the trichloroacetic esters, but dichloroacetic anhydride (DCAA) was used as a derivatizing reagent. The molar ratio of DCAA to CBD was 0.77: 0.03. The above procedure is optimal and results from studying the influence of time, temperature and analyte concentrations on the formation of THC-DCA esters in its DCM solution.

2.3. Plasma protein precipitation procedure

TCA, the precipitation agent (25 μL), was added to 475 μL of human plasma containing CBD (10 μg/mL). The samples were vortex mixed, incubated for 1 h, and centrifuged for 5 min at 18,600 x g. To 400 μL of the obtained supernatant 400 μL of hexane was added. After vortexing the mixture was centrifuged and hexane phase was subjected to GC-MS.

Protein precipitation in the experiments was performed using excess amount of TCA, as provided for the precipitation procedures.

2.4. GC–MS measurements

Qualitative analyses of CBD and CBD transformation products were conducted using a gas chromatograph hyphenated with a triple quadrupole tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan). GC–MS conditions were as follows: capillary column - Zebron ZB5-MSi (30 m x 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Torrance, CA, USA); carrier gas: helium (grade 5.0); flow rate: 1.0 mL/min; splitlesst injection mode (sampling time: 1.00 min); injector temperature: 260; 285 and 310 °C; injection volume: 1 μL; temperature program - initial temperature 60 °C held for 3 min and subsequently increased to 310 °C at a rate of 12 °C/min. The final temperature was held for 15 min. Mass spectrometer parameters: normalized electron energy of 70 eV; transfer line temperature: 285 °C; ion source temperature: 225 °C. The full SCAN mode with range 40–550 *m/z* was used.

In order to analyze extracts from the supernatants centrifuged from human plasma, samples spiked with CBD after their preliminary protein precipitation by TCA, multiple reaction monitoring (MRM) mode was used. GC-MS/MS analysis was performed using characteristic MRM transitions at optimal collision energies (CE) for the examined compounds. Three MRM transitions (*m/z* = > *m/z*) of the highest intensity were selected for further experiments:

1. CBD: 314 = > 246 (CE = 20 eV), 314 = > 231 (CE = 15 eV) and 314 = > 193 (CE = 12 eV);
2. Δ8-THC: 314 = > 246 (CE = 20 eV), 314 = > 231 (CE = 15 eV) and 314 = > 193 (CE =12 eV);
3. Δ9-THC: 314 = > 246 (CE = 20 eV), 314 = > 231 (CE = 15 eV) and 314 = > 193 (CE = 12 eV);
4. CBN: 310 = > 295 (CE = 20 eV), 310 = > 238 (CE = 15 eV) and 295 = > 238 (CE = 12 eV);
5. Δ8-THC-DCA: 425 = > 409 (CE = 22 eV), 425 = > 297 (CE = 18 eV) and 297 = > 229 (CE = 12 eV);
6. Δ9-THC-DCA: 425 = > 409 (CE = 22 eV), 425 = > 297 (CE = 18 eV) and 297 = > 229 (CE = 12 eV);

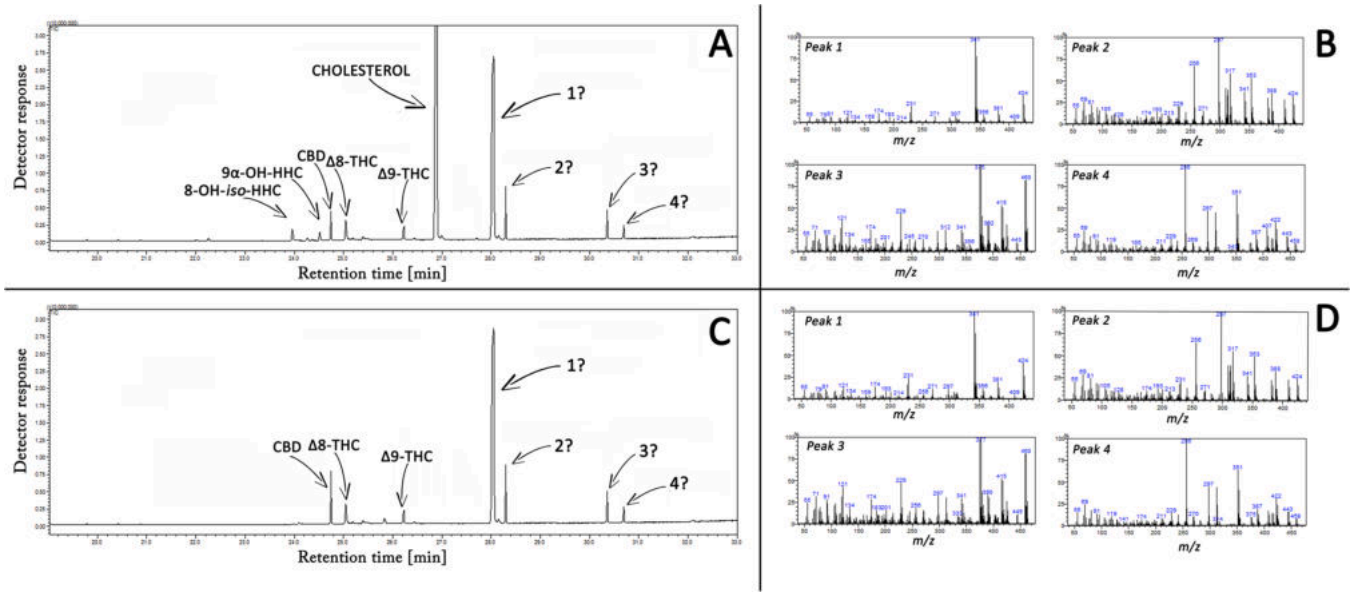


Fig. 1. GC-MS chromatograms (in Scan mode) of the supernatant centrifuged from human plasma samples spiked with CBD (10 μg/mL) after its preliminary protein precipitation by TCA (A), and CBD solution (10 μg/mL) in acetonitrile containing TCA (C). GC-MS spectra (B) and (D) correspond to unidentified substances (peaks 1–4) from (A) and (C) chromatograms, respectively.

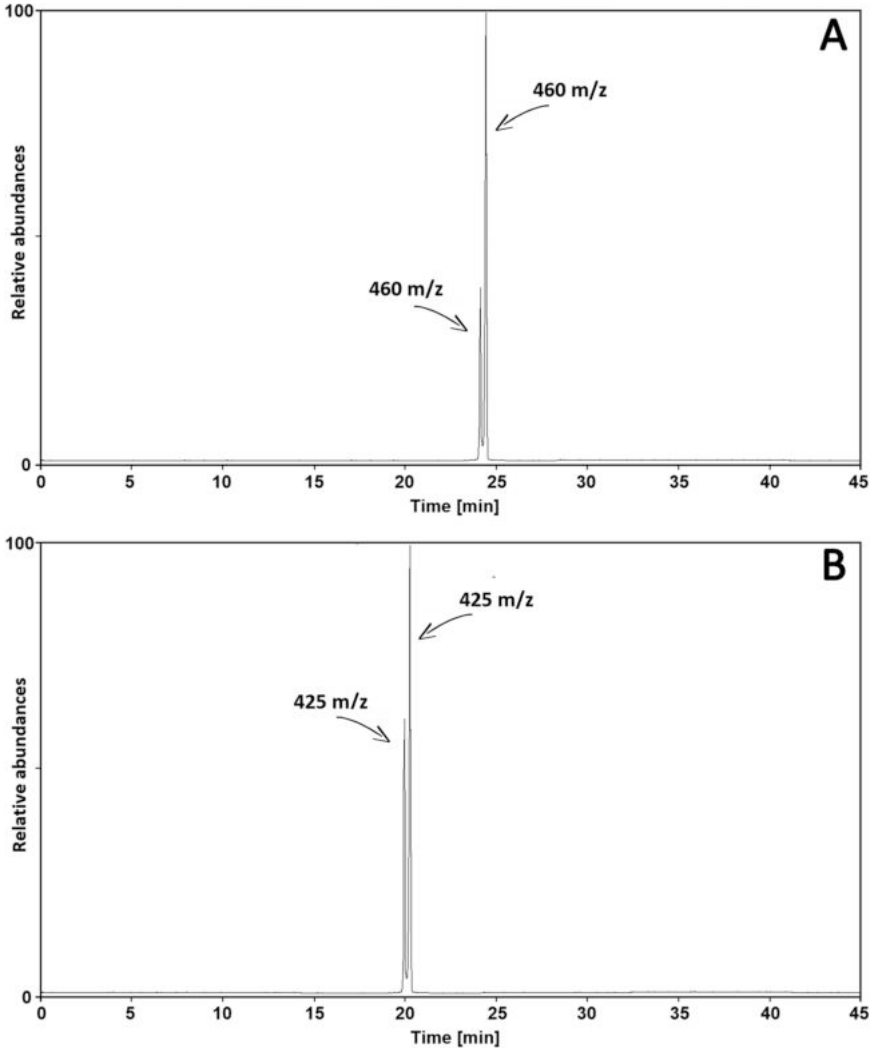
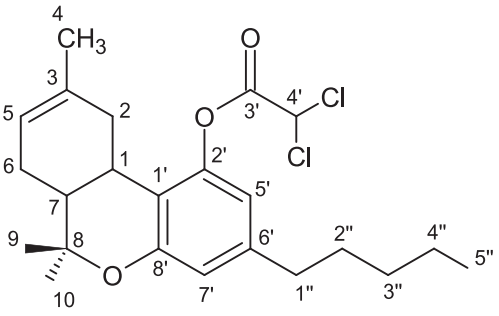
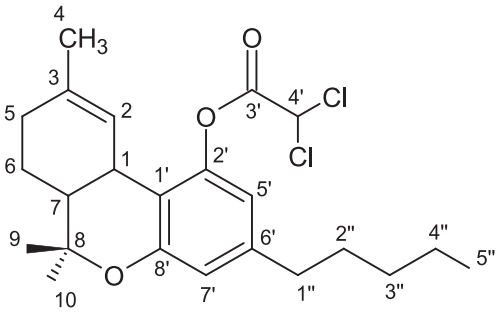


Fig. 2. LC-MS chromatograms (in Scan mode) of the mixture of trichloroacetic (A) and dichloroacetic (B) derivatives of Δ8- and Δ9-THC.

Table 1
^{1}H and ^{13}C NMR data of Δ8-THC-DCA (A) and Δ9-THC-DCA (B) in $CDCl_3$.



A



B

No.	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	31.6, CH	2.59, td (10.9, 4.8)	33.8, CH	3.10, m
2	36.4, CH_2	2.84, dd (16.7, 4.0)	123.3, CH	5.93, m
		1.90, m		
3	134.1, C	-	134.6, C	-
4	23.5, CH_3	1.67, s	23.2, CH_3	1.65, s[c]
5	119.6, CH	5.42, m	30.9, CH_2	2.14, m
6	27.6, CH_2	2.14, m	24.9, CH_2	1.90, m
		1.81, m[a]		1.35, m
7	44.9, CH	1.80, m[a]	45.4, CH	1.68, m[c]
8	77.2, C	-	77.4, C	-
9	27.5, CH_3	1.39, s	27.4, CH_3	1.42, s
10	18.6, CH_3	1.10, s	19.3, CH_3	1.10, s
1′	110.3, C	-	110.8, C	-
2′	149.2, C	-	149.3, C	-
3′	162.5, C	-	162.0, C	-
4′	64.2, $CHCl_2$	6.15, s	64.1, $CHCl_2$	6.16, s
5′	113.3, CH	6.45, d (1.6)	113.1, CH	6.47, d (1.6)
6′	143.4, C	-	143.2, C	-
7′	116.2, CH	6.62, d (1.6)	116.0, CH	6.60, d (1.6)
8′	154.7, C	-	154.4, C	-
1′	35.5, CH_2	2.51, t (7.7)	35.3, CH_2	2.52, t (7.7)
2′	30.6, CH_2	1.58, quint (7.5)	30.8, CH_2	1.57, quint (7.5)
3′	29.7, CH_2	1.27, m[b]	29.7, CH_2	1.27, m[d]
4′	22.4, CH_2	1.30, m[b]	22.6, CH_2	1.29, m[d]
5′	14.2, CH_3	0.89, t (7.0)	14.0, CH_3	0.88, t (7.1)

a-d Overlapping signals.

7. Δ8-THC-TCA: 462 = > 446 (CE = 22 eV), 462 = > 297 (CE = 18 eV) and 297 = > 229 (CE = 12 eV);
8. Δ9-THC-TCA: 462 = > 446 (CE = 22 eV), 462 = > 297 (CE = 18 eV) and 297 = > 229 (CE = 12 eV);
9. *exo*-THC: 314 = > 299 (CE = 20 eV), 314 = > 271 (CE = 15 eV) and 314 = > 231 (CE = 12 eV);
10. 9α-OH-HHC: 332 = > 314 (CE = 20 eV), 332 = > 299 (CE = 15 eV) and 314 = > 231 (CE = 12 eV).

2.5. LC-MS measurements

An LC-MS system composed of an UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) and a linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) was applied for the chromatographic analyses of the examined samples. Electrospray ionization source operating in the positive ionization mode at needle potential of 4.5 kV was employed. Nitrogen (>99.98%) was used as sheath gas (at 40 arbitrary units), auxiliary gas (at 10 arbitrary units) and sweep gas (at 10 arbitrary units). Capillary temperature was maintained at 320 °C. The resolution of MS was 60,000. Separations were performed at 25 °C on a Gemini C18 column (4.6 ×100 mm, 3 μm; Phenomenex) using gradient elution. Mobile phase A was 25 mM formic acid in water; mobile phase B was 25 mM formic acid in acetonitrile. The gradient program started at 30% B increasing to 90% for 40 min, and ended with isocratic elution (90% B) for 20 min. The total run time was 60 min at the mobile phase flow rate 0.4 mL/min.

Analyzing the examined samples, the SIM function was used to better visualize the chromatographic separation and to remove the signals from insignificant mixture components like the plasma components and the precipitation agent. Pseudo molecular ions $[M+H]^+$ of m/z = 425, and 460, corresponding with dichloroacetic and trichloroacetic esters of Δ8-THC and Δ9-THC, were monitored.

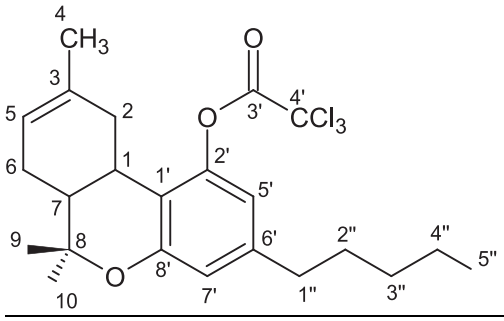
2.6. NMR measurements

NMR experiments were performed using Ascend 600 MHz instrument (Bruker, Bremen, Germany). The $CDCl_3$ solutions of the obtained samples were examined using ^{1}H, ^{13}C, ^{1}H–^{13}C HSQC, ^{1}H–^{1}H COSY and selective 1D TOCSY techniques.

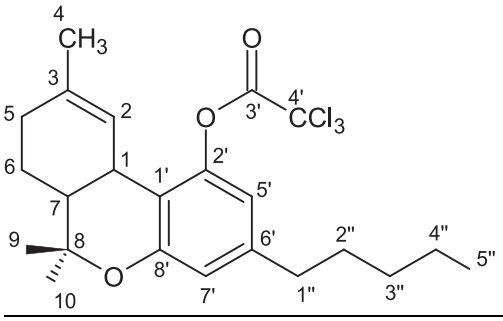
3. Results and discussion

To find out whether TCA esters with Δ9-THC and Δ8-THC are formed when protein precipitation process is used as sample preparation procedure in estimating CBD presence in human plasma, the supernatants centrifuged from its samples spiked with CBD (10 μg/mL) after their preliminary protein precipitation by TCA were examined using GC-MS working in SCAN mode. In order to facilitate the identification of CBD transformation products during GC analysis, plasma samples containing a high concentration of the analyte were used deliberately. The results of the GC-MS analyses are shown in Fig. 1A. As can be seen, in addition to

Table 2
^{1}H and ^{13}C NMR data of Δ8-THC-TCA (A) and Δ9-THC-TCA (B) in $CDCl_3$.



A



B

No.	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	31.6, CH	2.62 td (10.7, 4.7)	33.9, CH	3.14, m
2	36.4, CH_2	2.89, dd (16.9, 3.9) 1.93, dd (16.0, 10.7)	123.5, CH	5.99, m
3	134.0, C	-	134.7, C	-
4	23.4, CH_3	1.66, s	23.1, CH_3	1.63, s
5	119.7, CH	5.42, m	30.9, CH_2	2.14, m
6	27.8, CH_2	2.15, m 1.82, m[a]	24.8, CH_2	1.91, m 1.39, m
7	44.9, CH	1.82, m[a]	45.4, CH	1.69, m
8	77.6, C	-	77.8, C	-
9	27.4, CH_3	1.40, s	27.4, CH_3	1.43, s
10	18.6, CH_3	1.22, s	19.3, CH_3	1.11, s
1′	110.5, C	-	110.1, C	-
2′	149.7, C	-	149.3, C	-
3′	160.2, C	-	159.9, C	-
4′	89.7, CCl_3	-	89.9, CCl_3	-
5′	113.0, CH	6.51, d (1.6)	112.7, CH	6.50, d (1.6)
6′	143.4, C	-	143.2, C	-
7′	116.4, CH	6.65, d (1.6)	116.3, CH	6.63, d (1.6)
8′	154.6, C	-	154.7, C	-
1′	35.5, CH_2	2.53, t (7.4)	35.4, CH_2	2.51, t (7.4)
2′	30.5, CH_2	1.59, quint (7.6)	30.6, CH_2	1.58, quint (7.5)
3′	31.6, CH_2	1.31, m[b]	31.6, CH_2	1.30, m[c]
4′	22.5, CH_2	1.32, m[b]	22.5, CH_2	1.31, m[c]
5′	14.1, CH_3	0.89, t (6.9)	14.7, CH_3	0.88, t (7.0)

a-c Overlapping signals.

the peaks corresponding with CBD transformation products typically forming in the GC injector when an acidified CBD sample is analyzed (i.e. 9α-OH-HHC, 8-OH-iso-HHC, CBD, Δ9-THC, Δ8-THC and CBN), the chromatogram additionally contains four peaks belonging to unknown substances (peaks 1–4). Their GC-MS spectra are shown in Fig. 1B. The same compounds appear on the chromatogram of CBD solutions (10 μg/mL) in acetonitrile containing TCA (see Fig. 1C and D), which excludes their relationship with plasma components.

Compounds identification by the GC-MS method comes down to establishing their retention and spectroscopic data compared with the data collected in appropriate databases. This basic way of compounds identification cannot be applied to compounds 1–4 due to the absence of their data in the bases and literature. Nevertheless, the detailed analysis of the obtained GC-MS data (see Fig. 1) suggests that these are di- and trichloroacetic esters of Δ8-THC (peak 1 and 3, respectively) and di- and trichloroacetic esters of Δ9-THC (peak 2 and 4, respectively). This identification is supported by the following arguments:

1. the intensities of fragmentation ions in the spectrum of Δ8-THC are similar to those in the spectrum of compound 3, and the intensities of fragmentation ions in the spectrum of Δ9-THC are similar to those in the spectrum of compound 4;
2. *m/z* of molecular ion of compounds 3 and 4 is greater from the mass of THC by 145, which equals to the molar mass of trichloroacetyl moiety;
3. *m/z* of molecular ion of compounds 1 and 2 is greater from the mass of THC by 110, which equals to the molar mass of dichloroacetyl moiety;
4. on the spectra of compounds 1–4, the presence of the isotopic envelope of molecular and fragment ions corresponding to the appropriate number of atoms (containing chlorine 35 and 37) with M and M+ 2 isotopes in 3:1 ratio is observed;
5. the difference in *m/z* for compounds 3 and 1 and for compounds 4 and 2 is 35/37 Da, which indicates the elimination of chlorine from trichloroacetyl moiety;
6. the elution sequences of compounds 1 and 2 as well as 3 and 4 correspond to the elution sequences of their precursors, Δ8-THC and Δ9-THC, and are in agreement with the change in the physicochemical properties of these derivatives (i.e. with the increase in their molar mass and boiling point).

In the absence of any independent GC-MS data on the formed THC derivatives, the above identification requires additional research, the more so that when TCA was used as a protein precipitation agent, mainly dichloroacetic derivatives of both THCs were obtained, and not - as assumed - their trichloroacetic derivatives, what is surprising given the results reported in [29] about trifluoroacetic derivatives of THC.

In order to confirm the presence of di- and trichloroacetic derivatives of THC in the examined samples (see Fig. 1), two types of their mixtures were prepared on preparative scale in separate experiments (see 2.2. Experimental):

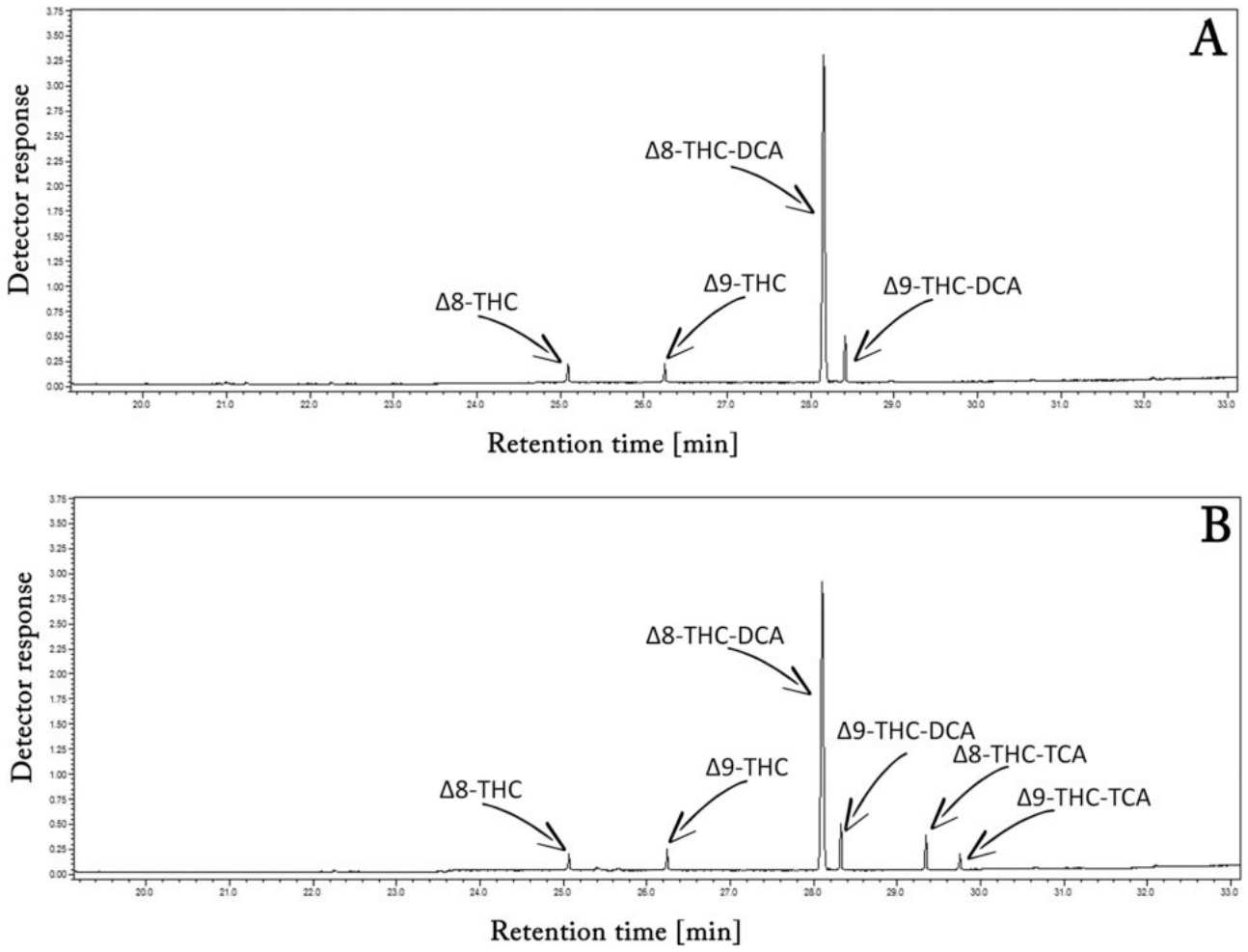


Fig. 3. GC-MS chromatograms (in Scan mode) of the mixture of dichloroacetic (A) and trichloroacetic (B) derivatives of Δ8- and Δ9-THC.

1. the mixture of dichloroacetic derivatives of Δ8- and Δ9-THC, and
2. the mixture of trichloroacetic derivatives of Δ8- and Δ9-THC

The LC-MS chromatograms and NMR spectra of these mixtures are presented in Fig. 2 and Fig. 1S (see supplementary materials), respectively, whereas the NMR data of individual di- and trichloroacetic derivatives of THC are collected in Tables 1 and 2.

Following Fig. 1S (see supplementary materials), each mixture, apart from the main components, i.e. dichloroacetic or trichloroacetic derivatives of Δ8- and Δ9-THC, contains also trace amount of their precursors, i.e. Δ8- and Δ9-THC.

The GC-MS chromatograms of the obtained mixtures are presented in Fig. 3. The chromatogram corresponding with the mixture of dichloroacetic derivatives of Δ8- and Δ9-THC (see Fig. 3A) consists of 4 peaks. The two small peaks are identified as Δ8- and Δ9-THC and the two main peaks as their dichloroacetic derivatives. The retention data and spectra of the main peaks are the same as for compounds 1 and 2 (see Fig. 1). Hence, it can be concluded that they belong to the dichloroacetic esters of Δ8-THC and Δ9-THC. This conclusion is additionally supported by Fig. 3B showing the chromatogram for the mixture of trichloroacetic derivatives of Δ8- and Δ9-THC. It consists of 6 peaks. The first two ones are identified as Δ8- and Δ9-THC, the last two as their trichloroacetic derivatives, and the two dominating middle peaks as belonging to their dichloroacetic derivatives. This somewhat surprising result indicates that trichloroacetic derivatives of Δ8- and Δ9-THC decompose in the hot GC injector forming dichloroacetic derivatives of these cannabinoids and explains why mainly dichloroacetic esters of Δ8-THC and Δ9-THC are formed in the GC injector during CBD analysis in blood/plasma samples by GC when TCA is used as their deproteinizer.

Fig. 2S (see supplementary materials) shows the influence of GC injector temperature on the signal ratio of dichloroacetic to trichloroacetic esters of Δ8-THC and Δ9-THC. As can be seen, the increase of GC injector temperature favors the formation of dichloroacetic derivatives of these cannabinoids in relation to their trichloroacetic derivatives.

At this point, attention should be directed to the physicochemical properties of the trichloroacetic acid itself. As known in the literature [30], trichloroacetic acid in reaction systems subjected to even a slight energetic action is transformed into dichloroacetic acid. Such transformation has not been reported so far in relation to trichloroacetic derivatives of organic compounds. The present paper demonstrates that trichloroacetic derivatives of Δ8- and Δ9-THC do undergo this type of transformation in the hot GC injector, leading to the formation of dichloroacetic derivatives of these cannabinoids.

Additionally GC analyses performed with trichloroacetic derivatives of other organic compounds indicate that they are also transformed in GC injector into their dichloroacetic derivatives. The results of the additional experiments are not discussed here. It is worth mentioning at this point that such instability of trichloroacetic derivatives of both Δ8-/Δ9-THC and many other organic substances under GC injection conditions is probably the reason not only for the lack of their GC-MS spectra in the databases, but also for erroneous spectra of many other trichloroacetic derivatives present in the databases, e.g. trichloroacetic acid decyl ester (CAS 65611–33–8), trichloroacetic acid hexadecyl ester (CAS 74339–54–1).

The results presented in Fig. 1 were obtained on samples containing CBD in a significant concentration, not found in routine medical

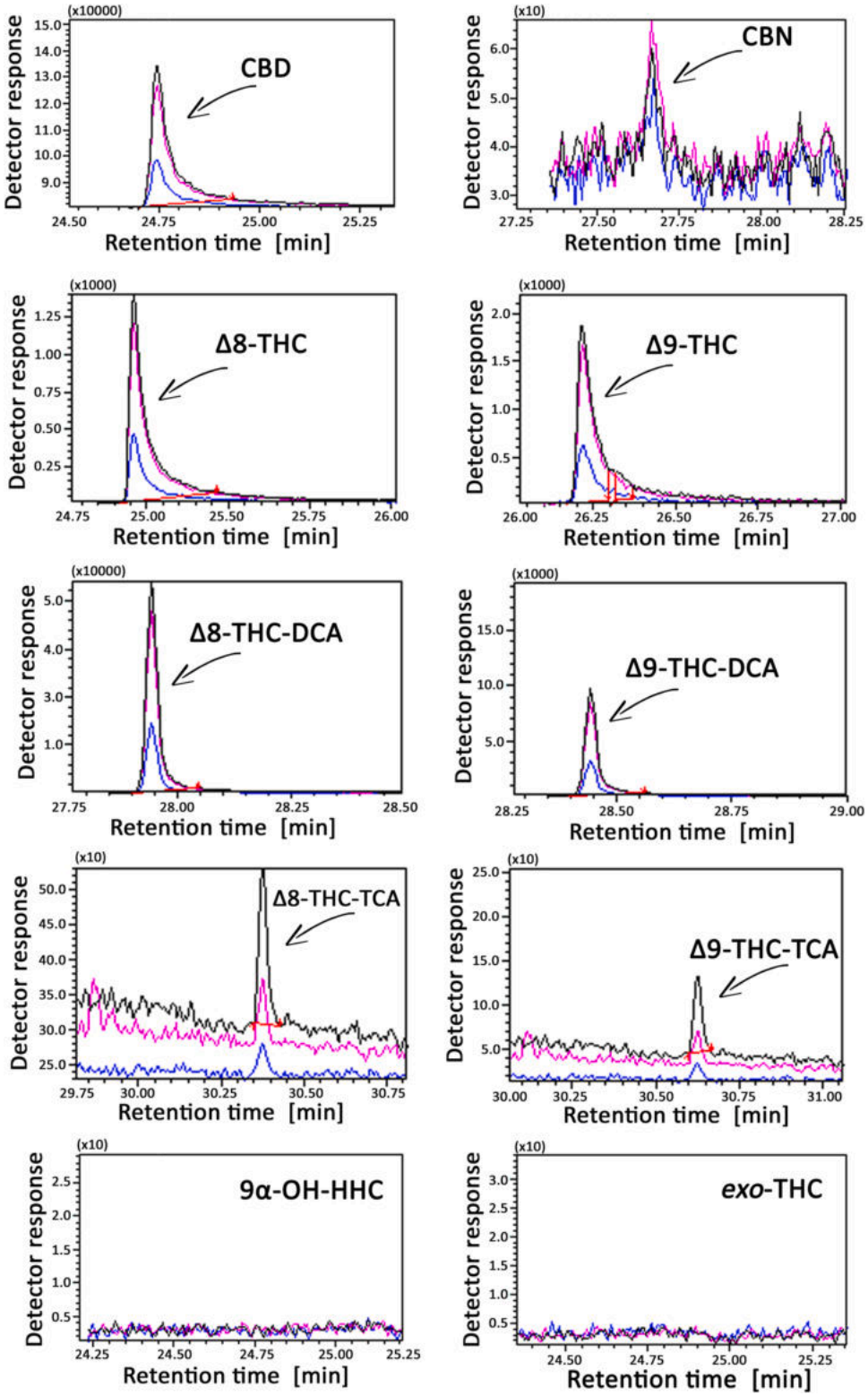


Fig. 4. GC-MS/MS chromatograms (in MRM mode) of supernatants centrifuged from human plasma samples spiked with CBD (30 ng/mL) after their preliminary protein precipitation by TCA.

practice. In order to check:

1. whether di- and trichloroacetic esters of Δ8- and Δ9-THC are formed in the GC injector during analysis of blood/plasma samples from people using this cannabinoid in medical purposes, and if so,
2. whether the formation of di- and trichloroacetic esters of Δ8- and Δ9-THC may affect the accuracy of CBD estimation,

the experiment from Fig. 1 was repeated using plasma samples containing a therapeutic level of CBD concentration. Fig. 4 shows GC-MS chromatograms (using MRM function) of the supernatants centrifuged

from human plasma samples spiked with CBD (30 ng/mL) after their preliminary protein precipitation by TCA. In the course of chromatographic separation, all possible derivatives of CBD (see Fig. 1) were searched for. As results from the presented chromatograms, not only all of them are registered by the detection system, but they form in significant amounts (especially dichloroacetic ester of Δ8-THC). Such CBD transformations diminishes its amount in the examined sample and precludes the estimation of accurate concentration of this cannabinoid.

4. Conclusions

The knowledge of compounds stability in the process of sample preparation for analysis and during analysis itself helps assess the accuracy and precision of estimating their concentration in the tested samples. As reported in [29], if protein precipitation by TFA is applied as a sample preparation method in blood/plasma analysis, a significant part of CBD contained in the sample analyzed by GC transforms not only to 9α-OH-HHC, 8-OH-iso-HHC, Δ9-THC, Δ8-THC and CBN (i.e. typical transformation products of CBD in any acidic environmental), but also to two trifluoroacetic esters of THC: Δ9-THC-TFA and Δ8-THC-TFA. The present study takes this knowledge further by demonstrating the formation of four other THC derivatives in the GC injector if TCA is used for protein precipitation. Two of them, Δ8-THC-TCA and Δ9-THC-TCA, can be treated as analogues of Δ8-THC-TFA and Δ9-THC-TFA, and two others - Δ8-THC-DCA and Δ9-THC-DCA - are the products of Δ8-THC-TCA and Δ9-THC-TCA decomposition in the hot GC injector. The amount of each trichloroacetic and dichloroacetic ester of Δ8-THC and Δ9-THC strongly depends on the GC injector temperature. The obtained results demonstrate why the use of trichloroacetic acid for plasma samples deproteinization should be avoided when CBD and/or THC are determined by GC. The transformation of trichloroacetic derivatives of organic compounds into their dichloroacetic derivatives in GC injector has not been described yet. The instability of trichloroacetic derivatives of both THC, Δ8-/Δ9-THC during their GC analysis is probably the reason for the lack of their GC-MS spectra in the databases.

CRediT authorship contribution statement

Andrzej L. Dawidowicz: Conceptualization, Writing – original draft, Investigation. **Michal P. Dybowski:** Writing – original draft, Writing – review & editing, Investigation, Methodology, Data curation, Visualization. **Rafal Typek:** Writing – original draft, Investigation, Methodology, Data curation. **Michal Rombel:** Writing – original draft, Investigation, Data curation. **Piotr Holowinski:** Writing – original draft, Investigation, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Acknowledgments

We gratefully acknowledge the use of the apparatus in ECOTECH-COMPLEX in Lublin, purchased under the project “ECOTECH-COMPLEX Man, Environment, Production” project No POIG.02.01.00-06-212/09-03 funded from the European Regional Development Fund 2007–2013 by the National Center for Research and Development in Poland.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115388.

References

[1] H. Li, Y. Liu, D. Tian, L. Tian, X. Ju, L. Qi, Y. Wang, C. Liang, Overview of cannabidiol (CBD) and its analogues: structures, biological activities, and neuroprotective mechanisms in epilepsy and Alzheimer’s disease, Eur. J. Med. Chem. 192 (2020), 112163, https://doi.org/10.1016/j.ejmech.2020.112163.
[2] L. Song, S. Carlson, G. Valenzuela, M. Chao, S.B. Pathipaka, Development of a validated method for rapid quantification of up to sixteen cannabinoids using ultra-high-performance liquid chromatography diode-array detector with optional electrospray ionization time-of-flight mass spectrometry detection, J. Chromatogr. A 1670 (2022), 462953, https://doi.org/10.1016/J.CHROMA.2022.462953.
[3] F. Palazzoli, C. Citti, M. Licata, A. Vilella, L. Manca, M. Zoli, M.A. Vandelli, F. Forni, G. Cannazza, Development of a simple and sensitive liquid chromatography triple quadrupole mass spectrometry (LC–MS/MS) method for the determination of cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC) and its metabolites in rat whole blood after oral administration of a single high dose of CBD, J. Pharm. Biomed. Anal. 150 (2018) 25–32, https://doi.org/10.1016/J.JPBA.2017.11.054.
[4] A.L. Dawidowicz, M.P. Dybowski, M. Rombel, R. Typek, Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: oleamide-induced transient matrix effect, J. Pharm. Biomed. Anal. 204 (2021), 114265, https://doi.org/10.1016/J.JPBA.2021.114265.
[5] N. Mashmoushi, J. Larry Campbell, R. di Lorenzo, W. Scott Hopkins, Rapid separation of cannabinoid isomer sets using differential mobility spectrometry and mass spectrometry, Analyst 147 (2022) 2198–2206, https://doi.org/10.1039/D1AN02327F.
[6] P. Fadda, L. Robinson, W. Fratta, R.G. Pertwee, G. Riedel, Differential effects of THC- or CBD-rich cannabis extracts on working memory in rats, Neuropharmacology 47 (2004) 1170–1179, https://doi.org/10.1016/J.NEUROPHARM.2004.08.009.
[7] R.G. Pertwee, Cannabidiol as a Potential Medicine, in: Cannabinoids as Ther, Birkhäuser-Verlag, 2005, pp. 47–65, https://doi.org/10.1007/3-7643-7358-x_3.
[8] O. Devinsky, M.R. Cilio, H. Cross, J. Fernandez-Ruiz, J. French, C. Hill, R. Katz, V. Di Marzo, D. Jutras-Aswad, W.G. Notcutt, J. Martinez-Orgado, P.J. Robson, B. G. Rohrback, E. Thiele, B. Whalley, D. Friedman, Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders, Epilepsia 55 (2014) 791–802, https://doi.org/10.1111/epi.12631.
[9] R. Mechoulam, L. Hanuš, Cannabidiol: an overview of some chemical and pharmacological aspects. Part I: chemical aspects, Chem. Phys. Lipids 121 (2002) 35–43, https://doi.org/10.1016/S0009-3084(02)00144-5.
[10] A.F. Lo Faro, B. Venanzi, G. Pilli, U. Ripani, G. Basile, S. Pichini, F.P. Busardò, Ultra-high-performance liquid chromatography-tandem mass spectrometry assay for quantifying THC, CBD and their metabolites in hair. Application to patients treated with medical cannabis, J. Pharm. Biomed. Anal. 217 (2022), 114841, https://doi.org/10.1016/J.JPBA.2022.114841.
[11] M.R. Amin, D.W. Ali, Pharmacology of medical cannabis, Adv. Exp. Med. Biol. 1162 (2019) 151–165, https://doi.org/10.1007/978-3-030-21737-2_8/COVER.
[12] C. Marchioni, T.M. Vieira, A.E. Miller Crotti, J.A. Crippa, M.E. Costa Queiroz, In-tube solid-phase microextraction with a dummy molecularly imprinted monolithic capillary coupled to ultra-performance liquid chromatography-tandem mass spectrometry to determine cannabinoids in plasma samples, Anal. Chim. Acta 1099 (2020) 145–154, https://doi.org/10.1016/j.aca.2019.11.017.
[13] E.M. Rock, D. Bolognini, C.L. Limebeer, M.G. Cascio, S. Anavi-Goffer, P.J. Fletcher, R. Mechoulam, R.G. Pertwee, L.A. Parker, Cannabidiol, a nonpsychotropic component of cannabis, attenuates vomiting and nausea-like behaviour via indirect agonism of 5-HT 1A somatodendritic autoreceptors in the dorsal raphe nucleus, Br. J. Pharm. 165 (2012) 2620–2634, https://doi.org/10.1111/j.1476-5381.2011.01621.x.
[14] B. Palmieri, C. Laurino, M. Vadalà, Short-term efficacy of CBD-enriched hemp oil in girls with dysautonomic syndrome after human papillomavirus vaccination, Isr. Med. Assoc. J. 19 (2017) 79–84. ⟨https://europepmc.org/article/med/28457055⟩. accessed October 7, 2022.
[15] M.M. Caffarel, C. Andradas, E. Pérez-Gómez, M. Guzmán, C. Sánchez, Cannabinoids: a new hope for breast cancer therapy? Cancer Treat. Rev. 38 (2012) 911–918, https://doi.org/10.1016/j.ctrv.2012.06.005.
[16] P. Massi, M. Solinas, V. Cinquina, D. Parolaro, Cannabidiol as potential anticancer drug, Br. J. Clin. Pharmacol. 75 (2013) 303–312, https://doi.org/10.1111/J.1365-2125.2012.04298.X.
[17] V. Pilařová, Z. Hadysová, F. Švec, L. Nováková, Supercritical fluids in analysis of cannabinoids in various Cannabis products, Anal. Chim. Acta 1232 (2022), 340452, https://doi.org/10.1016/J.ACA.2022.340452.
[18] R.A. Vlad, L. Farczádi, C.M. Toma, S. Imre, P. Antonoaea, E.M. Rédai, D. L. Muntean, A. Ciurba, Cannabidiol content evaluation in commercial dietary supplements and stability in oil vehicle, Stud. Univ. Babes-Bolyai Chem. 66 (2021) 299–308, https://doi.org/10.24193/SUBBCHEM.2021.2.26.
[19] V. Morais, H. Massaldi, A model mechanism for protein precipitation by caprylic acid: Application to plasma purification, Biotechnol. Appl. Biochem. 59 (2012) 50–54, https://doi.org/10.1002/BAB.68.

[20] A. Bhati, R.P. Desai, C.N. Ramchand, Enhancement in recovery of drugs with high protein binding efficiency from human plasma using magnetic nanoparticles, J. Pharm. Biomed. Anal. 143 (2017) 277–284, https://doi.org/10.1016/J.JPBA.2017.06.009.

[21] J. Klawitter, C. Sempio, S. Mörlein, E. De Bloois, J. Klepacki, T. Henthorn, M. A. Leehey, E.J. Hoffenberg, K. Knupp, G.S. Wang, C. Hopfer, G. Kinney, R. Bowler, N. Foreman, J. Galinkin, U. Christians, J. Klawitter, An atmospheric pressure chemical ionization ms/ms assay using online extraction for the analysis of 11 cannabinoids and metabolites in human plasma and urine, Ther. Drug Monit. 39 (2017) 556–564, https://doi.org/10.1097/FTD.0000000000000427.

[22] A.L. Dawidowicz, R. Kalitynski, M. Kobielski, J. Pieniadz, Influence of propofol concentration in human plasma on free fraction of the drug, Chem. Biol. Interact. 159 (2006) 149–155, https://doi.org/10.1016/J.CBI.2005.10.108.

[23] L. Taylor, J. Crockett, B. Tayo, G. Morrison, A phase 1, open-label, parallel-group, single-dose trial of the pharmacokinetics and safety of cannabidiol (CBD) in subjects with mild to severe hepatic impairment, J. Clin. Pharm. 59 (2019) 1110–1119, https://doi.org/10.1002/JCPH.1412.

[24] M.J. Roslawski, R.P. Remmel, A. Karanam, I.E. Leppik, S.E. Marino, A.K. Birnbaum, Simultaneous quantification of 13 cannabinoids and metabolites in human plasma by liquid chromatography tandem mass spectrometry in adult epilepsy patients, Ther. Drug Monit. 41 (2019) 357–370, https://doi.org/10.1097/FTD.0000000000000583.

[25] K. Watanabe, Y. Itokawa, S. Yamaori, T. Funahashi, T. Kimura, T. Kaji, N. Usami, I. Yamamoto, Conversion of cannabidiol to Δ9-tetrahydrocannabinol and related cannabinoids in artificial gastric juice, and their pharmacological effects in mice, Forensic Toxicol. 25 (2007) 16–21, https://doi.org/10.1007/s11419-007-0021-y.

[26] M.P. Dybowski, A.L. Dawidowicz, R. Typek, M. Rombel, Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – Troubles with reliable CBD quantitation when acidic precipitation agents are applied, Talanta 220 (2020), 121390, https://doi.org/10.1016/j.talanta.2020.121390.

[27] P. Marzullo, F. Foschi, D.A. Coppini, F. Fanchini, L. Magnani, S. Rusconi, M. Luzzani, D. Passarella, Cannabidiol as the substrate in acid-catalyzed intramolecular cyclization, J. Nat. Prod. 83 (2020) 2894–2901, https://doi.org/10.1021/ACS.JNATPROD.0C00436/SUPPL_FILE/NP0C00436_SI_001.PDF.

[28] R. Typek, P. Holowinski, A.L. Dawidowicz, M.P. Dybowski, M. Rombel, Chromatographic analysis of CBD and THC after their acylation with blockade of compound transformation, Talanta 251 (2023), 123777, https://doi.org/10.1016/J.TALANTA.2022.123777.

[29] P. Holowinski, R. Typek, A.L. Dawidowicz, M. Rombel, M.P. Dybowski, Formation of trifluoroacetic artefacts in gas chromatograph injector during cannabidiol analysis, J. Chromatogr. A 1671 (2022), 463020, https://doi.org/10.1016/J.CHROMA.2022.463020.

[30] M.M. Ketcha, D.K. Stevens, D.A. Warren, C.T. Bishop, W.T. Brashear, Conversion of trichloroacetic acid to dichloroacetic acid in biological samples, J. Anal. Toxicol. 20 (1996) 236–241, https://doi.org/10.1093/JAT/20.4.236.

Supplementary materials

Unexpected formation of dichloroacetic and trichloroacetic artefacts in gas chromatograph injector during Cannabidiol analysis

Andrzej L. Dawidowicz, Michał P. Dybowski, Rafał Typek, Michał Rombel, Piotr Hołowiński

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031, Lublin, Poland

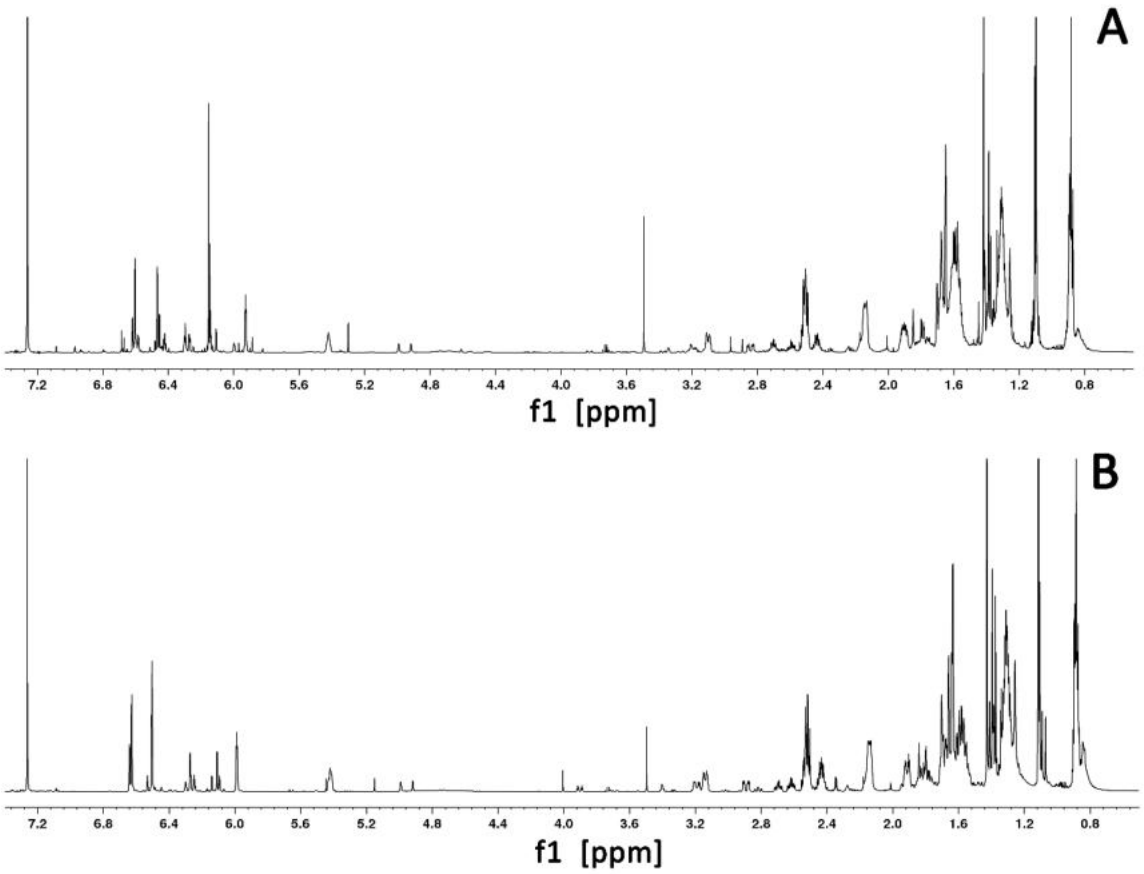


Fig. 1S. LC-MS chromatograms (in Scan mode) of the mixture of dichloroacetic (A) and trichloroacetic (B) derivatives of Δ8- and Δ9-THC.

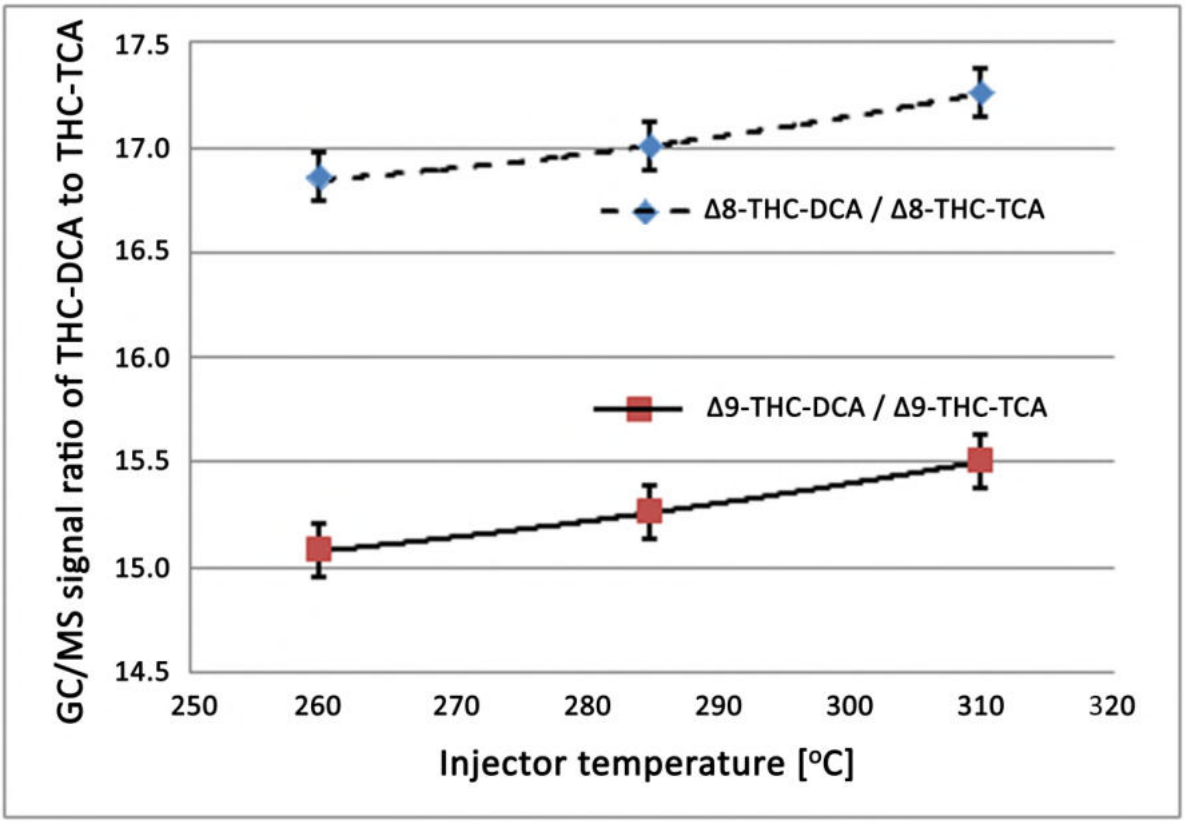


Fig. 2S. The influence of the GC injector temperature on the ratio of GC-MS signal magnitude of dichloroacetic to trichloroacetic derivative of Δ8- (dashed line with diamonds) and Δ9-THC (solid line with squares).

Publikacja D7

A.L. Dawidowicz, R. Typek, M.P. Dybowski, P. Hołowiński, **M. Rombel**

Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry

Forensic Toxicol. *In Press*

IF 2-letni: 2,2; MEiN: 100 pkt

Date: 31 Aug 2023
To: "Andrzej L. Dawidowicz" andrzej.dawidowicz@mail.umcs.pl
From: "Akira Namera" namera@hiroshima-u.ac.jp
Subject: Your Submission FOTO-D-23-00035R5

Dear Prof. Dawidowicz,

We are pleased to inform you that your manuscript, "Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry", has been accepted for publication in Forensic Toxicology.

Please remember to quote the manuscript number, FOTO-D-23-00035R5, whenever inquiring about your manuscript.

With best regards,

Akira Namera
Chief Editor

Reviewer #2: It would be even better if you could write about the signal increase and decrease of figure 6 in the Results section.
line 207: TIC mode -> scan mode

Please note that this journal is a Transformative Journal (TJ). Authors may publish their research with us through the traditional subscription access route or make their paper immediately open access through payment of an article-processing charge (APC). Authors will not be required to make a final decision about access to their article until it has been accepted.

Authors may need to take specific actions to achieve compliance with funder and institutional open access mandates. If your research is supported by a funder that requires immediate open access (e.g. according to Plan S principles) then you should select the gold OA route, and we will direct you to the compliant route where possible. For authors selecting the subscription publication route our standard licensing terms will need to be accepted, including our self-archiving policies. Those standard licensing terms will supersede any other terms that the author or any third party may assert apply to any version of the manuscript.

Find out more about compliance: https://www.springernature.com/gp/open-research/funding/policy-compliance-faqs

This letter contains confidential information, is for your own use, and should not be forwarded to third parties.

Recipients of this email are registered users within the Editorial Manager database for this journal. We will keep your information on file to use in the process of submitting, evaluating and publishing a manuscript. For more information on how we use your personal details please see our privacy policy at https://www.springernature.com/production-privacy-policy. If you no longer wish to receive messages from this journal or you have questions regarding database management, please contact the Publication Office at the link below.

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: https://www.editorialmanager.com/foto/login.asp?a=r). Please contact the publication office if you have any questions.

Forensic Toxicology

Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry

--Manuscript Draft--

Manuscript Number:	FOTO-D-23-00035
Full Title:	Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry
Article Type:	Original Article
Funding Information:	
Abstract:	Purpose While the analytical sensitivity of CBD, Δ9-THC or CBN estimation by GC-MS is similar and sufficiently high, it is exceptionally low in the case of CBG (ca. 25 times lower than for the other mentioned cannabinoids). The purpose of this study is to explain the reasons for the extremely low analytical sensitivity of GC-MS in estimating CBG and to present possible ways of its improvement. Methods GC-MS responses to CBG and its various derivatization and transformation products were studied. The structures of the obtained CBG derivatization and transformation products were estimated by NMR. Results The validation data of individual derivatives of CBG and its transformation products were established. CBG silylation/acylation or hydration allows to decrease LOD about 3-times, whereas the formation of pyranic CBG derivative leads to 10-times decrease of LOD in the analytical procedure of CBG estimation. The paper enriches the literature of the subject by providing MS and NMR spectra, not published so far, for derivatives of CBG and its transformation products. The most likely cause of low GC-MS response to CBG is also presented in the discussion. Conclusions The presented results shows that although the signal increase of CBG can be obtained through its derivatization by silylation and/or acylation, the greatest increase is observed in the case of its cyclization to the pyranic CBG form during the sample preparation process. The CBG cyclization procedure is very simple and workable in estimating this cannabinoid in blood/plasma samples and plant extracts.
Corresponding Author:	Andrzej L. Dawidowicz Maria Curie-Sklodowska University: Uniwersytet Marii Curie-Sklodowskiej POLAND
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Maria Curie-Sklodowska University: Uniwersytet Marii Curie-Sklodowskiej
Corresponding Author's Secondary Institution:	
First Author:	Andrzej L. Dawidowicz
First Author Secondary Information:	
Order of Authors:	Andrzej L. Dawidowicz
	Rafal Typek
	Michal P. Dybowski

	Piotr Holowinski
	Michal Rombel
Order of Authors Secondary Information:	
Author Comments:	Dear Prof. Seto, Please find enclosed the manuscript entitled “Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry” which we would like to publish in Forensic Toxicology. A very important feature of the analytical procedure is its sensitivity towards the examined compound. While the analytical sensitivity of CBD, Δ9-THC or CBN estimation by GC-MS is similar and sufficiently high, it is exceptionally low in the case of CBG (ca. 25 times lower than for the other mentioned cannabinoids). The most frequent way of increasing the chromatographic analysis sensitivity of examined compound is their preliminary derivatization. The present paper discusses the effects of various methods of CBG derivatization (classical silylation/acylation and more sophisticated saturation by hydrogen and CBG cyclization to its pyranic derivate) on the increase in GC-MS response in the analytical procedure of its estimation. The most likely cause of low GC-MS response to CBG is presented. It is also shown that the greatest sensitivity of CBG analysis is obtained by transforming this cannabinoid to its pyranic derivative (ca. 10 times higher than that for unmodified CBG). The proposed new way of CBG derivatization is very simple and compatible with the analytical procedures of CBG estimation in real samples. We believe that the innovation proposed in the manuscript is appropriate for the publication in Forensic Toxicology. The manuscript reports original research work and is not under consideration by any other journal. All authors approved the manuscript and its submission, and have no conflicts of interest to disclose. We hope you’ll find the manuscript worth the reviewing process and are looking forward to your response. With kind regards, Andrzej L. Dawidowicz

Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry

Andrzej L. Dawidowicz*, Rafal Typek, Michal P. Dybowski, Piotr Holowinski, Michal Rombel

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031 Lublin, Poland

Andrzej L. Dawidowicz *,

* Corresponding author. Tel.: +48 81 537 55 45; fax: +48 81 533 33 48

E-mail address: andrzej.dawidowicz@mail.umcs.pl

Abstract

Purpose

According to recent reports CBG concentration level in blood and body fluids may have forensic utility as a highly specific albeit insensitive biomarker of recent cannabis smoking. While the analytical sensitivity of CBD, Δ^9-THC, CBC or CBN estimation by GC-MS is similar and sufficiently high, it is exceptionally low in the case of CBG (ca. 25 times lower than for the other mentioned cannabinoids). The purpose of this study is to explain the reasons for the extremely low analytical sensitivity of GC-MS in estimating CBG and to present possible ways of its improvement.

Methods

NMR data and GC-MS responses to CBG and its various derivatization and transformation products were studied.

Results

The validation data of individual derivatives of CBG and its transformation products were established. CBG silylation/acylation or hydration allows to decrease LOD about 3-times, whereas the formation of pyranic CBG derivative leads to 10-times decrease of LOD. The paper enriches the literature of the subject by providing MS and NMR spectra, not published so far, for derivatives of CBG and its transformation products. The most likely cause of low GC-MS response to CBG is also presented.

Conclusions

The presented results shows that although the signal increase of CBG can be obtained through its derivatization by silylation and/or acylation, the greatest increase is observed in the case of its cyclization to the pyranic CBG form during the sample preparation process. The CBG cyclization procedure is very simple and workable in estimating this cannabinoid in blood/plasma samples.

Keywords:

GC-MS, signal enhancement, cannabigerol, sample derivatization, CBG cyclization

Introduction

Cannabinoids constitute a group of at least 150 diverse organic compounds exhibiting bioactive properties due to their ability to interact with CB1 and CB2 receptors of the endocannabinoid system that is very important one for various health systems [1–3]. Although psychoactive Δ^9-tetrahydrocannabinol (Δ^9-THC) is still the most famous cannabinoid in this group [4–6], cannabidiol (CBD), devoid of psychotropic effect, has become nowadays just as popular due to its medicinal properties [7, 8]. In addition to its potential activity in the treatment of epileptic syndromes [9–11], preclinical studies demonstrate wider bio-activity of CBD, suggesting its possible usage in the treatment of many other disease entities [12, 13]. Lately, more and more attention has also been paid to the bioactive properties of another cannabinoid, cannabigerol (CBG). The literature on its bioactive properties is not as extensive as on CBD, but it indicates, *inter alia*, its cytotoxic effect against epithelioid carcinoma and breast cancer, and its stimulating effect on a number of receptors important for pain, inflammations, and heat sensitization [14]. Recently, CBG has also become an interesting object for forensic analysis researchers. The reports [15–18] show that it may be treated as a highly specific, though insensitive, biomarker of recent cannabis smoking. Because Δ^9-THC can persist in the blood after the psychoactive effects of intoxication have subsided, especially in regular users, short-lived minor cannabinoids such as CBG have merited investigation as additional indicators of recent cannabis inhalation. Another reason for the interest of forensic analysts in the determination of CBG in physiological fluids is related to its alpha-2-adrenergic action promoting hypotension and bradycardia [19], what can cause fatal descent.

Growing interest in the mentioned cannabinoids and their bioactive properties, as well as a marked increase in the use of dietary supplements containing these cannabinoids in self-healing therapies [20], require the development of reliable and sensitive analytical procedures for their quantitative determination in different types of matrices. While both gas chromatography (GC) or high performance liquid chromatography (HPLC) can be used for this purpose, GC is a more pragmatic option as the choice a simpler, cheaper and a more sensitive technique. In this technique gas chromatography-tandem mass spectrometry (GC-MS/MS) is becoming a routine analytical instrument, advisable especially when analyzing compounds in very complex matrices (e.g. in plasma/plant material) [21–23].

A very important feature of any analytical procedure is its sensitivity towards the examined compound. In forensic and clinical analysis, high sensitivity analytical methods are usually

preferred. The more sensitive the analytical method, the lower concentration level of given xenobiotic can be determined in examined sample. As results from the previous reports [24, 25] and our experiences, the sensitivity of analytical estimation of CBD and Δ^9-THC (or such cannabinoids like CBC and CBN) using GC-MS is similar and sufficiently high, but it is exceptionally low for CBG. The aim of this study is to explain the reasons for the extremely low analytical sensitivity of GC-MS in estimating CBG and to present possible ways of its improvement.

Materials and methods

Materials

Acetonitrile (ACN) (LC/MS grade), the standards (certified reference materials) of CBC, CBN, CBG, CBD and Δ^9-THC (1.0 mg/mL in methanol - Cerilliant), trifluoroacetic anhydride (TFAA), hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS), palladium on carbon (Pd/C) and p-toluenesulfonic acid (PTSA) were acquired from Merck (Merck KGaA, Darmstadt, Germany). Dichloromethane (DCM) and sodium dicarbonate were purchased from the Polish Chemical Plant POCh (Gliwice, Poland). $CDCl_3$ was purchased from Armar AG (Döttingen, Switzerland). CBG crystal (>99%) was a gift from CannLAB (Kraków, Poland). Hydrogen and nitrogen were obtained from Air Liquide Polska (Kraków, Poland). Deionized water was purified by the Milli-Q system (Merck Millipore, Merck KGaA, Darmstadt, Germany).

Human plasma samples were obtained by the centrifugation of human blood samples. The samples were collected by a registered nurse from volunteers, after obtaining their informed consent, using a single closed system containing an S-Monovette coagulation activator (Citrate 3.2%), following the manufacturer's instructions (Sarstedt AG, Nümbrecht, Germany), and thoroughly mixed in order to maintain their homogeneity. The plasma samples were CBG free.

***CBG silylation procedure* (*formation of CBG-2TMS*)**

The CBG silylation was carried out according to the optimal procedure described in the reports [25, 26]. The mixture consisting of 500 μL of CBG solution in ACN (1 mg/mL) and 500 μL of silylation mixture (HMDS/TMCS/ACN 1:1:1 v/v/v) was heated at 35 °C for 60 min. The mixture was subsequently centrifuged at 12,000 rpm for 5 min and the obtained supernatant was divided into two parts. One part was diluted 500 times using ACN and subjected to GC/MS analysis. The other one was evaporated under nitrogen stream, dissolved in $CDCl_3$ (500 μL) and subjected to nuclear magnetic resonance (NMR) analysis.

***CBG acylation procedure by TFAA* (*formation of CBG-2TFA*)**

The CBG acylation was carried out according to the optimal procedure described in the reports [27, 28]. The mixture consisting of 500 μL of CBG solution in ACN (1 mg/mL) and TFAA (130 μL) was heated at 65 °C for 60 min. After that, the high volatile components of the reaction mixture were evaporated under nitrogen stream and the obtained dry residue was dissolved in an appropriate solvent before GC/MS and NMR analysis. In GC/MS analysis, the dry residue was dissolved in ACN (1 mL) and then diluted 1000 times. In NMR analysis, the dry residue was dissolved in $CDCl_3$ (500 μL).

CBG hydrogenation (formation of CBG-4H)

The suspensions consisting of Pd/C (1 mg) and 1 mL of CBG solution in DCM (5 mg/mL) were mixed for 3 hours in hydrogen atmosphere at ambient temperature and then centrifuged at 12,000 rpm for 2 min. The obtained supernatant, before its GC/MS analysis, was diluted 5000 times using DCM, whereas before NMR analysis, the supernatant was evaporated to dryness and the obtained dry residue was dissolved in $CDCl_3$ (500 μL). The above procedure is the result of optimizing the time and temperature of the process.

***Formation of pyranic CBG structure* (*Pyr-CBG*) *in chemical process* (*chemical CBG cyclization*)**

The mixture consisting of 500 µL of CBG solution in ACN (5 mg/mL) and 500 µL of PTSA solution in ACN (1 mg/mL) was heated at 75°C for 2 hours. In the case of the reaction product estimation by GC-MS, the obtained mixture was diluted 2500 times using ACN and subjected to GC-MS analysis. The above procedure was optimized studying the influence of time and temperature on the course of CBG cyclization in its ACN solution.

The estimation of the reaction product by NMR requires the removal of PTSA from the mixture. To do this, the obtained mixture was evaporated to dryness under nitrogen stream. Next, the dry residue was dissolved in DCM (500 µL) and extracted 3 times by the saturated water solution of $NaHCO_3$ (500 µL), removing water phase each time. Finally, the cleaned organic phase was evaporated under nitrogen stream and the obtained dry residue was dissolved in $CDCl_3$ (500 µL).

***Silylation of pyranic CBG structure* (*formation of Pyr-CBG-TMS*)**

The mixture consisting of 500 µL of CBG solution in ACN (5 mg/mL) and 500 µL of PTSA solution in ACN (1 mg/mL) was heated at 75 °C for 2 hours and then evaporated to dryness under nitrogen stream. Next, the dry residue was dissolved in DCM (500 µL) and extracted 3 times by saturated water solution of $NaHCO_3$ (3x500 µL), removing water phase each time. Finally, the cleaned organic phase was evaporated under nitrogen stream and the obtained dry residue was silylated by heating at 35 °C for 60 min with 1 mL of silylation mixture (HMDS/TMCS/ACN 1:1:1 v/v/v). The mixture was subsequently centrifuged at 12,000 rpm for 5 min and the obtained supernatant, after its prior 2500 fold dilution by ACN, was subjected to GC-MS analysis.

***Formation of pyranic CBG structure in physical processes* (*physical CBG cyclization*)**

To find out if an energetic physical action on CBG can transform it into its pyrenic form, separate CBG samples were exposed to high temperature, UV and electron stream radiation.

CBG thermal treatment Thermal treatment of the CBG sample was performed in a closed glass vessel containing argon atmosphere at 270 °C for 2 hour using a classical laboratory oven.

CBG exposion to UV radiation The CBG sample, placed in a thin quartz tube, was exposed to UV radiation for 5 min using a high energetic UV lamp emitting 2.0 kW energy

CBG exposion to electron stream radiation The CBG sample was exposed to electron stream for 10 hours using the electron source of energy 7 eV. UHV Flood Gun (PreVac, Rogów, Poland) was used for this purpose.

After the physical treatment, each sample was dissolved in DCM and subjected to GC-MS analysis.

Preparation of plasma samples for GC-MS analysis of CBG, CBN, CBD, Δ9-THC and CBC mixture using QuEChERS procedure

The version without CBG cyclization

$MgSO_4$ (200 mg) and NaCl (50 mg) were added to a plasma sample (700 µL) spiked properly with the mixture of CBG/CBN/CBC/CBD/Δ^9-THC. The concentration of individual cannabinoids (CBG, CBN, CBD, Δ^9-THC and CBC) was 20 ng/mL, similar to the concentration of that determined in the real blood samples from cannabis/hemp consumers [29, 30]. After vortexing for 1 min, ACN (700 µL) was introduced and the whole suspension was vortexed again and then centrifuged at 12,000 rpm for 3 min. The isolated aliquot was subjected to GC-MS analysis.

The version with CBG cyclization

$MgSO_4$ (200 mg) and NaCl (50 mg) were added to a plasma sample (700 µL) spiked properly with the mixture of CBG/CBN/CBC/CBD/Δ^9-THC – the concentrations of individual cannabinoids were the same as in the version without CBG cyclization. After vortexing for 1 min, 700 µL of PTSA solution in ACN (0.5 mg/mL) was introduced and the whole suspension was vortexed again and then centrifuged at 12,000 rpm for 3 min. The isolated aliquot (600 µL) was heated at 75 ^oC for 2 hour. After cooling, the obtained solution was subjected to GC-MS analysis.

GC-FID measurements

ACN solution of CBG, CBD, THC, CBC and CBN mixture was analysed using gas chromatograph with the flame ionization detector GC-FID model GC-2010 (Shimadzu, Kyoto, Japan). 1 µL sample was injected by an AOC – 20i type autosampler into a ZB5-MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) (Phenomenex, USA). Separation conditions were the following: carrier gas - hydrogen (grade 5.0); flow rate - 1.0 ml/min; split injection mode; injector temperature – 310 °C. The temperature program involved: initial temperature 150 °C held for 5 min; temperature increase to 260 °C (at a rate of 9 °C/min) and maintained for 4 min; further temperature increase to 300 °C (at a rate of 6 °C/min).

GC-MS/MS measurements

Qualitative analyses of the examined cannabinoids and CBG transformation products were conducted using a gas chromatograph hyphenated with a triple quadrupole tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan). GC–MS conditions were as follows: capillary column - Zebron ZB5-MSi (30 m x 0.25 mm i.d., 0.25 µm film thickness, Phenomenex, Torrance, CA, USA); carrier gas helium (grade 5.0); flow rate 1.0 ml/min; splitless/split injection mode (sampling time: 1.00 min); injector temperature 310 °C; injection volume 1 µL; temperature program - initial temperature 60 °C held for 3 min subsequently increased to 310 °C at the rate of 12 °C/min and held for 15 min. Mass spectrometer parameters: normalized electron energy of 70 eV; ion source temperature 225 °C. The TIC mode with range 40–750 m/z was used in analysing solutions of cannabinoids and CBG transformation products. In order to analyse QuEChERS extracts from the supernatants centrifuged from human plasma samples spiked with CBG, CBN, CBD, Δ9-THC and CBC, multiple reaction monitoring (MRM) mode was used. GC-MS/MS analysis was performed using characteristic MRM transitions at optimal collision energies (CE) for the examined compounds. Three MRM transitions (m/z => m/z) of the highest intensity were selected for further experiments:

314 > 231 (CE = 24eV), 314 > 193 (CE = 21eV) and **246 > 231** (CE = 21eV) for CBD,
316 > 231 (CE = 15eV), 316 > 193 (CE = 15eV) and 316 > 123 (CE = 6eV) for CBG,
314 > 299 (CE = 12eV), 314 > 271 (CE = 15eV) and 314 > 231 (CE = 18eV) for Δ^9-THC,

314 > 299 (CE = 12eV), 314 > 271 (CE = 15eV) and 314 > 231 (CE = 18eV) for Δ^8-THC,
316 > 231 (CE = 15eV), 316 > 193 (CE = 15eV) and 316 > 123 (CE = 6eV) for Pyr-CBG,
314 > 299 (CE = 15eV), **314 > 231** (CE = 15eV) and 299 > 231 (CE = 6eV) for CBC,
310 > 295 (CE = 21eV), 310 > 238 (CE = 30eV) and **295 > 238** (CE = 27eV) for CBN.
MRM transitions selected for quantification of individual cannabinoids were bolded and underlined.

NMR measurements

NMR experiments were performed using the Ascend 600 MHz instrument (Bruker, Bremen, Germany) equipped with 5 mm BBO probe at 298 K. Before 1H and 13C spectra acquisition, examined samples were dissolved in $CDCl_3$. ^1H–^1H Double Quantum Filtered COSY and ^1H–^{13}C Multiplicity Edited HSQC techniques were employed to facilitate assignment of proton and carbon resonances. Acquisition and processing of considered spectra were performed using TopSpin 3.5 and IconNMR software (Bruker, Bremen, Germany). Tetramethylsilane was used as an internal standard for calibration of ^1H and ^{13}C chemical shifts. NMR data are gathered in Table 1S and 2S (see supplementary materials).

Method validation

To validate the analytical methods, certified standards of individual CBG derivatives and Pyr-CBG should be purchased. Such substances are not commercially available. For this reason, purified CBG derivatives and Pyr-CBG obtained for NMR analysis were used as validation standards. Their purity was 95.3, 96.4, 99.2 and 98.7% for CBG-2TMS, CBG-2TFA, CBG-4H and Pyr-CBG, respectively. These values were estimated by GC-MS using peak normalization method. The compounds used for methods validation should be treated as Type II standards.

The methods were validated in terms of linearity, the limit of detection (LOD), the limit of quantification (LOQ) and the intraday and interday precision and accuracy measurements. To evaluate the method linearity, five replicated analytical procedures were performed for each examined concentration level. The LOD and LOQ were considered to be signal-to-noise ratios equal to 3 and 10, respectively. The intra- and interday precisions and accuracies were evaluated by statistical analysis of the quantitative results (obtained on the same day and on

three different days) for five independent samples containing test compounds (20 ng/ mL). The linearity of the assay was calculated by the least squares method and expressed as the coefficient of determination (R^2). Calibration plots were prepared using ACN spiked with the target analytes at concentration levels of 10, 25, 50, 75 and 100 ng/mL for CBG, 5, 10, 25, 50 and 75 ng/mL for CBG-2TMS, CBG-2TFA and CBG-4H, and 1, 2.5, 5, 10, 25 ng/mL for Pyr-CBG. The solutions were prepared in triplicate.

Results

Fig. 1 shows exemplary chromatograms of CBG, CBD, CBC, Δ^9-THC and CBN mixture obtained using GC with FID and MS detector (a and b, respectively). The molar concentrations of the components in the mixture were the same (0.5 mM). As can be seen, the GC-MS response for CBD, CBC, Δ^9-THC or CBN is similar and high, it is exceptionally low in the case of CBG (ca. 25 times lower than for the other mentioned cannabinoids).

The most frequent way of increasing the chromatographic analysis sensitivity to the examined compounds is their preliminary derivatization, usually performed by silylation or acylation. Fig. 2 shows the size of the GC-MS response to CBG itself and to its silyl and acyl derivative obtained after CBG silylation with TMCS/HMDS (a) and acylation with TFAA (b). MS and NMR spectra of the obtained TMS derivative of CBG are provided in c and e, respectively, and of the obtained TFA derivatives of CBG in d and f, respectively.

The size change of the GC-MS signal resulting from the saturation of alkyl-diene chain in the CBG molecule by hydrogen is shown in Fig. 3a, whereas Fig. 3b and ∈ c present, respectively, MS and NMR spectra of the obtained saturated derivative of CBG.

The presence of alkyl-diene chain and phenolic groups in CBG allows the formation of its pyranic derivative (Pyr-CBG). The diagram of such a transformation illustrates Fig. 4. The change in the GC-MS signal size resulting from CBG transformation into Pyr-CBG is presented in Fig. 5a, whereas MS and NMR spectra of this compound are shown in Fig. 5b and c, respectively.

Fig. 1S (see supplementary materials) shows the chromatograms of DCM solutions of the CBG samples exposed to high temperature, UV and electron stream radiation. These

chromatograms indicates that CBG can be cyclized to its pyranic form by energetic physical action.

According to the results presented in Fig. 2a and 5a, the silylation of CBG increases the sensitivity of its GC-MS estimation about 3 times, whereas the cyclization of CBG about 9 times. Fig. 2S (see supplementary materials) shows GC-MS response to pyranic derivative of CBG before and after its silylation by TMCS/HMDS mixture.

The validation data for CBG, CBG derivatives (CBG-2TMS, CBG-2TFA, CBG-4H) and Pyr-CBG are gathered in Table 1.

The practical utility of the signal enhancement of CBG by its cyclization is shown in Fig. 6 illustrating the results of GC-MS analyses of CBG, CBN, CBD, Δ^9-THC and CBC mixture in plasma samples. Classical QuEChERS and QuEChERS with the CBG cyclization process were used as blood sample preparation methods in the analytical experiments.

Discussion

As results from Fig. 1 showing exemplary chromatograms of CBG, CBD, CBC, Δ^9-THC and CBN mixture obtained by GC with FID and MS detector, FID responses to individual cannabinoids are similar. This is not a surprising effect in the analysed compounds and results from the same number of carbon atoms in each of them and from their structural similarity. A slightly different chromatographic image of the tested mixture is observed in GC-MS. While the MS responses to CBD, CBC, Δ^9-THC and CBN are similar, the MS signal for CBG is extremely low (ca. 25 times lower than for the other mentioned cannabinoids), which confirms experimentally our earlier observations and sparse literature reports [24, 25] about poor response of the MS detector to CBG.

The most frequent way of increasing the chromatographic analysis sensitivity to the examined compounds is their preliminary derivatization, usually performed by silylation or acylation. As can be seen from the spectroscopic data presented in Fig. 2c-f, the use of excessive amounts of silylating or acylating reagents in the CBG derivatization process, which is common practice in the case of the same processes with other compounds, leads to the derivatization of both phenolic groups of this cannabinoid. As seen in Fig. 2a and b, the silyl and acyl derivative of CBG evokes a GC-MS signal about 3 times higher than

unmodified CBG. Thus, these ways of CBG derivatization allow to lower LOD in the analytical estimation of CBG (see Table 1), but it still remains much greater than the LOD for non-derivatized CBD, CBC, Δ^9-THC or CBN. In the case of the equipment used in these experiments LOD for the last mentioned cannabinoids does not exceed 0.1 ng/mL. It is worth noting here that the figure also enriches the literature of the subject by providing MS and NMR spectra of the obtained CBG-2TMS derivatives (c and e, respectively) and CBG-2TFA derivatives (d and f, respectively).

A characteristic feature of the CBG molecule, in contrast to CBD, CBC, Δ^9-THC and CBN, is the presence of an alkyl-diene chain. The saturation of multiple bonds in the analyte with hydrogen is difficult to classify as a classical derivatization procedure, but it was intended to raise the CBG signal in GC-MS analysis. According to NMR data presented in Fig. 3c and Table 1S (see supplementary materials), 3-hour hydrogenation process of CBG provides total saturation of this cannabinoid – the obtained saturated CBG derivative (CBG-4H) does not contain double bonds in alkyl chain. As it can be seen from Fig. 3a, GC-MS signal increment resulting from the saturation of both double bonds in alkyl-diene chain of CBG is similar to that observed after CBG silylation and/or acylation i.e. the saturated derivate of CBG (i.e. CBG-4H) evokes a GC-MS signal about 3 times higher than unmodified CBG. However, this effect is much less practical as it was achieved after a 3-hour saturation process, much longer than the 1-hour silylation/acylation classical derivatization.

Due to the presence of alkyl-diene chain and phenolic groups, CBG can be transformed into its pyranic form (see Fig. 4). The molecular structure of this transformation product is more similar to the molecular structure of CBD, CBC, Δ^9-THC and CBN than to CBG. Following the results presented in Fig. 5a, CBG cyclization evokes ca. 9-fold increase of the GC-MS signal, significantly higher than classical CBG derivatization (silylation/acylation) or CBG saturation. The pyranic derivative of CBG presented in this study is a new compound obtained by cyclization of CBG, and this is why it can be call either cyclo-CBG or pyro-CBG (because it has a pyran ring). The structure of the obtained Pyr-CBG, presented in Fig. 4, is confirmed by NMR data (Fig. 5c and Table 2S in supplementary materials). It is worth noting here that another cyclic derivative of CBG has been described in the literature [31], resulting from the metabolism of CBG by human Cytochrome P450s, which was briefly called cyclo-CBG. It should have been named hydroxycyclo-CBG or furan-CBG because it has a furan ring. To avoid confusion, the cyclic derivative of CBG identified and synthetized by us will be referred to as Pyr-CBG in the further part of this paper.

A more detailed analysis of the results shows that the MS spectrum of Pyr-CBG (see Fig. 5b) is almost identical to the MS spectrum of CBG itself (see MS library). Various intensity of the CBG molecular ion is the only difference in these spectra, and it is visibly higher in the spectrum of Pyr-CBG. These findings suggest that when CBG is analysed, all its molecules entering into the ionization chamber are transformed to its pyranic form. Only a small part of the molecules of this compound undergo ionization and fragmentation processes evoking low GC-MS response. It can be assumed that when CBG is analyzed, most of the energy from the EI source is consumed in its cyclization process. Hence, the GC-MS signal from its pyranic structure is greater than that of CBG itself (see Fig. 5a), as all the energy of the EI source is used to ionize Pyr-CBG molecules.

To confirm that CBG can be cyclized to its pyranic form by energetic physical action, separate CBG samples were exposed to high temperature, UV and electron stream radiation. As can be seen from Fig. 1S (see supplementary materials), UV and electron stream radiation cause CBG transformation to its pyranic form, which does not happen in the thermally treated CBG sample. It is worth noting that only a small part of the thermally treated CBG sample dissolves in DCM, and that the GC-MS signal of its solution is lower than that of the solution of the unheated CBG sample. These observations are probably connected with CBG polymerization and transformation to CBC [32] during its heating process – see CBC peak in Fig. 1Sa (see supplementary materials). Taking into account the results of two other experiments, CBG exposures to UV and electron stream, CBG transformation to the pyranic form in the GC-MS ionization chamber is very likely.

According to the results presented in Fig. 2a and 5a, the silylation of CBG increases the sensitivity of its GC-MS estimation about 3 times, whereas the cyclization of CBG about 9 times. NMR data for Pyr-CBG (Fig. 5c and Table 2S in supplementary materials) prove that it has one free phenolic group (see Fig. 4), which enables a two-step CBG transformation: cyclization (step one) and silylation/acylation (step two). The related question arises whether, for example, the silylation of Pyr-CBG will make CBG analysis sensitivity higher than cyclization of CBG itself? As follows from Fig. 2S (see supplementary materials), showing GC-MS responses to Pyr-CBG before and after its silylation, the silylation process in this particular case does not lead to the increase of the GC-MS signal.

The presence of 3,7-dimethylocta-2,6-dien chain in the CBG molecule allows also for the Diels-Alder reaction to be carried out and to form six other transformation products of this

cannabinoid. The possible products of CBG transformation which can be obtained in the Diels-Alder reaction are shown in Fig. 3S (see supplementary materials). Diels-Alder reaction was not tested in the present study because the conditions required for its performance (high temperature and expensive catalyst) make it impractical in the routine CBG analyses in blood/plasma samples.

As results from the performed experiments, the greatest sensitivity of CBG estimation by GC-MS is observed in the procedure involving cyclization of the cannabinoid to its pyranic form (see Table 1). Fig. 6 showing the results of GC-MS analyses of plasma samples containing CBG, CBN, CBD, Δ^9-THC and CBC mixture proves the practical utility of the CBG cyclization. The comparison of the chromatograms of supernatants from plasma samples obtained by classical QuEChERS and by QuEChERS with the CBG cyclization process, which were used as sample preparation methods, indicates that the novel procedure of CBG transformation is applicable in the analytical procedures of real samples. The presented figure shows that the cyclization process causes:

- a multiple increase of the CBG signal;
- remaining of CBC and CBN signals at the same level;
- the decrease of CBD signal. It is obvious as the cyclization of CBG to its pyranic form requires an acidic environment, in which CBD transform to Δ^9-/Δ^8-THC [23]
- the increase of Δ^9-THC signal, which results from CBD transformation in acidic environment (see above comment)
- the appearance of Δ^8-THC signal, which results from CBD transformation to Δ^9-/Δ^8-THC and Δ^9-THC isomerization to Δ^8-THC in acidic environment (see above comments)

Although the presented chromatograms indicate that the cyclization process causes an additional CBD and THC transformations, nevertheless, the method has great potential in increasing the CBG signal making the quantification of this bio-marker in real samples easier.

Conclusions

It is suggested that due to the relatively rapid metabolism of CBG, the estimation of its concentration in blood or oral fluid can be used to determine cannabis use. A low CBG signal in its GC-MS analysis seriously limits accurate estimation of the cannabinoid in the tested samples. The performed experiments indicate that the low response of the MS detector towards CBG results from its transformation to the pyranic CBG form, which occurs as an effect of electrons energy absorption by CBG molecules in the MS ionization chamber. The most frequent method of increasing the chromatographic analysis sensitivity to the examined compounds is their preliminary derivatization. The presented results shows that although the signal increase of CBG can be obtained through its derivatization by silylation and/or acylation, the greatest increase is observed in the case of its cyclization to the pyranic CBG form during the sample preparation process. The CBG cyclization procedure is very simple and workable in estimating this cannabinoid in blood/plasma samples.

The presented transformation of CBG to its pyranic form seems to be one of the best ways to increase the analytical sensitivity of GC-MS to CBG, but it has one disadvantage. The cyclization of CBG to its pyranic form requires an acidic environment, in which not only CBG undergoes structural transformation in such an environment, but so does CBD (to Δ^9-/Δ^8-THC) and Δ^9-THC (to Δ^8-THC). Therefore, if it is necessary to accurately determine the concentration of THC/CBD in the test sample, these cannabinoids should be determined before and then CBG using the described method. Although the cyclization process causes an additional CBD and THC transformations, nevertheless, the method has great potential in increasing the CBG signal making the quantification of this bio-marker in real samples easier.

Compliance with Ethical Standards

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

All those who provided biological samples provided written informed consent. This study was approved by the University Ethics Committee for research on human blood samples.

CRediT authorship contribution statement

Andrzej L. Dawidowicz: Conceptualization, Writing- Original draft preparation, Writing- Reviewing and Editing, Methodology, Investigation. **Michal P. Dybowski:** Writing- Original draft preparation, Writing- Reviewing and Editing, Investigation, Methodology, Data curation, Visualization. **Rafal Typek:** Writing- Original draft preparation, Investigation, Methodology, Data curation. **Michal Rombel:** Writing- Original draft preparation, Investigation, Data curation. **Piotr Holowinski:** Writing- Original draft preparation, Investigation, Methodology, Data curation.

Data availability

All data generated or analyzed during this study are included in this article

Acknowledgments

We gratefully acknowledge the use of the facilities of the Ecotech-Complex in Lublin. Contract number: UDA-POIG.02.01.00-06-212/09-03.

References

1. Ramzy V, Priefer R (2021) THC detection in the breath. Talanta 222:121528. https://doi.org/10.1016/J.TALANTA.2020.121528

2. Morales P, Hurst DP, Reggio PH (2017) Molecular Targets of the Phytocannabinoids: A Complex Picture. Prog Chem Org Nat Prod 103:103–131. https://doi.org/10.1007/978-3-319-45541-9_4

3. Delgado-Povedano MM, Sánchez-Carnerero Callado C, Priego-Capote F, Ferreiro-Vera C (2020) Untargeted characterization of extracts from Cannabis sativa L. cultivars by gas and liquid chromatography coupled to mass spectrometry in high resolution mode. Talanta 208:120384. https://doi.org/10.1016/j.talanta.2019.120384

4. Kimura T, Takaya M, Usami N, Watanabe K, Yamamoto I (2019) Δ9-Tetrahydrocannabinol, a major marijuana component, enhances the anesthetic effect of pentobarbital through the CB1 receptor. Forensic Toxicol 37: 207–214. https://doi.org/10.1007/s11419-018-0457-2

5. Dybowski MP, Dawidowicz AL (2018) Application of the QuEChERS procedure for analysis of Δ^9-tetrahydrocannabinol and its metabolites in authentic whole blood samples by GC–MS/MS. Forensic Toxicol 36:415–423. https://doi.org/10.1007/S11419-018-0419-8

6. Burnier C, Esseiva P, Roussel C (2019) Quantification of THC in Cannabis plants by fast-HPLC-DAD: A promising method for routine analyses. Talanta 192:135–141. https://doi.org/10.1016/J.TALANTA.2018.09.012

7. Pichini S, Malaca S, Gottardi M, Pérez-Acevedo AP, Papaseit E, Perez-Maña C, Farré M, Pacifici R, Tagliabracci A, Mannocchi G, Busardò FP (2021) UHPLC-MS/MS analysis of cannabidiol metabolites in serum and urine samples. Application to an individual treated with medical cannabis. Talanta 223:121772. https://doi.org/10.1016/J.TALANTA.2020.121772

8. Nelson KM, Bisson J, Singh G, Graham JG, Chen S-N, Friesen JB, Dahlin JL, Niemitz M, Walters MA, Pauli GF (2020) The Essential Medicinal Chemistry of Cannabidiol (CBD). J Med Chem 63:12137–12155. https://doi.org/10.1021/acs.jmedchem.0c00724

9. Devinsky O, Cilio MR, Cross H, Fernandez-Ruiz J, French J, Hill C, Katz R, Di Marzo V, Jutras-Aswad D, Notcutt WG, Martinez-Orgado J, Robson PJ, Rohrback BG, Thiele E, Whalley B, Friedman D (2014) Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. Epilepsia 55:791–802. https://doi.org/10.1111/epi.12631

10. Crippa JA, Guimarães FS, Campos AC, Zuardi AW (2018) Translational Investigation of the Therapeutic Potential of Cannabidiol (CBD): Toward a New Age. Front Immunol 9:2009. https://doi.org/10.3389/FIMMU.2018.02009

11. Tzadok M, Uliel-Siboni S, Linder I, Kramer U, Epstein O, Menascu S, Nissenkorn A, Yosef OB, Hyman E, Granot D, Dor M, Lerman-Sagie T, Ben-Zeev B (2016) CBD-enriched medical cannabis for intractable pediatric epilepsy: The current Israeli experience. Seizure 35:41-44. https://doi.org/10.1016/j.seizure.2016.01.004

12. Pertwee RG (2005) Cannabidiol as a potential medicine. In: Mechoulam R (ed) Cannabinoids as Therapeutics. Birkhäuser-Verlag, Basel, pp 47–65

13. Deville M, Charlier C (2023) Cannabidiol in urine is not a proof of CBD consumption—lesson learned from urine sample analysis in routine caseworks. Forensic Toxicol 41:213–220. https://doi.org/10.1007/s11419-022-00652-8

14. Deiana S (2017) Potential Medical Uses of Cannabigerol: A Brief Overview. In: Preedy VR (ed) Handbook of Cannabis and Related Pathologies. Elsevier, Amsterdam, pp 958–967 https://doi.org/10.1016/B978-0-12-800756-3.00115-0

15. Rague JM, Ma M, Dooley G, Wang GS, Friedman K, Henthorn TK, Brooks-Russell A, Kosnett MJ (2023) The minor cannabinoid cannabigerol (CBG) is a highly specific blood biomarker of recent cannabis smoking. Clin Toxicol 61:363–369. https://doi.org/10.1080/15563650.2023.2173076

16. Jastrząb A, Jarocka-Karpowicz I, Skrzydlewska E (2022) The Origin and Biomedical Relevance of Cannabigerol. Int J Mol Sci 23:7929. https://doi.org/10.3390/IJMS23147929

17. Hidvégi E, Somogyi GP (2010) Detection of cannabigerol and its presumptive metabolite in human urine after Cannabis consumption. Pharmazie 65:408–411. https://doi.org/10.1691/PH.2010.0035R

504 18. Krämer M, Schäper M, Dücker K, Philipsen A, Losacker M, Dreimüller N, Engelmann
505 J, Madea B, Hess C (2021) Detectability of cannabinoids in the serum samples of
506 cannabis users: Indicators of recent cannabis use? A follow-up study. Drug Test Anal
507 13:1614–1626. https://doi.org/10.1002/DTA.3110

508 19. Déléaval M, Burri H, Bakelants E (2022) Harmless herbs? A case report of acquired
509 long QT syndrome and torsades de pointes in a patient taking herbal supplements.
510 HeartRythm Case Rep 8:309–312. https://doi.org/10.1016/J.HRCR.2022.03.018

511 20. Vlad RA, Farczádi L, Toma CM, Imre S, Antonoaea P, Rédai EM, Muntean DL,
512 Ciurba A (2021) Cannabidiol content evaluation in commercial dietary supplements
513 and stability in oil vehicle. Stud Univ Babes-Bolyai Chem 2:299–308.

514 21. Andrenyak DM, Moody DE, Slawson MH, O'Leary DS, Haney M (2017)
515 Determination of Δ-9-Tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-
516 carboxy-THC and Cannabidiol in Human Plasma using Gas Chromatography–Tandem
517 Mass Spectrometry. J Anal Toxicol 41:277–288. https://doi.org/10.1093/JAT/BKW136

518 22. Zekič J, Križman M (2020) Development of Gas-Chromatographic Method for
519 Simultaneous Determination of Cannabinoids and Terpenes in Hemp. Molecules
520 25:5872. https://doi.org/10.3390/MOLECULES25245872

521 23. Dybowski MP, Dawidowicz AL, Typek R, Rombel M (2020) Conversion of
522 cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations
523 prior to plasma samples analysis by chromatography – Troubles with reliable CBD
524 quantitation when acidic precipitation agents are applied. Talanta 220:121390.
525 https://doi.org/10.1016/j.talanta.2020.121390

526 24. Mareck U, Fusshöller G, Geyer H, Huestis MA, Scheiff AB, Thevis M (2021)
527 Preliminary data on the potential for unintentional antidoping rule violations by
528 permitted cannabidiol (CBD) use. Drug Test Anal 13:539–549.
529 https://doi.org/10.1002/DTA.2959

530 25. Antunes M, Barroso M, Gallardo E (2023) Analysis of Cannabinoids in Biological
531 Specimens: An Update. Int J Environ Res Public Health 20:2312.
532 https://doi.org/10.3390/IJERPH20032312

533 26. Dawidowicz AL, Dybowski MP, Rombel M, Typek R (2021) Improving the sensitivity

of estimating CBD and other xenobiotics in plasma samples: Oleamide-induced transient matrix effect. J Pharm Biomed Anal 204:114265. https://doi.org/10.1016/J.JPBA.2021.114265

27. Typek R, Holowinski P, Dawidowicz AL, Dybowski MP, Rombel M (2023) Chromatographic analysis of CBD and THC after their acylation with blockade of compound transformation. Talanta 251:123777. https://doi.org/10.1016/J.TALANTA.2022.123777

28. Fodor B, Molnár-Perl I (2017) The role of derivatization techniques in the analysis of plant cannabinoids by gas chromatography mass spectrometry. TrAC Trends Anal Chem 95:149–158. https://doi.org/10.1016/J.TRAC.2017.07.022

29. Pacifici R, Pichini S, Pellegrini M, Rotolo MC, Giorgetti R, Tagliabracci A, Busardò FP, Huestis MA (2020) THC and CBD concentrations in blood, oral fluid and urine following a single and repeated administration of "light cannabis." Clin Chem Lab Med 58:682–689. https://doi.org/10.1515/CCLM-2019-0119

30. Newmeyer MN, Swortwood MJ, Barnes AJ, Abulseoud OA, Scheidweiler KB, Huestis MA (2016) Free and Glucuronide Whole Blood Cannabinoids' Pharmacokinetics after Controlled Smoked, Vaporized, and Oral Cannabis Administration in Frequent and Occasional Cannabis Users: Identification of Recent Cannabis Intake. Clin Chem 62:1579–1592. https://doi.org/10.1373/CLINCHEM.2016.263475

31. Roy P, Dennis DG, Eschbach MD, Anand SD, Xu F, Maturano J, Hellman J, Sarlah D, Das A (2022) Metabolites of Cannabigerol Generated by Human Cytochrome P450s Are Bioactive. Biochemistry 61:2398-2408. https://doi.org/10.1021/acs.biochem.2c00383

32. García-Valverde MT, Sánchez-Carnerero Callado C, Díaz-Liñán MC, Sánchez de Medina V, Hidalgo-García J, Nadal X, Hanuš L, Ferreiro-Vera C (2022) Effect of temperature in the degradation of cannabinoids: From a brief residence in the gas chromatography inlet port to a longer period in thermal treatments. Front Chem 10:1038729. https://doi.org/10.3389/fchem.2022.1038729

Figures and tables

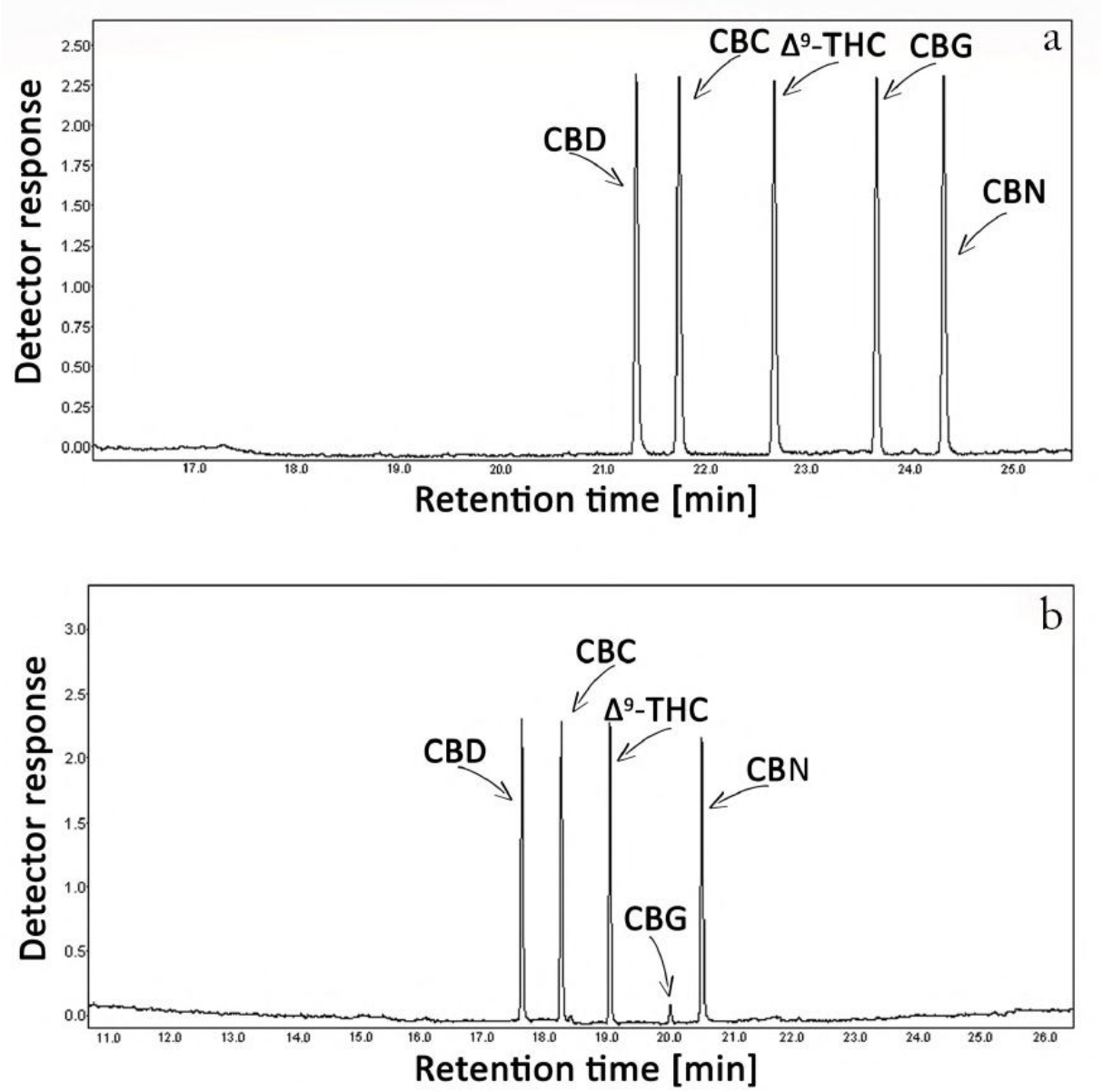
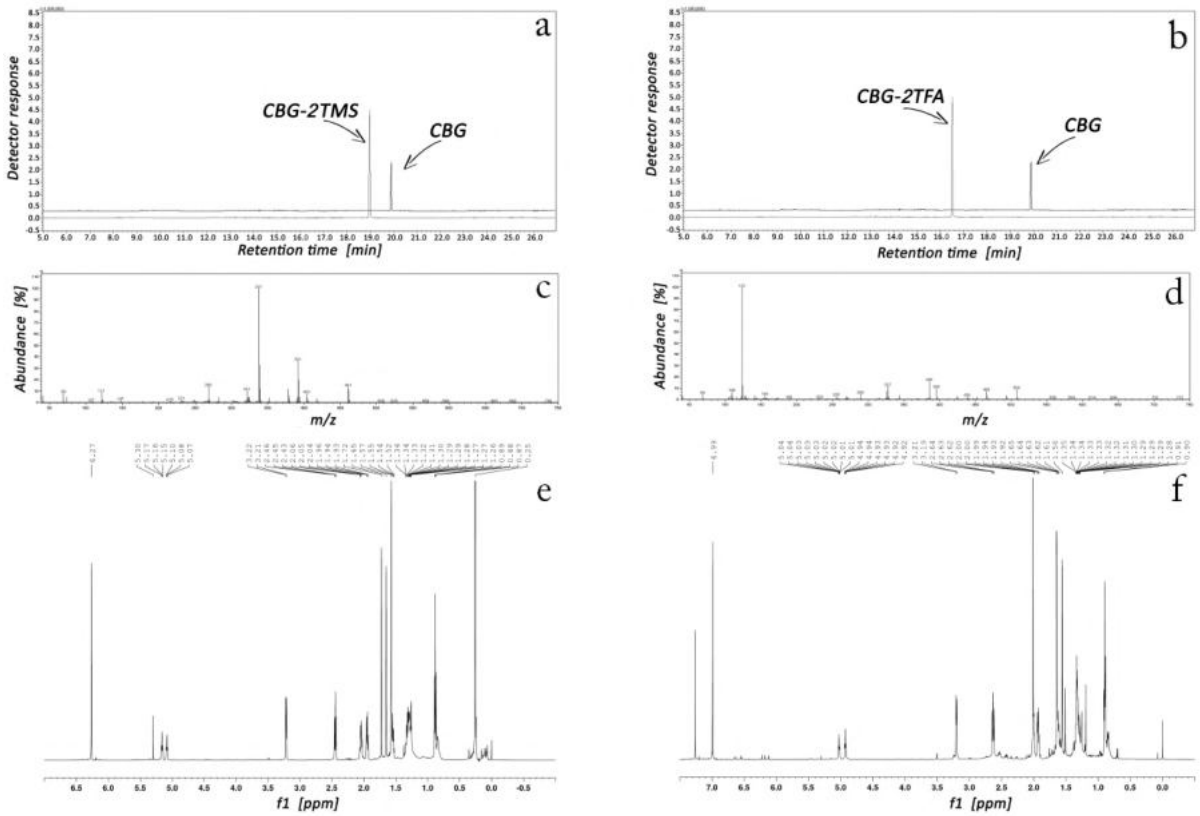
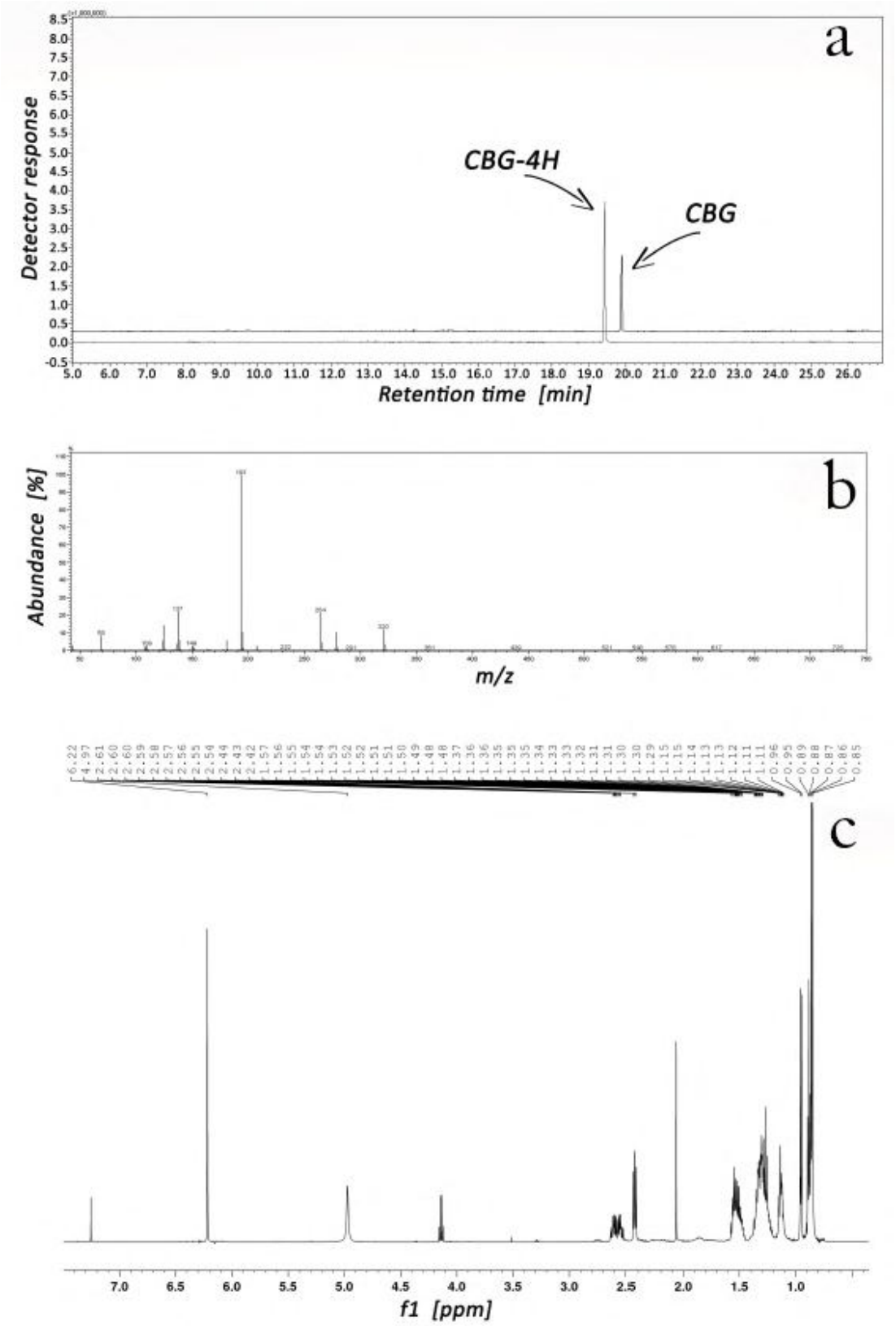


Fig. 1 Exemplary chromatogram obtained by GC-FID (**a**) and total ion current chromatogram (TICC) by GC-MS (**b**) of CBG, CBD, CBC, Δ9-THC and CBN mixture dissolved in ACN. The molar concentration of each mixture component was the same and amounted 0.5 mM.



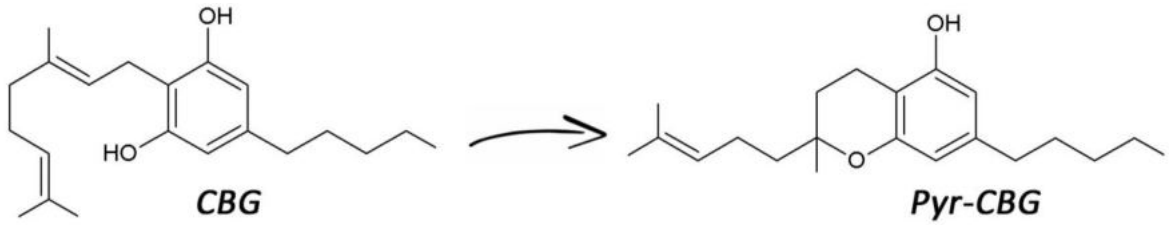
570

571 Fig. 2 TICCs of CBG and CBG-2TMS (**a**), CBG and CBG-2TFA (**b**) corresponding before
572 and after derivatization, respectively. MS spectra of CBG-2TMS (**c**) and CBG-2TFA
573 (**d**). NMR spectra of CBG-2TMS (**e**) and CBG-2TFA (**f**).



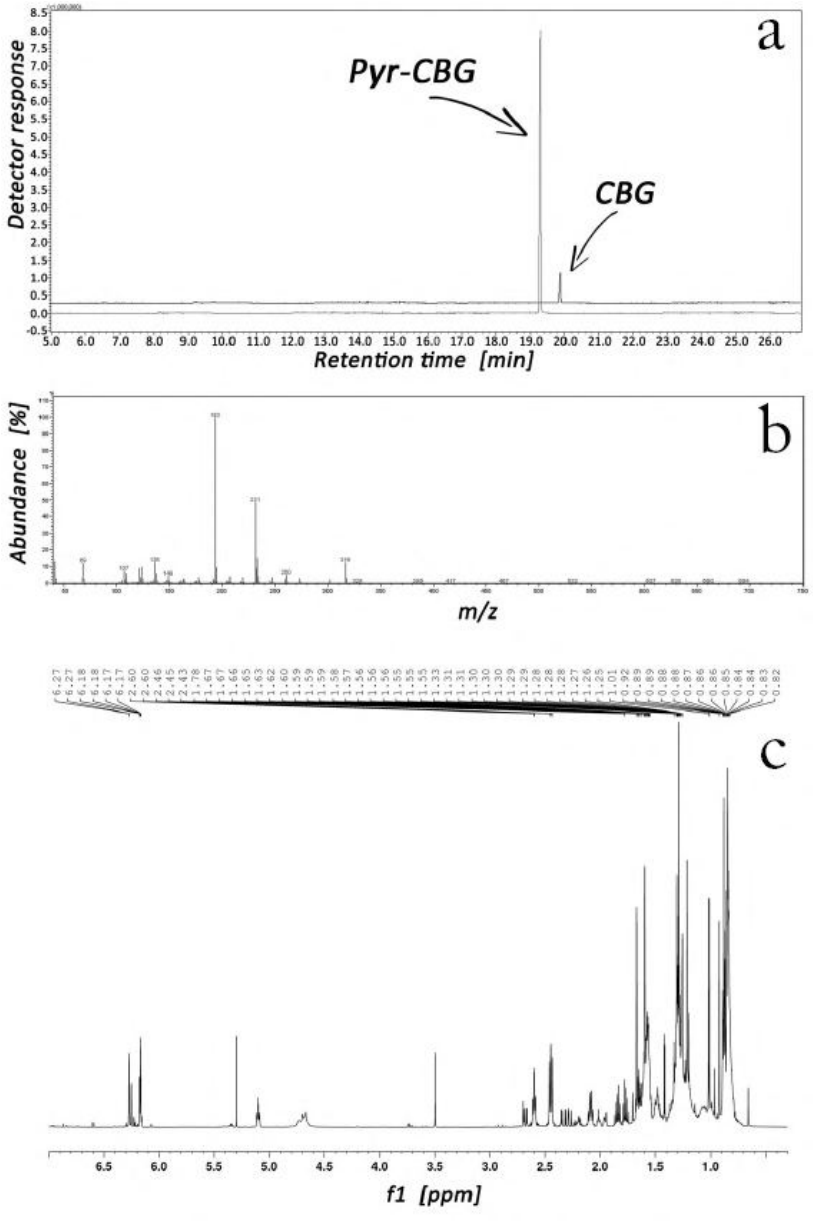
574

575 Fig. 3 TICCs of CBG and CBG-4H corresponding before and after hydrogenation (**a**). MS
576 spectrum (**b**) and NMR spectrum (**c**) of CBG-4H, respectively.



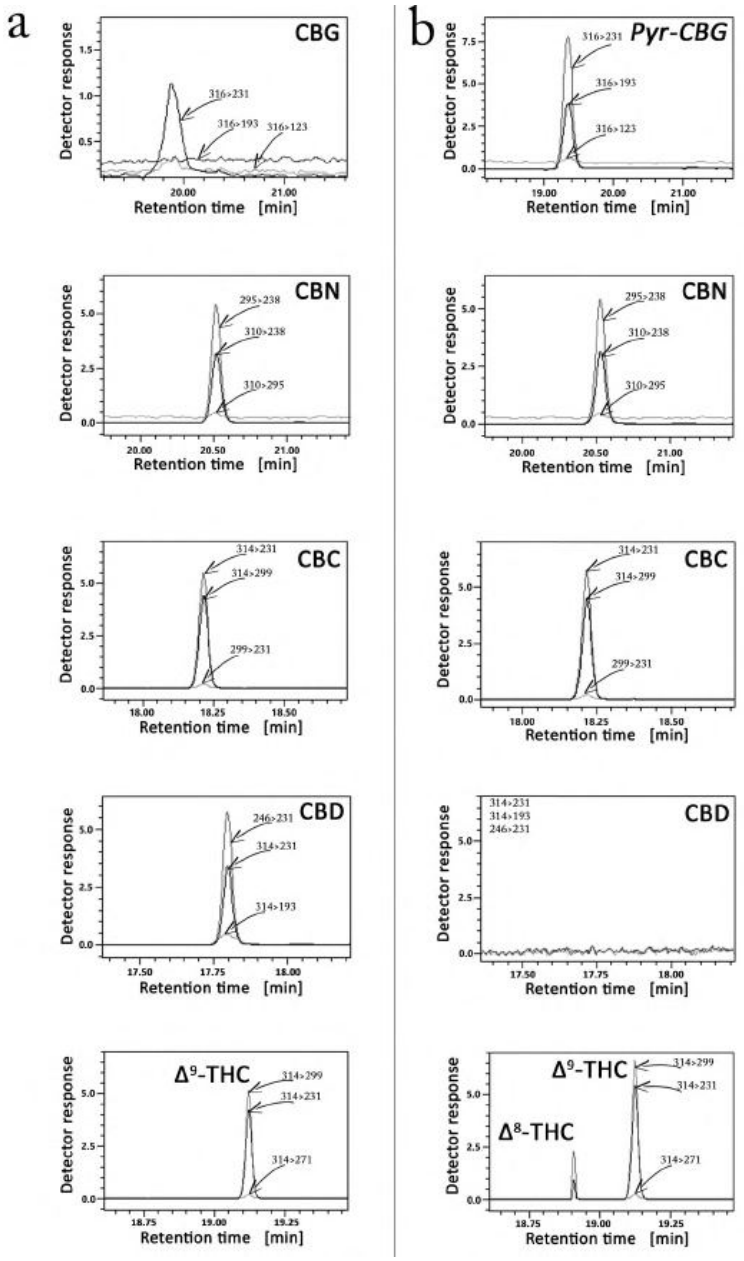
577

578 Fig. 4 Cyclization reaction of CBG to its pyranic form (Pyr-CBG).



579

580 Fig. 5 TICCs of CBG and Pyr-CBG corresponding before and after cyclization (**a**). MS
581 spectrum (**b**) and NMR spectrum (**c**) of Pyr-CBG, respectively.



582

583 Fig. 6 MRM chromatograms of blood sample containing CBG, CBN, CBD, Δ9-THC and
584 CBC mixture before (**a**) and after cyclization (**b**). Arrows indicate signals with
585 corresponding MRM transitions.

586

587 Table 1. Linearities, intra- and interday precisions and accuracies, limits of detection (LOD),
588 limits of quantification (LOQ) of CBG, CBG-2TMS, CBG-2TFA, CBG-4H and Pyr-
589 CBG, respectively.

Tested parameter	Compound				
	CBG	**CBG-2TMS**	**CBG-2TFA**	**CBG-4H**	**Pyr-CBG**
Linearity (R^2)	0.9991	0.9969	0.9975	0.9988	0.9987
Intraday precision (% RSD)	3.17	4.56	4.61	3.74	3.88
Interday precision (% RSD)	3.32	4.82	4.79	3.95	4.21
Intraday accuracy (%)	98.7	97.9	97.1	98.2	97.9
Interday accuracy (%)	97.6	96.2	96.6	97.1	96.9
LOD (ng/mL)	2.78	0.93	0.91	0.83	0.26
LOQ (ng/mL)	9.26	3.09	3.03	2.76	0.86

590 R^2 coefficient of determination, RSD relative standard deviation, LOD limit of detection, LOQ limit of quantification

591

Supplementary materials

Cannabigerol (CBG) signal enhancement in its analysis by gas chromatography coupled with tandem mass spectrometry

Andrzej L. Dawidowicz, Rafał Typek, Michał P. Dybowski, Piotr Hołowiński, Michał Rombel

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Sklodowska University in Lublin, 20-031, Lublin, Poland

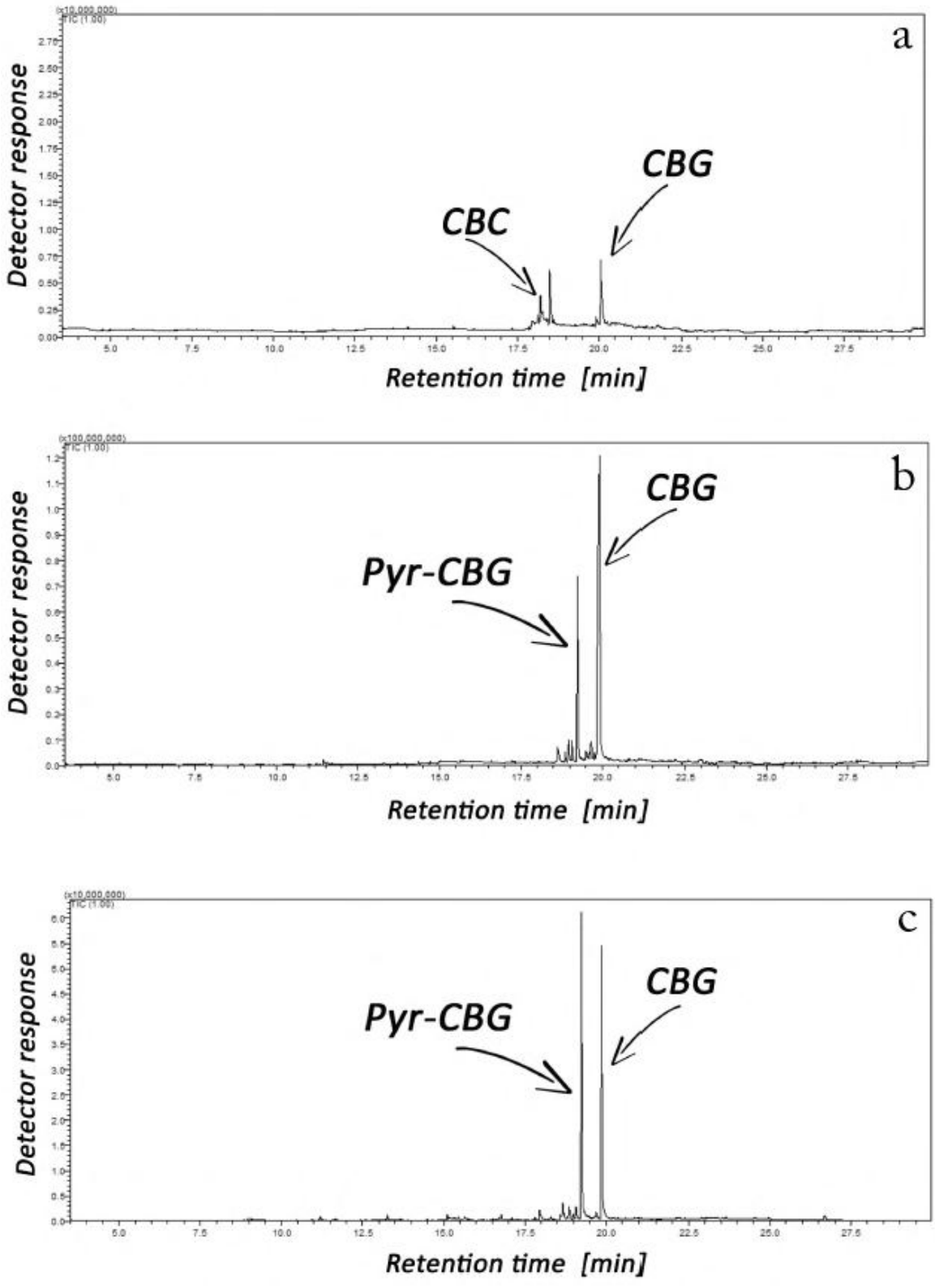


Fig. 1S. TICCs of DCM solutions of CBG samples exposed to thermal treatment (**a**), UV radiation (**b**) and electron stream radiation (**c**).

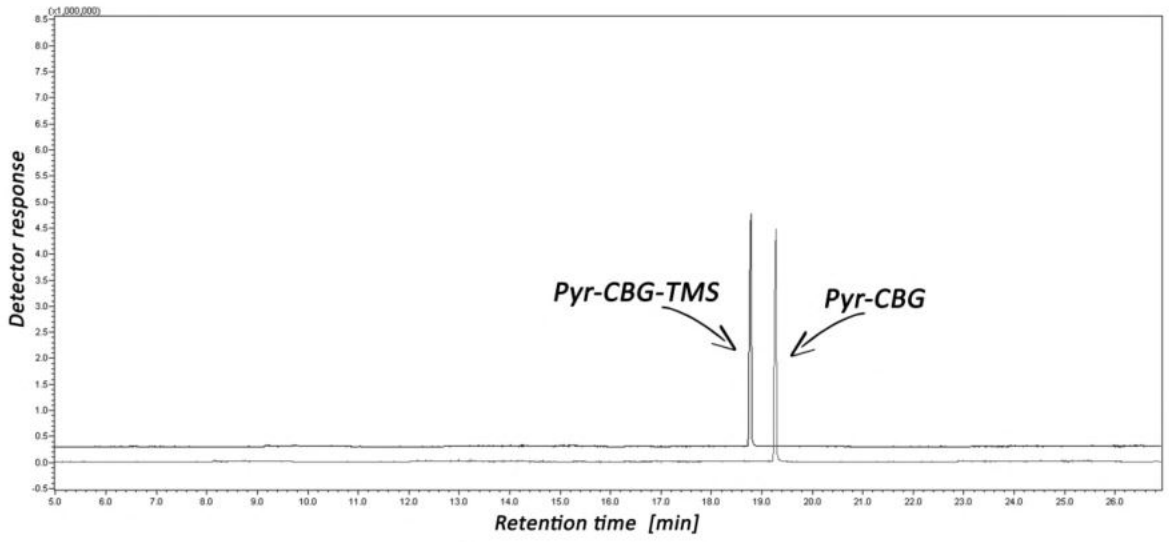


Fig. 2S. TICCs of Pyr-CBG and Pyr-CBG-TMS corresponding before and after derivatization.

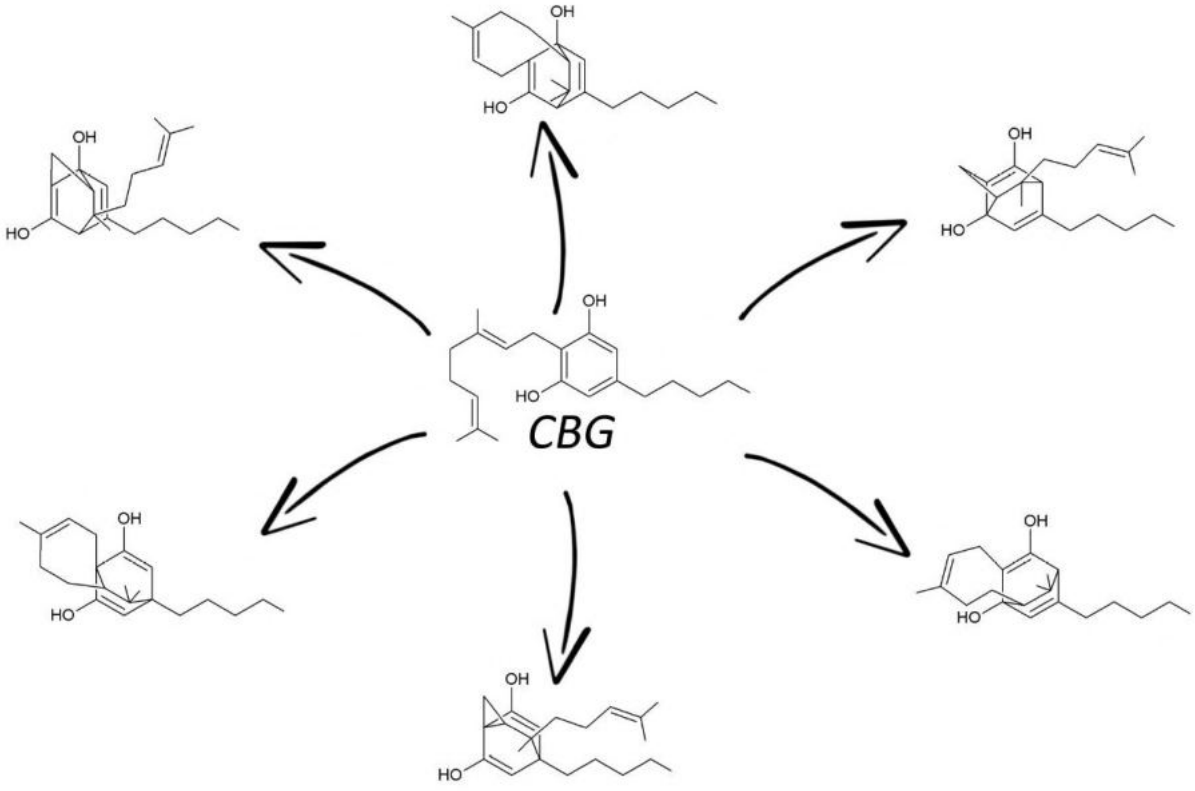
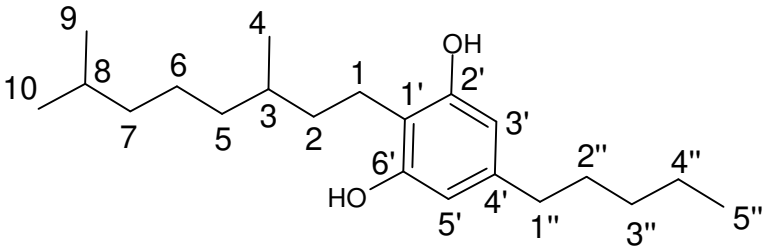


Fig. 3S. Possible CBG cyclization pathways in Diels-Alder reaction.

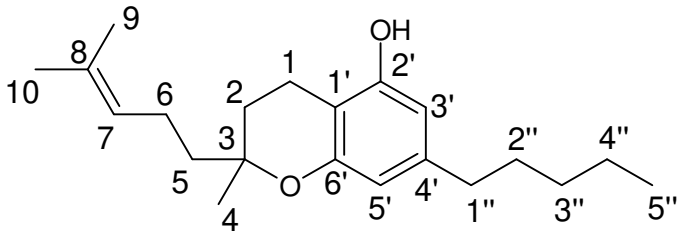
Table 1S. 1H and 13C NMR data of CBG-4H derivative in $CDCl_3$

No.	δ_C, type	δ_H (*J* in Hz)
1	20.7, CH_2	2.60, m
		2.54, m
2	36.4, CH_2	1.53, m[a]
		1.35, m[b]
3	33.2, CH	1.48, m[a]
4	19.6, CH_3	0.95, d (6.5)
5	37.2, CH_2	1.33, m[b]
		1.13, m[c]
6	39.4, CH_2	1.12, m[c]
7	22.5, CH_2	1.31, m[b]
8	28.0, CH	1.51, m[a]
9	22.7, CH_3	0.86, d (6.8)
10		
1′	113.0, C	-
2′	154.4, C	-
3′	108.0, CH	6.22, s
4′	142.0, C	-
5′	108.0, CH	6.22, s
6′	154.4, C	-
2′-OH	-	4.97, brs
6′-OH	-	4.97, brs
1″	35.5, CH_2	2.43, t (7.8)
2″	30.9, CH_2	1.55, m[a]
3″	31.6, CH_2	1.29, m[b]
4″	22.7, CH_2	1.30, m[b]
5″	14.1, CH_3	0.88, t (7.0)

[a-c] Overlapping signals.

Table 2S. 1H and 13C NMR data of Pyr-CBG derivative in $CDCl_3$.

No.	δ_C, type	δ_H (*J* in Hz)
1	16.5, CH_2	2.60, td (6.8, 2.5)
2	30.4, CH_2	1.84, m
		1.77, m
3	75.7, C	-
4	24.0, CH_3	1.28, s
5	39.2, CH_2	1.62, m
6	22.3, CH_2	2.09, q (7.9)
7	124.3, CH	5.10, m
8	131.6, C	-
9	25.7, CH_3	1.67, s
10	17.7, CH_3	1.59, s[a]
1′	105.6, C	-
2′	153.4, C	-
3′	106.3, CH	6.17, d (1.3)
4′	142.5, C	-
5′	109.8, CH	6.27, d (1.2)
6′	154.6, C	-
2′-OH	-	4.72, brs
1″	35.6, CH_2	2.44, t (7.5)
2″	30.9, CH_2	1.56, m[a]
3″	31.7, CH_2	1.29, m[b]
4″	22.7, CH_2	1.31, m[b]
5″	14.2, CH_3	0.88, t (7.0)

[a-b] Overlapping signals.

Publikacja D8

M.P. Dybowski, A.L. Dawidowicz, **M. Rombel**, R. Typek

GC vs. HPLC in quantitation of CBD, CBG, Δ9-THC and CBN in plasma using different sample preparation methods

J. Pharm. Biomed. Anal. 234 (2023) 115563

https://doi.org/10.1016/J.JPBA.2023.115563

IF 2-letni: 3,4; MEiN: 100 pkt

Journal of Pharmaceutical and Biomedical Analysis 234 (2023) 115563

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Short communication

GC vs. HPLC in quantitation of CBD, CBG, Δ9-THC and CBN in plasma using different sample preparation methods

Michal P. Dybowski *, Andrzej L. Dawidowicz, Michal Rombel, Rafal Typek

Department of Chromatography, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie Sklodowska University in Lublin, 20-031 Lublin, Poland

ARTICLE INFO

Keywords:
Cannabinoids quantitation
Sample preparation methods
SPE
QuEChERS
Protein precipitation
LLE

ABSTRACT

The sensitivity of complex analytical procedures depends not only on the sensitivity of the analytical instrument used, but also on the recovery degree of the examined analyte by the employed sample preparation method. The recovery degrees of individual cannabinoids reported in literature, estimated using the same sample preparation method, are unexpectedly divergent. Therefore, the aim of this study was a thorough assessment of the most commonly used sample preparation methods, such as protein precipitation, LLE, QuEChERS and SPE, in the context of the reliability of the obtained results. The presented report shows that the highest sensitivity, precision and reliability of the chromatographic analysis of CBG, CBD, Δ9-THC and CBN in human plasma can be obtained using SPE. The recovery degrees of these cannabinoids by SPE are highly repeatable and exceed 95 %, while they are significantly lower for such sample preparation methods as protein precipitation, LLE and QuEChERS (ca. 80, 65 and 87, respectively). Moreover, the supernatants obtained by the latter methods contain interferents evoking matrix-effect, which makes reliable quantification of the listed cannabinoids by GC difficult. To our knowledge, the paper is the first such extensive comparison of sample preparation procedures used for the determination of cannabinoids in plasma by GC-MS and HPLC-MS. The presented results and the discussion allow to understand why different recovery degrees for the same xenobiotic can be find in literature despite they have been estimated using the same or different sample preparation method or different chromatography types.

1. Introduction

Out of over 150 known cannabinoids, CBD, CBG, Δ9-THC and CBN are the most often mentioned ones today as components of pharmaceutical preparations helpful in medical therapies or dietary supplements supporting the functioning of the human body. Their great popularity results from scientific reports [1] indicating their potential in treating many diseases and ailments. According to [2,3], CBD may be applicable in the management of epilepsy and some other central motor disorders; in the treatment of inflammation states, anxiety, psychotic illnesses, and neurotoxicity (for instance associated with stroke); for the attenuation of unwanted side-effects of Δ9-THC used as a medicine, and in other conditions. CBG, in turn, is credited with cytotoxic effect against epithelioid carcinoma and breast cancer, or with stimulating effect on a number of receptors important in pain, inflammations, and heat sensitization [4]. CBN, unlike CBD and CBG, has weak psychoactive properties (about 10 times weaker than Δ9-THC), yet many countries allow its usage in sleep aiding remedies due to its sedative effect on humans. Its anticonvulsant, anti-inflammatory, antibiotic, and anti-MRSA (methicillin-resistant Staphylococcus aureus) properties are also well known [5].

The interest of potential users (doctors, quacks and people interested in autotherapy) in specific cannabinoids has led to their huge and uncontrolled supply. There are many products on the market today that contain the four cannabinoids dealt with in this article. They are available not only in the form of drugs officially approved by pharmaceutical supervision, but also in various products (cannabinoid oils, cosmetics, dietary supplements, drinks and others) not subjected to strict control in many countries. Consequently, in research works concerning controlled cannabinoids use (e.g. to establish dose-therapeutic effect relationships) as well as the effects of their accidental or uncontrolled consumption (poisoning, allergies, forensic investigations), the determination of cannabinoids in samples of blood/plasma is becoming more and more important.

CBG, CBD, Δ9-THC and CBN are highly hydrophobic molecules of high affinity to bind with plasma proteins. Several analytical procedures have been developed for their estimation in plasma or whole blood applying GC-MS [6], two-dimensional GC-MS [7], and LC-MS/MS [8]

* Corresponding author.
E-mail address: michal.dybowski@mail.umcs.pl (M.P. Dybowski).

https://doi.org/10.1016/j.jpba.2023.115563
Received 14 April 2023; Received in revised form 19 June 2023; Accepted 3 July 2023
Available online 4 July 2023

equipment. They employ liquid-liquid extraction (LLE), solid-phase extraction (SPE), QuEChERS or protein precipitation as sample preparation methods before the cannabinoid analysis [9,10]. Although there are reports in the literature on the determination of cannabinoids in plasma using different sample preparation methods [6–10], there is no unequivocal comparison of these methods in terms of their reliability and usefulness. Due to the fact that there are distinct differences in recovery degree of a given cannabinoid reported in the literature with the use of same sample preparation method, such a comparison seems to be exceptionally justified. This paper presents and discusses the usefulness of the above methods in the context of the sensitivity and precision of the cannabinoids analysis in plasma by CG-MS and LC-MS.

2. Materials and methods

2.1. Reagents and standards

Acetonitrile, dichloromethane and methanol (all LC/MS grade), phosphate buffered saline, pH 7.2 (PBS), the standards of cannabigerol (CBG), cannabidiol (CBD), tetrahydrocannabinol (Δ9-THC), and cannabinol (CBN), all of 1.0 mg/mL in methanol (Cerilliant), were purchased from Merck (Darmstadt, Germany). The Sepra C18-E sorbent (50 μm, 65 Å) was obtained from Phenomenex (Torrance, CA, USA). Acetone (99.5 %), n-hexan (99 %), sodium dihydrogen phosphate dihydrate and di-sodium hydrogen phosphate anhydrous, anhydrous magnesium sulfate (99.5 % powder), and sodium chloride were purchased from Avantor Performance Materials Poland (Gliwice, Poland). Deionized water was purified by the Milli-Q system (Millipore Sigma, Bedford, MA, USA).

100 mL blood samples were collected by a registered nurse from 10 volunteers, after obtaining their informed consent, using a single closed system containing an S-Monovette coagulation activator, according to the manufacturer's instructions (Sarstedt AG, Nümbrecht, Germany), and thoroughly mixed in order to maintain their homogeneity. The samples were centrifuged and the separated plasma was pooled to obtain one representative plasma matrix.

2.2. Plasma sample preparation methods for GC-MS and LC-MS analysis of the examined cannabinoids

Four plasma sample preparation methods were used in the experiments: protein precipitation (PP), liquid-liquid extraction (LLE), QuEChERS, and solid phase extraction (SPE).

2.2.1. Protein precipitation procedure

Numerous compounds and mixtures can be used as protein precipitation agents in the analytical procedures of drug assay in plasma. In general, they fall into three groups: organic solvents (e.g. acetonitrile, methanol, acetone), acidic agents (e.g. H_2SO_4, CF_3COOH, CCl_3COOH, $ZnSO_4$, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, $HClO_4$, $CHCl_3$) and neutral salts (e.g. $MgSO_4$, Na_2SO_4, NaCl, $MgCl_2$, CH_3COONH_4, $HCOONH_4$) [11–13]. As demonstrated in [14,15], if an acidic precipitation agent is used, a significant amount of CBD or Δ9-THC in a sample analyzed by GC transforms to their derivatives, making it difficult to accurately quantify these cannabinoids. This is why organic solvent, acetonitrile, was used as precipitation agent in the reported experiments.

Acetonitrile (2 mL) was added to human plasma (1 mL) containing mixture of CBG, CBD, Δ9-THC and CBN (concentration of each cannabinoid for GC-MS and LC-MS measurements is given in the figures or captions), vortexed and centrifuged for 10 min at 4000 rpm. The separated supernatant was analyzed by GC-MS. Before LC-MS analysis, the supernatant was evaporated and the obtained dry residue was reconstituted in MeOH.

2.2.2. Liquid-liquid extraction

Two extraction procedures were used in the experiments: with and without the addition of protein precipitation agent.

Extraction without protein participation agent (classical extraction). To 1 mL of plasma sample containing mixture of CBG, CBD, Δ9-THC and CBN (for each cannabinoid concentration see figures and their captions), 3.0 mL of n-hexane was added. After 5 min vortexing or vortexing and sonification, the mixture was centrifuged (4000 rpm for 10 min) and the withdrawn organic phase was evaporated using nitrogen stream. The obtained residue was dissolved in an appropriate solvent (ACN for GC-MS or MeOH for LC-MS) and subjected to chromatographic analysis.

Extraction with the addition of protein precipitation agent. Some extraction procedures of cannabinoid from plasma samples recommend the addition of a protein precipitation agent to the sample just before the extraction process [16]. It is sometimes advisable in the case of drugs strongly binding with plasma proteins. To 1 mL of human plasma sample with the mixture of CBG, CBD, Δ9-THC and CBN (for each cannabinoid concentration see figures and their captions), ACN (2 mL) and hexane (3 mL) were added. The mixture, after 5 min vortexing or vortexing and sonification, was centrifuged (4000 rpm for 10 min), and the withdrawn organic phase was evaporated using nitrogen stream. The obtained residue was dissolved in appropriate solvent (ACN for GC-MS or MeOH for LC-MS) and subjected to chromatographic analysis.

2.2.3. QuEChERS

To 1 mL of plasma sample containing the mixture of CBG, CBD Δ9-THC and CBN (for each cannabinoid concentration see figures and their captions), $MgSO_4$ (200.0 mg) and NaCl (50.0 mg) were added. After vortexing for 1 min, acetonitrile (1 mL) was introduced to the vial and the whole suspension was vortexed again and centrifuged at 4000 rpm for 10 min. Finally, the aliquot (1800 μL) was cleaned-up by d-SPE with 15 mg of C18 (Sepra C18-E sorbent) and centrifuged at 4000 rpm for 10 min. The separated supernatant was diluted three-fold in ACN and analyzed by GC-MS. Before LC-MS, the supernatant (1 mL) was evaporated and the obtained dry residue was dissolved in MeOH (3 mL).

2.2.4. Solid phase extraction

1 mL of plasma sample with mixture of CBG, CBD, Δ9-THC and CBN (for each cannabinoid concentration see figures and their captions) was diluted with 4 mL of phosphate buffer (0.1 M, pH 6), carefully mixed and applied to a solid-phase extraction column (Sepra C18-E sorbent, 500 mg) coupled with a vacuum manifold (SPE-12 G processor, J.T. Baker, N.J., USA). Prior to SPE procedure, the SPE column was conditioned by flushing with 2 × 3 mL of methanol, 2 mL of water, 2 × 2 mL water/methanol (80:20; v/v) and dried for 10 min. The cannabinoids were eluted with 7 mL of dichloromethane/acetone (50:50; v/v). The obtained extract was evaporated under a slight stream of nitrogen, reconstituted in 3 mL of appropriate solvent (ACN for GC-MS or MeOH for LC-MS) and subjected to analysis.

The methods described above result from preliminary research aimed at determining their optimal performance in relation to the tested cannabinoids. All sample preparation procedures were performed in glass laboratory vessels and vials.

2.3. Determination of free CBG, CBD, Δ9-THC and CBN concentration - equilibrium dialysis

The binding of CBG, CBD, Δ9-THC and CBN to plasma proteins was determined using equilibrium dialysis [17]. 1 mL of plasma was dialyzed against an equal volume of isotonic PBS (pH 7.2). The dialysis was performed using a self-made two-compartment dialyzers containing YM-10 membranes (product no. 40424, Millipore, Bedford, MA, USA) of 10 kDa molecular mass cutoff in previously established optimal conditions (37 ^{o}C for 6 hr). Aliquots from the buffer cells were diluted with methanol (1:9 v/v) and transferred to LC-MS analysis. The binding degree of individual cannabinoids was estimated according to [18].

2.4. Chromatographic measurements

Qualitative analyses of the examined cannabinoids were conducted by means of gas and liquid chromatography.

GC-MS measurements. A gas chromatograph hyphenated with a triple quadruple tandem mass spectrometer detector (GCMS-TQ8040; Shimadzu, Kyoto, Japan) was used. GC–MS conditions were as follows: capillary column - Zebron ZB5-MSi (30 m x 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Torrance, CA, USA); carrier gas - helium (grade 5.0); flow rate 1.0 mL/min; splitless/split injection mode (sampling time 1.00 min); injector temperature 310 ^{o}C; injection volume 1 μL; temperature program - initial temperature 60 ^{o}C held for 3 min and then increased to 310 ^{o}C at the rate of 12 ^{o}C/min. The final temperature was held for 15 min. Mass spectrometer parameters: normalized electron energy of 70 eV; ion source temperature 225 ^{o}C. In order to analyse supernatants from human plasma samples spiked with cannabinoids, multiple reaction monitoring (MRM) mode was used. GC-MS/MS analysis was performed using characteristic MRM transitions at optimal collision energies (CE) for the examined compounds.

Three MRM transitions ($m/z > m/z$) of the highest intensity were selected for further experiments:

314 > 231 (CE = 24 eV), 314 > 193 (CE = 21 eV) and 246 > 231 (CE = 21 eV) for CBD,
314 > 299 (CE = 12 eV), 314 > 271 (CE = 15 eV) and 314 > 231 (CE = 18 eV) for Δ9-THC,
316 > 231 (CE = 15 eV), 316 > 193 (CE = 15 eV) and 316 > 123 (CE = 6 eV) for CBG,
310 > 295 (CE = 21 eV), 310 > 238 (CE = 30 eV) and 295 > 238 (CE = 27 eV) for CBN.

LC-MS measurements. LC-MS system composed of an UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) and a linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) was applied for the chromatographic analyses of the examined supernatants. ESI ionization source operating in the positive polarization mode at needle potential equal to 4.5 kV was employed. Nitrogen (>99.98 %) was used as sheath gas (at 40 arbitrary units), auxiliary gas (at 10 arbitrary units) and sweep gas (at 10 arbitrary units). Capillary temperature was 320 ^{o}C. The scan cycle used a full-scan event at the resolution of 60,000. Chromatographic separations were performed on a Gemini C18 column (4.6 ×100 mm, 3 μm; Phenomenex, USA). The mobile phase components were: A – 25 mM formic acid in water, and B - 25 mM formic acid in acetonitrile. Isocratic elution was applied using mobile phase containing 40 % of B component. The total run time was 15 min at the mobile phase flow rate 0.5 mL/min.

When analyzing supernatants from plasma samples, the SIM function was used to better visualize the chromatographic separation and to remove the signals originating from the plasma components and the precipitation agent. The monitored ions were as follows:

315 m/z for CBD and Δ9-THC,
317 m/z for CBG,
311 m/z for CBN.

3. Results and discussion

The sensitivity of complex analytical procedures requiring the use of sample preparation method depends not only on the sensitivity of the analytical instrument used, but also on the recovery degree of the examined analyte by the employed method. The higher the recovery degree, the higher the analytical procedure sensitivity. A low detection limit of the examined analyte in analytical procedures is especially important in estimating drug concentration in complex biological matrices, e.g. in plasma, to establish a dose-therapeutic effect relationships. Drugs are known to exist in blood/plasma in either a free and a bound form. The drug binding degree with plasma proteins expresses the strength of its interaction with them. The higher the binding degree of drug to plasma proteins, the more difficult it is to achieve its 100 % recovery degree in the sample preparation procedure [19,20]. The experimentally determined binding degrees for individual cannabinoids with human plasma proteins are gathered in Table 1. The presented data prove that they are extremely high, ca. 99 %. The obtained data are in agreement with fragmentary literature dealing with CBD and Δ9-THC [17]. Table 2.

In the case of drugs strongly binding with plasma proteins, PP is frequently recommended as the method of choice for sample preparation. The recovery degrees of CBG, CBD, Δ9-THC, and CBN estimated on the basis of GC-MS and LC-MS analysis of PP supernatants from plasma are presented in Fig. 1A and B, respectively. The results established by GC-MS for individual cannabinoids are very divergent and significantly exceed 100 %. Such values of recovery degrees for the examined cannabinoids suggest that PP supernatant injected on the column contains, apart from the cannabinoids, also compounds evoking matrix effect [6, 21]. The diagram in Fig. 1C (part I), showing the influence of CBD concentration in plasma samples on its recovery degree calculated from GC-MS analyses of PP supernatants, confirms this supposition - the increase of CBD concentration in a plasma sample causes that the impact of matrix effect on the analyte signal magnitude less and less relevant [6, 21]. At high analyte concentration, the quantification errors resulting from matrix effect may be omitted. Additional evidence confirming the occurrence of matrix effect in the analytical procedure of cannabinoids using the GC involving PP is the result presented in part II of Fig. 1C. It shows that the degree of CBD recovery from plasma estimated by GC employing PP, after which d-SPE sample clean-up was performed to decrease the concentration of interferents evoking matrix effect, equals about 80 %. This value was established for plasma samples containing 2.0 μg/mL of CBD, the concentration at which the influence of matrix effect on the magnitude of the CBD signal is very pronounced when d-SPE clean-up after PP is omitted (compare the bars marked with one asterisk in part I and II of Fig. 1C). It is compatible with that estimated from HPLC analysis of non-cleaned PP supernatant (compare part II of Fig. 1C and Fig. 1B). It should be remembered that matrix effect does not occur in HPLC.

The data presented in Fig. 1 indicate that:

- the recovery degrees of the individual cannabinoids in the analytical procedure involving PP as the method of plasma sample preparation are about 80 %. Ca. 20 % of the cannabinoids contained in the sample is co-precipitated and occluded in the centrifuged protein sediment. ACN as plasma protein precipitating agent was used in these experiments. A similar recovery degrees of the studied cannabinoids from plasma are registered with the use of neutral precipitating salts (e.g. $MgSO_4$, Na_2SO_4, NaCl, $MgCl_2$). The use of acidic precipitating agents (in PP procedure) might allow to receive higher recovery degrees of the examined cannabinoids; yet they should not be used in CBD and Δ9-THC quantification, as an acidic environmental catalyses the transformation of CBD to Δ9-THC and the isomerization of Δ9-THC to Δ8-THC [14,15]
- HPLC is a more convenient and reliable analytical method than GC to quantitate the examined cannabinoids in the PP supernatant. The

Table 1
Binding degree of CBG, CBD, Δ9-THC and CBN (in %) with human plasma proteins.

Compound	Protein binding degree [%]
CBD	99,82
Δ9-THC	99,75
CBG	99,71
CBN	99,84

Table 2
Relative standard deviation in quantification of examined cannabinoids by GC and HPLC procedures involving different sample preparation methods.

		Compound	Sample preparation method			
			LLE	PP	QuEChERS	SPE
			Relative standard deviation RSD [%]			
Analytical method	GC	CBD	7.3 %	6.1 %	4.5 %	3.8 %
		Δ9-THC	7.1 %	6.0 %	4.3 %	3.6 %
		CBG	7.5 %	6.2 %	4.4 %	3.7 %
		CBN	7.2 %	5.8 %	4.6 %	3.8 %
	LC	CBD	6.9 %	5.8 %	4.0 %	3.1 %
		Δ9-THC	6.7 %	5.4 %	4.3 %	3.2 %
		CBG	6.8 %	5.5 %	4.2 %	3.5 %
		CBN	7.1 %	5.5 %	4.1 %	3.4 %

occurrence of matrix effect during GC analysis of cannabinoids in PP supernatants limits the use of this technique to plasma samples containing cannabinoids to high concentration levels at which the influence of matrix effect on the final result is irrelevant (see bars with two asterisks in part I of Fig. 1C). The use of GC for the analysis of PP supernatants from plasma samples containing low levels of cannabinoids, usually found in human blood, requires the removal of substances that cause matrix effect (e.g. by performing d-SPE after the PP procedure), or the use of calibration methods eliminating errors resulting from matrix effect (e.g. the use of deuterated analytes as internal standards in the internal calibration method [14, 15]).

The recovery degrees of CBG, CBD Δ9-THC and CBN estimated on the basis of GC-MS and LC-MS analysis of LLE supernatants from plasma

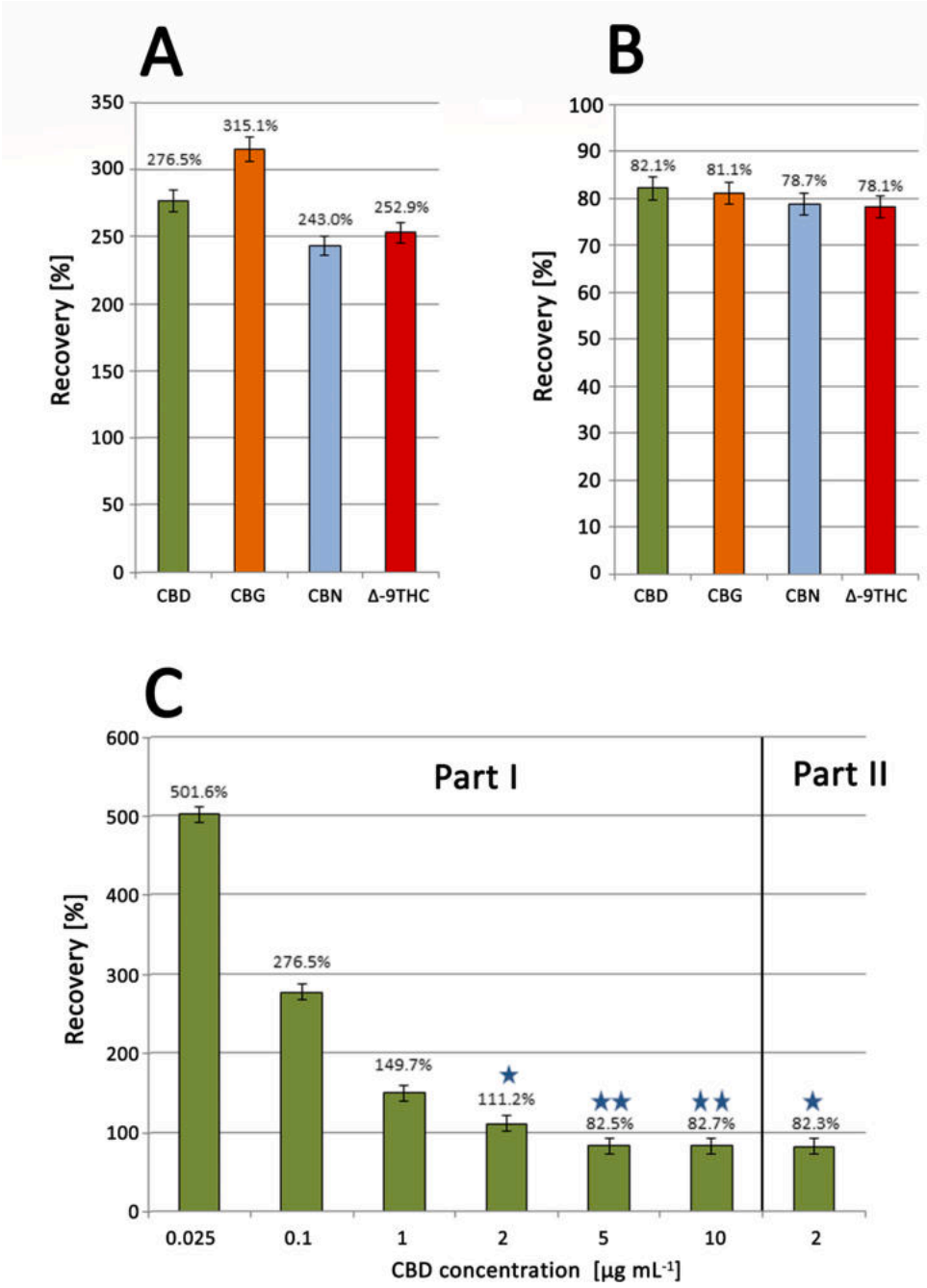


Fig. 1. Recovery degree of CBD (green bar), CBG (orange bar), CBN (blue bar) and Δ9-THC (red bar) calculated from GC-MS (A) and LC-MS (B) analysis of PP supernatants from human plasma samples containing 0.1 μg/mL (for GC-MS) or 0.2 μg/mL (for LC-MS) of each cannabinoid. Recovery degree of CBD calculated from GC-MS analysis of PP supernatants from plasma samples with different cannabinoid concentration (diagram C - part I) and recovery degree of CBD calculated from GC-MS analysis of d-SPE cleaned-up PP supernatant from plasma sample containing 2.0 μg/mL CBD (diagram C - part II).

are presented in Fig. 2A and B, respectively. The analysis of these diagrams shows that the recovery degree of individual cannabinoids by LLE is similar but very low (ca. 30 % and 25 % from GC-MS and LC-MS data, respectively). In these experiments hexane was applied as extracting liquid. It is worth mentioning that almost the same recovery of cannabinoids from plasma is obtained with the hexane/ethyl acetate mixture (9/1 v/v) - another popular extractant applied for the isolation of hydrophobic drugs from plasma (data not presented in this paper). The main reason of the extremely low recovery degree of individual cannabinoids from plasma by LLE seems to be very strong binding of these hydrophobic analytes to plasma proteins. If it is true, any additional process preceding and/or assisting LLE which would facilitate the disintegration of protein-cannabinoid complex should increase the cannabinoids recovery degrees by this method. Fig. 2C presents the influence of ultrasounds and preliminary protein precipitation by ACN or NaCl on the recovery degree of CBD assumed in this experiment as a representative of the examined cannabinoids. Each of the used modifications of the classical LLE process enhances the recovery degree of CBD. The presented results confirm the literature reports recommending the initial precipitation of protein before the extraction process of cannabinoids from plasma [16], but they also indicate that the process (i.e. LLE preceded by protein precipitation) benefits from the support by ultrasounds. Hence, it can be assumed that strong binding of individual cannabinoids to plasma proteins is responsible for their low recovery degree by LLE (see Fig. 2A and B). The similarity of the recovery degrees of individual cannabinoids determined by GC and HPLC using ultrasound-assisted LLE preceded by protein precipitation (compare Fig. 2D and E) indicates the absence of interferents evoking matrix effect in the obtained supernatants. According to these results, about 35 % of each cannabinoid is in settled phase (i.e. in the mixture of protein sediment, rest of plasma and ACN). Hence, the loss of the examined cannabinoids in LLE preceded by precipitation is greater than in PP. It is also worth noting that the recovery degrees of individual cannabinoids, we determined using LLE, are consistent with the literature data for this procedure [22].

The recovery degrees of CBG, CBD Δ9-THC and CBN estimated on the base of GC-MS and LC-MS analysis of QuEChERS supernatants from plasma are presented in Fig. 3A and B, respectively. Its analysis shows that recovery degrees of individual cannabinoids by QuEChERS are very high, and that the recovery degrees determined from GC-MS data are slightly higher (on average ca. 7 %) than those from LC-MS data. It can results from a small influence of matrix effect on signal magnitudes of individual cannabinoids despite the use of d-SPE sample clean-up. The results presented in Fig. 3C confirm this supposition. At cannabinoid concentrations at which the impact of matrix effect is meaningful, the recovery degree estimated by GC-MS is the same as that estimated by LC-MS. It needs to be mentioned at this point that the standard amount of C-18 sorbent applied in our experiments (15 mg) is insufficient to remove interferents evoking GC matrix effect, whereas its increase causes the loss of the examined analytes (data not presented in this paper). Thus, as the amount and type of interfering compounds, and consequently the size of the matrix effect, depends on plasma origin, the amount of sorbent added in the sample clean-up step should be optimized individually to the examined matrix.

The recovery degrees of CBG, CBD Δ9-THC and CBN estimated on the base of GC-MS and LC-MS analysis of SPE extracts from plasma samples are presented in Fig. 4A and B, respectively. As appears from Fig. 4B, the recovery degrees of individual cannabinoids calculated from LC-MS data are the highest of those received by other sample preparation methods (compare with Figs. 1B, 2B, and 3B). Moreover, they are almost the same, within experimental error, as those calculated from GC-MS measurements. This indicates that the SPE extracts do not

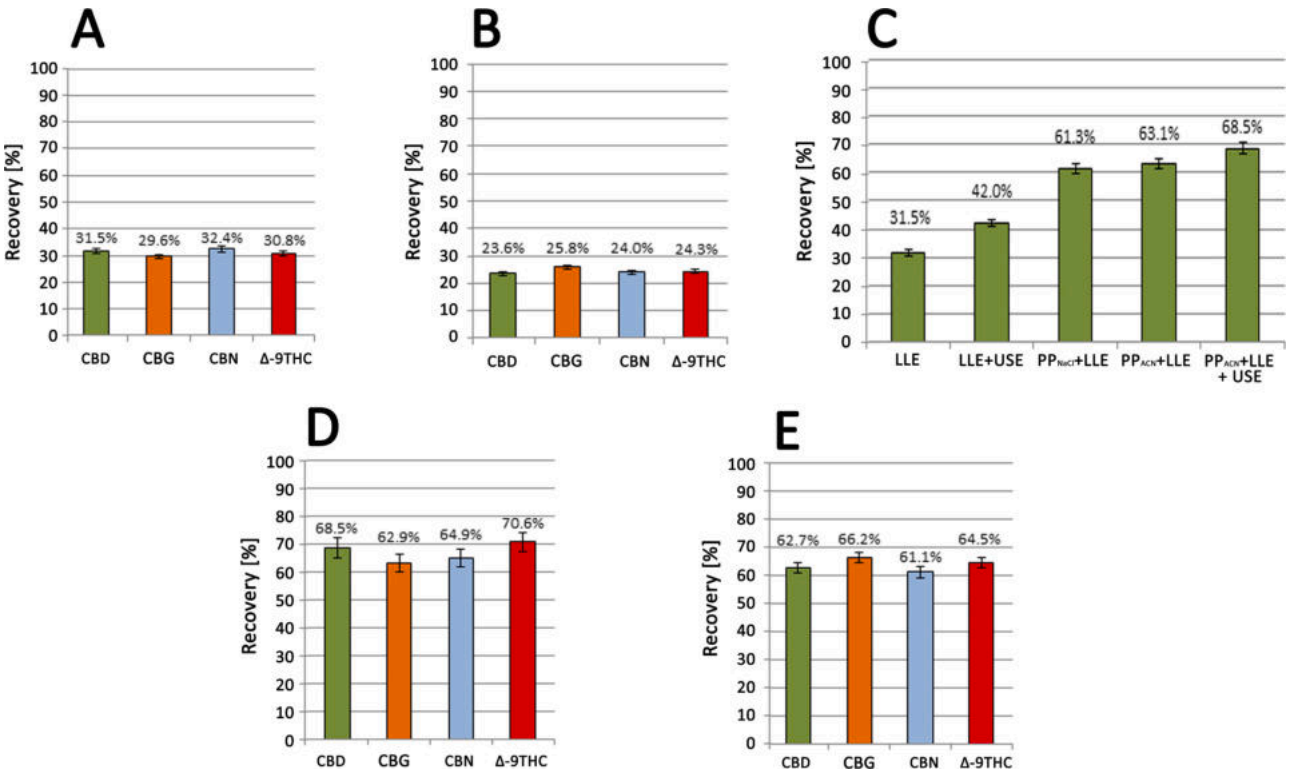


Fig. 2. Recovery degree of CBD (green bar), CBG (orange bar), CBN (blue bar) and Δ9-THC (red bar) calculated from GC-MS (A) and LC-MS (B) analysis of LLE supernatants from plasma samples containing 0.1 μg/mL (for GC-MS) or 0.2 μg/mL (for LC-MS) of each cannabinoid. Recovery degree of CBD calculated from GC-MS analysis of LLE supernatants obtained at the ultrasounds assistance or after preliminary protein precipitation (using NaCl or ACN) or at the ultrasounds assistance and preliminary protein precipitation (using ACN) from plasma samples containing 0.1 μg/mL of CBD (diagram C). Recovery degree of CBG, CBD, Δ9-THC and CBN calculated from GC-MS (D) and LC-MS (E) analysis of LLE supernatants obtained at the ultrasounds assistance and preliminary protein precipitation (using ACN) from plasma samples containing 0.1 μg/mL (for GC-MS) or 0.2 μg/mL (for LC-MS) of each cannabinoid.

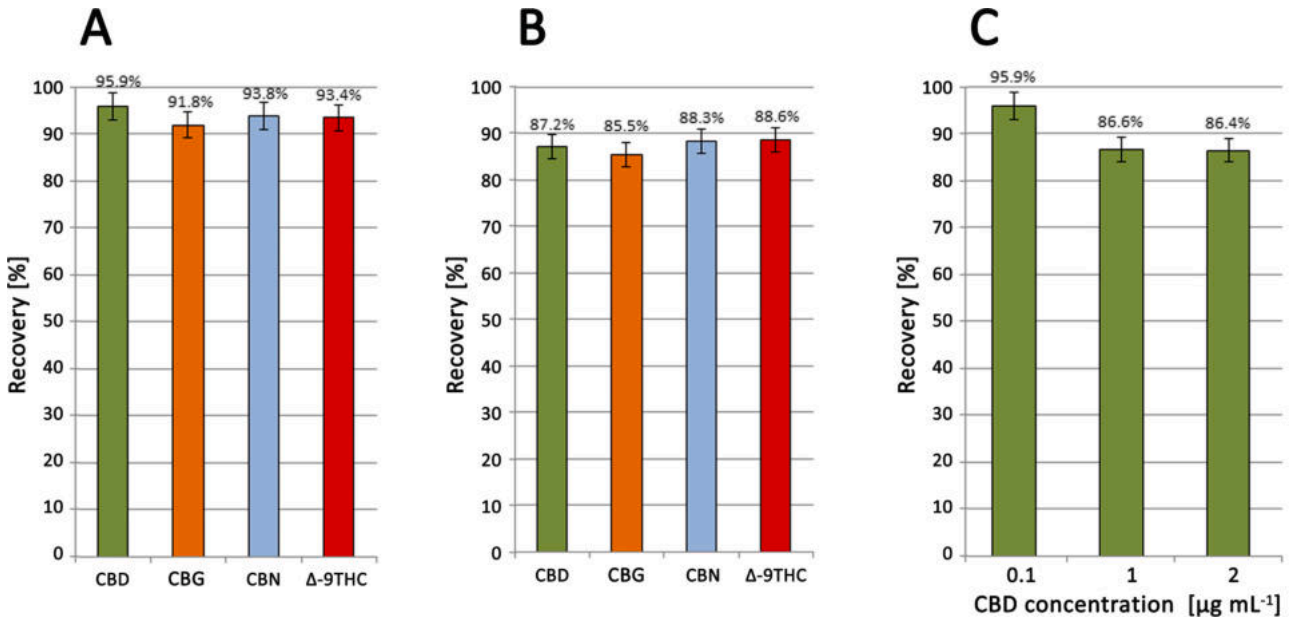


Fig. 3. Recovery degree of CBD (green bar), CBG (orange bar), CBN (blue bar) and Δ9-THC (red bar) calculated from GC-MS (A) and LC-MS (B) analysis of QuEChERS supernatants from plasma samples containing 0.1 µg/mL (for GC-MS) or 0.2 µg/mL (for LC-MS) of each cannabinoid. Recovery degree of CBD calculated from GC-MS analysis of QuEChERS supernatants from plasma samples with different cannabinoid concentration (diagram C).

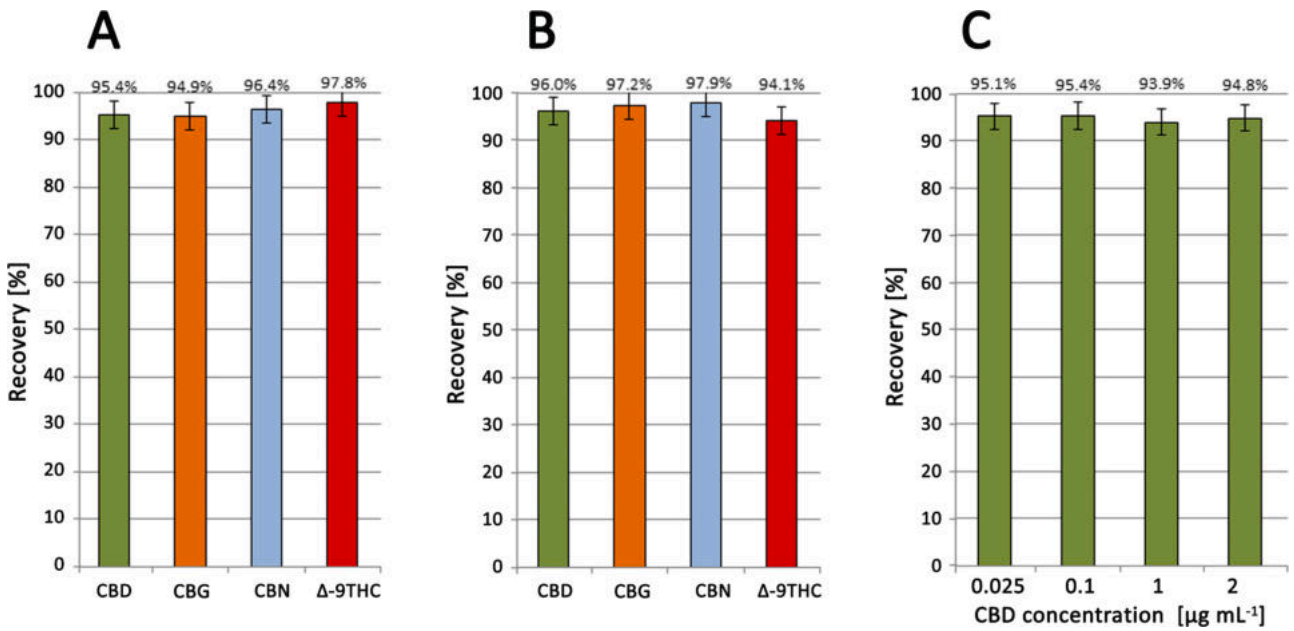


Fig. 4. Recovery degree of CBD (green bar), CBG (orange bar), CBN (blue bar) and Δ9-THC (red bar) calculated from GC-MS (A) and LC-MS (B) analysis of SPE extracts from plasma samples containing 0.1 µg/mL (for GC-MS) or 0.2 µg/mL (for LC-MS) of each cannabinoid. Recovery degree of CBD calculated from GC-MS analysis of SPE extracts from plasma samples with different cannabinoid concentration (diagram C).

contain compounds inducing matrix effect in the GC analysis of the tested cannabinoids. The absence of effect of the concentration changing of cannabinoid (CBD) on its recovery degree by SPE confirms the above statement (see Fig. 4C). It is worth stressing that the almost 100 % degree of SPE recovery of individual cannabinoids compares favorably with the 40–80 % degrees reported in the literature [23–25]. However, the explanation of this difference requires separate research, taking into account the impact of plasma sample storage conditions, the presence of xenobiotics and a few other factors.

4. Conclusions

The recovery degree of the analyte by the sample preparation method used in its quantitation is a key factor influencing the sensitivity of the analytical procedure. According to the performed research, the highest sensitivity of the chromatographic analysis of CBG, CBD, Δ9-THC and CBN in plasma can be obtained using SPE as a sample preparation method. It can be performed without restrictions using GC as well as HPLC in the final stage of the analysis. The recovery degrees of individual cannabinoids with SPE is similar and close to 100 %. In the case of other sample preparation methods– PP, LLE-assisted by ultrasounds

after protein precipitation and QuEChERS– the recovery degrees of individual cannabinoids within a given method are also similar, but their values are visibly lower than those obtained using SPE. In addition, the supernatants obtained in this way contain interferents evoking matrix effect, which makes reliable quantification of the examined cannabinoids by GC difficult. The latter problem is especially important because the amount and type of interfering compounds, and thus the size of the matrix effect, depends on plasma origin.

To our knowledge, the paper is the first such extensive comparison of sample preparation procedures used for the determination of cannabinoids in plasma by GC-MS and HPLC-MS.

CRediT authorship contribution statement

Michal P. Dybowski: Writing- Original draft preparation, Writing-Reviewing and Editing, Investigation, Methodology, Data curation, Visualization. **Andrzej L. Dawidowicz:** Conceptualization, Writing-Original draft preparation, Writing- Reviewing and Editing, Methodology, Investigation. **Michal Rombel:** Writing- Original draft preparation, Investigation, Data curation. **Rafal Typek:** Writing- Original draft preparation, Investigation, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

References

[1] K.M. Nelson, J. Bisson, G. Singh, J.G. Graham, S.N. Chen, J.B. Friesen, J.L. Dahlin, M. Niemitz, M.A. Walters, G.F. Pauli, The essential medicinal chemistry of cannabidiol (CBD), J. Med. Chem. 63 (2020) 12137–12155, https://doi.org/10.1021/acs.jmedchem.0c00724.

[2] R.G. Pertwee, Cannabidiol as a potential medicine, in: Cannabinoids as Ther., Birkhäuser-Verlag, 2005, pp. 47–65, https://doi.org/10.1007/3-7643-7358-x_3.

[3] A.F. Lo Faro, B. Venanzi, G. Pilli, U. Ripani, G. Basile, S. Pichini, F.P. Busardò, Ultra-high-performance liquid chromatography-tandem mass spectrometry assay for quantifying THC, CBD and their metabolites in hair. Application to patients treated with medical cannabis, J. Pharm. Biomed. Anal. 217 (2022), 114841, https://doi.org/10.1016/J.JPBA.2022.114841.

[4] H. Clarke, P. Roychoudhury, S.N. Narouze, Other phytocannabinoids, Cannabinoids Pain. (2021) 87–92, https://doi.org/10.1007/978-3-030-69186-8_12.

[5] E.B. Russo, J. Marcu, Cannabis pharmacology: the usual suspects and a few promising leads, Adv. Pharm. 80 (2017) 67–134, https://doi.org/10.1016/BS.APHA.2017.03.004.

[6] A.L. Dawidowicz, M.P. Dybowski, M. Rombel, R. Typek, Improving the sensitivity of estimating CBD and other xenobiotics in plasma samples: Oleamide-induced transient matrix effect, J. Pharm. Biomed. Anal. 204 (2021), 114265, https://doi.org/10.1016/J.JPBA.2021.114265.

[7] R. Andrews, S. Paterson, A validated method for the analysis of cannabinoids in post-mortem blood using liquid-liquid extraction and two-dimensional gas chromatography-mass spectrometry, Forensic Sci. Int. 222 (2012) 111–117, https://doi.org/10.1016/j.forsciint.2012.05.007.

[8] F. Palazzoli, C. Citti, M. Licata, A. Vilella, L. Manca, M. Zoli, M.A. Vandelli, F. Forni, G. Cannazza, Development of a simple and sensitive liquid chromatography triple quadrupole mass spectrometry (LC–MS/MS) method for the determination of cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC) and its metabolites in rat whole blood after oral administration of a single high dose of CBD, J. Pharm. Biomed. Anal. 150 (2018) 25–32, https://doi.org/10.1016/J.JPBA.2017.11.054.

[9] M.P. Dybowski, A.L. Dawidowicz, Application of the QuEChERS procedure for analysis of Δ9-tetrahydrocannabinol and its metabolites in authentic whole blood samples by GC–MS/MS, Forensic Toxicol. 36 (2018) 415–423, https://doi.org/10.1007/s11419-018-0419-8.

[10] M.J. Roslawski, R.P. Remmel, A. Karanam, I.E. Leppik, S.E. Marino, A.K. Birnbaum, Simultaneous quantification of 13 cannabinoids and metabolites in human plasma by liquid chromatography tandem mass spectrometry in adult epilepsy patients, Ther. Drug Monit. 41 (2019) 357–370, https://doi.org/10.1097/FTD.0000000000000583.

[11] J. Klawitter, C. Sempio, S. Mörlein, E. De Bloois, J. Klepacki, T. Henthorn, M. A. Leehey, E.J. Hoffenberg, K. Knupp, G.S. Wang, C. Hopfer, G. Kinney, R. Bowler, N. Foreman, J. Galinkin, U. Christians, J. Klawitter, An atmospheric pressure chemical ionization MS/MS assay using online extraction for the analysis of 11 cannabinoids and metabolites in human plasma and urine, Ther. Drug Monit. 39 (2017) 556–564, https://doi.org/10.1097/FTD.0000000000000427.

[12] L. Jiang, L. He, M. Fountoulakis, Comparison of protein precipitation methods for sample preparation prior to proteomic analysis, J. Chromatogr. A. 1023 (2004) 317–320, https://doi.org/10.1016/j.chroma.2003.10.029.

[13] A.L. Dawidowicz, A. Fijalkowska, Determination of propofol in blood by HPLC. Comparison of the extraction and precipitation methods, J. Chromatogr. Sci. 33 (1995) 377–382, https://doi.org/10.1093/CHROMSCI/33.7.377.

[14] K. Watanabe, Y. Itokawa, S. Yamaori, T. Funahashi, T. Kimura, T. Kaji, N. Usami, I. Yamamoto, Conversion of cannabidiol to Δ9-tetrahydrocannabinol and related cannabinoids in artificial gastric juice, and their pharmacological effects in mice, Forensic Toxicol. 25 (2007) 16–21, https://doi.org/10.1007/s11419-007-0021-y.

[15] M.P. Dybowski, A.L. Dawidowicz, R. Typek, M. Rombel, Conversion of cannabidiol (CBD) to Δ9-tetrahydrocannabinol (Δ9-THC) during protein precipitations prior to plasma samples analysis by chromatography – Troubles with reliable CBD quantitation when acidic precipitation agents are applied, Talanta 220 (2020), 121390, https://doi.org/10.1016/j.talanta.2020.121390.

[16] E. Hidvégi, G.P. Somogyi, Determination of main tetrahydrocannabinoids by GC-MS: Impact of protein precipitation by acetonitrile on solid phase extraction of cannabinoids from human serum, Pharmazie 69 (2014) 417–419, https://doi.org/10.1691/ph.2014.3229R.

[17] D.E. Brenneman, D. Petkanas, W.A. Kinney, Pharmacological comparisons between cannabidiol and KLS-13019, J. Mol. Neurosci. 66 (2018) 121–134, https://doi.org/10.1007/S12031-018-1154-7/FIGURES/5.

[18] H. Liu, P.P. Wu, M.J. Yang, L. Men, H.L. Lin, Y.L. Zhao, X. Tang, Z.G. Yu, Application of a UPLC-MS/MS method to the protein binding study of TM-2 in rat, human and beagle dog plasma, J. Pharm. Anal. 6 (2016), https://doi.org/10.1016/j.jpha.2015.08.001.

[19] A.L. Dawidowicz, R. Kalitynski, M. Kobielski, J. Pieniadz, Influence of propofol concentration in human plasma on free fraction of the drug, Chem. Biol. Interact. 159 (2006) 149–155, https://doi.org/10.1016/J.CBI.2005.10.108.

[20] A.L. Dawidowicz, R. Kalityński, HPLC investigation of free and bound propofol in human plasma and cerebrospinal fluid, Biomed. Chromatogr. 17 (2003) 447–452, https://doi.org/10.1002/BMC.269.

[21] A.L. Dawidowicz, M.P. Dybowski, M. Rombel, R. Typek, Oleamide as analyte protectant in GC analysis of THC and its metabolites in blood, J. Pharm. Biomed. Anal. 215 (2022), https://doi.org/10.1016/J.JPBA.2022.114800.

[22] D.M. Andrenyak, D.E. Moody, M.H. Slawson, D.S. O'Leary, M. Haney, Determination of Δ-9-Tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC and cannabidiol in human plasma using gas chromatography–tandem mass spectrometry, J. Anal. Toxicol. 41 (2017) 277, https://doi.org/10.1093/JAT/BKW136.

[23] T. Rosado, L. Fernandes, M. Barroso, E. Gallardo, Sensitive determination of THC and main metabolites in human plasma by means of microextraction in packed sorbent and gas chromatography-tandem mass spectrometry, J. Chromatogr. B. Anal. Technol. Biomed. Life Sci. 1043 (2017) 63–73, https://doi.org/10.1016/J.JCHROMB.2016.09.007.

[24] R.A. Gustafson, E.T. Moolchan, A. Barnes, B. Levine, M.A. Huestis, Validated method for the simultaneous determination of Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy-THC in human plasma using solid phase extraction and gas chromatography–mass spectrometry with positive chemical ionization, J. Chromatogr. B. 798 (2003) 145–154, https://doi.org/10.1016/J.JCHROMB.2003.09.022.

[25] E.W. Schwilke, E.L. Karschner, R.H. Lowe, A.M. Gordon, J.L. Cadet, R.I. Herning, M.A. Huestis, Intra- and intersubject whole blood/plasma cannabinoid ratios determined by 2-dimensional, electron impact GC-MS with cryofocusing, Clin. Chem. 55 (2009) 1188–1195, https://doi.org/10.1373/CLINCHEM.2008.114405.