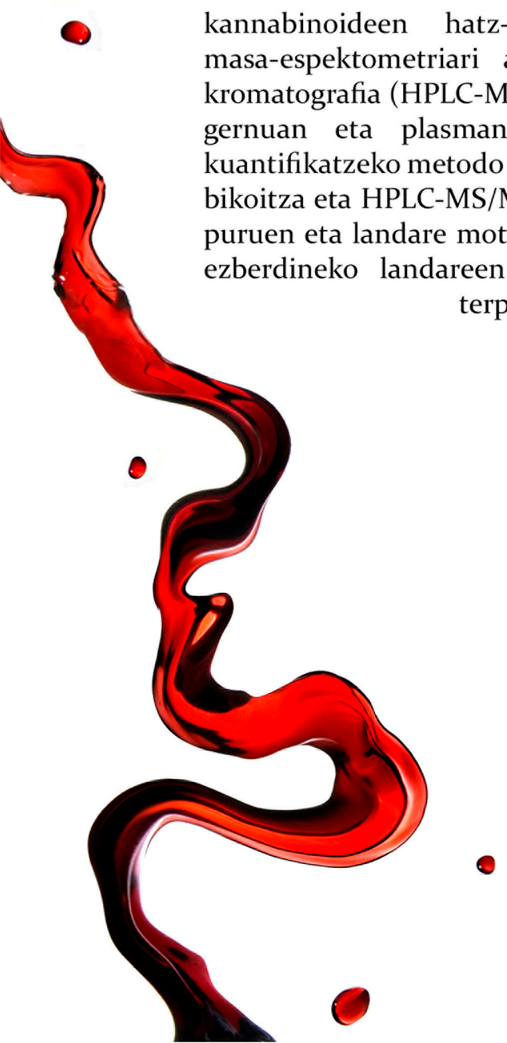
Tesi hau bi zutabe nagusitan banatzen da. Lehenengoan ardo hondakinen balioa handitzeko metodologia bat garatu da. Lehenik, ardo hondakinetatik polifenolak eta gantz azidoak bakoitza bere aldetik erauzteko jariakin gainkritiko bidezko erauzketa (SFE) optimizatu da. Polifenolak ez dira oso egonkorrak ordea eta gorputzean bioeskuragarritasun baxua dute. Arazo hauek konpontzeko polifenolak zurrusta-bibrazio bidezko mikrokapsularatzearen (VNM) bidez enkapsulatu dira.

Bigarren zutabean etorkizun hurbilean kannabisa sendagai bezala erabili ahal izateko 3 urrats eman dira. Alde batetik, landare ezberdinak efektu ezberdinekin lotzea ahalbidetu dezakeen landareen kannabinoideen hatz-marka osatzeko fragmentazio bikoitzeko masa-espektometriari akoplaturiko eraginkortasun handiko likido kromatografia (HPLC-MS/MS) bidezko metodo bat garatu da. Bestalde, gernuan eta plasman kannabinoideak eta euren metabolitoak kuantifikatzeko metodo bat ere garatu da, hidrolisi entzimatiko-alkalino bikoitza eta HPLC-MS/MS analisi teknika erabiliz. Azkenik, konposatu puruen eta landare mota jakinen produkzioa optimizatzeko, kemotipo ezberdineko landareen hazkuntzan zeharreko kannabinoideen eta terpenoen garapena aztertu da.



Konposatu bioaktiboen ustiapenerako metodo analitikoen garapena

UPV EHU

Oier Aizpurua Olaizola

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Development of analytical methods for the exploitation of bioactive compounds

Oier Aizpurua Olaizola

Abendua 2015

eman ta zabal zazu

universidad del país vasco

euskal herriko unibertsitatea

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KIMIKA ANALITIKOA SAILA

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Development of analytical methods for the exploitation of bioactive compounds

Report to complete for the European PhD degree

Oier Aizpurua Olaizola

2015

Mila esker. Mila esker bihotzez 4 urte hauetan zehar horrenbeste buruhauste sortu dizkidazuenei. Lehenik eta behin mila esker zuri krisia, gaur egungo errealitatearen gordintasuna Idokiren adibidearekin erakustearren, eta gauzatutako lana aurrera eramateko aurrekontua mugatzearren. Zugatik izan ez balitz ez nukeen ikasiko ikerketa lan bat gauzatzeak duen kostu erreala zein den, eta hori aurrera begira ezinbestekoa dela iruditzen zait. Mila esker zuri ere burokrazia, farmazietan gorputzarentzat askoz kaltegarriagoak diren botiken kiloak eta kiloak saltzen diren bitartean miligramo bateko "droga arriskutsuen" estandarrak erosteko horrenbeste zailtasun ipintzeagatik (eskumiñak bide batez oporretan nengoela aduanetatik deika eta deika aritu zitzaizkidan langileei). Zuri esker etxean askotan aditutako "las cosas del palacio van despacio" esaldiari askoz zentzu gehiago hartzen diot orain.

Mila esker nola ez fibromialgia duten gaixoengan kannabisa erabiltzeak duen eragina aztertzeko ikerketa talde, erakunde eta enpresa ezberdinen artean garatzen aritu ginen proiektua ezereztea lortu zuten politikari, epaile eta arduradunei ere. Lehen zalantzarik baldin banuen orain argi gelditu zait gai sozialak tabu politiko bihurtzeko duzuen gaitasuna, unibertsoa eta gizakiaren inozokeria bezala mugagabea dela. Nola ez, mila esker zuri ere Manolo, zientziarik garrantzitsuena pazientzia dela erakustearren. Guztiak eskertzea ez da erraza, beraz norbait ahaztu badut mila esker hari ere eta mesedez barka nazala. Eta barkatuko nauzue zuek ere, baina apur bat egolatra izan nahi nuke. Azken batean nire tesia da, eta nork sortu dizkit nire buruak baino buruhauste gehiago? Mila esker beraz Oier! Hanka sartutako aldi bakoitzagatik mila esker, guztia ondo egin ezkero ez nukeen ikasitakoaren erdirik ikasiko eta.

Izan ere tesia ikasketa prozesu bat bezala ikusten dut nik. Niretzat tesia ez da sukaldari aditu baten errezetak eta esanak hitzez hitz jarraituz platera zoragarriak prestatzea; hainbat aldiz zikindu, hatzak moztu, pikantearekin pasa, gatza botatzeaz ahaztu eta janaria erre ostean zure kabuz platera zoragarri eta berritzaileak prestatzeko gaitasuna lortzea baizik. Eta horretarako ere behar da sukaldari adituen laguntza, asko gainera. Lerro hauek erabili nahi nituzke beraz nire bi chef-ak eskertzeko, kasu honetan inongo ironiarik gabe. Eskerrik asko Aresatz eta Pati, gauzak nire kabuz egiteko eta hanka sartzeko askatasuna eman, baina aldi berean behar izan zaituztedan guztietan hor egotearren. Zalantzan dauden guztiei esango diet zuen sukaldetik pasatzeko, ez zaie damutuko eta. Eskerrik asko bihotzez.

Eskerrik asko baita gure culinary center-eko Mitxelin izarra izan den Nestorri. Eskerrik asko benetan emandako laguntzarengatik eta sukalde berrietarako ateak zabaltzeagatik. Muchas gracias también a l@s compañer@s de Foodomics por haberme hecho sentir como en casa y por darme la oportunidad de aprender tanto en tan poco tiempo. And of course, many thanks to AiFame GmbH people, specially to you Umut, for believing in me and for giving me such an opportunity. Eskerrak bihotzez Renovatioko kideei ere eurekin sukaldatzeko aukera eskeini izanagatik. Eta nola ez, eskerrik asko 4 urte hauetan zehar sukaldea banatu dugun gainontzeko chef eta chef ikasle guztiei eta bakoitzari. Eskerrik asko beti laguntzeko prest egoteaz gain gatza eta piperra eguneroko ogi bihurtu eta momenturik okerrenak azukrez goxatzearren.

Amantal eta txano zuriaren atzean pertsona bat ezkutatzen du baina sukaldariak. Eta ez da sukaldean ondo arituko bertatik kanpora gustura ez badago. Zorionez ez da nire kasua. Hargatik, besarkada bero bana eman nahi nieke urte hauetan zehar munduko hiriburuko bizitzan bidelagun izan

ditudan pisu-kide, bertso-kide, pala-kide, zine-kide, parranda-kide eta mota guztietako kide zoragarriei. Besarkada bero bana baita Motxiandarrei, koadrilakoei (bereziki txinatarren lana egiten eta magia egiten jarri ditudanei) eta une onak zein txarrak elkarbanatu ditudan gainerako tarta pusketei ere. Eta nola ez, besarkada bero bat baita sukaldean ezagutu eta azken urte hauetan etxe osoan zehar usain goxo-goxoa zabaltzen ari den nire brownie-ari ere.

Azkenik, eskerrak bihotz bihotzez eta besarkada bero bana, kafearen, ardoaren, azukrearen eta mahai gainean aurkitutako guztiaren soberakinekin nahasteak prestatzen nituenetik sukaldari bihurtzerainoko prozesuan ondoan izan zaituztedan etxekoei eta etxekotzat ditudanei. Esker hitzak ez dira aski zuen kasuan, beraz sukaldari txapela kentzen dut zuen aurrean.

AURKIBIDEA-CONTENTS

Sarrera

1. ATALA

Abiaduraren aroan bizi gara. Gauzak hamar urtean aurreko 50 urteetan baino gehiago aldatzen diren aroan. Mundua abiadura zorabiagarri batean biraka dabilenez eta bertatik ezin jaitsi garenez, geu ere erritmo horretan lasterka bizi behar garen aroan. Geure itzalak aurreratuko gaituen beldur garen eta infiniturantz zuzentzen den etengabeko estres egoera batean bizi garen aroan. Egunari orduak falta zaizkiolako McDonalds-ean korrika eta presaka hanburgesa bat jaten dugun bakoitzean psikologo adituren batek gaixotasun berriren bat diagnostikatzen duen aro eroan.

Dena den, pixkanaka ohartzen hasi gara aurrerapenak ez duela zertan izan penak aurreratzea, gelditu, arnas hartu eta oinarrirantz urrats bat ematea ere aurrera egiteko modua izan daitekeela batzuetan. Ohartzen hasi gara bizitza osasuntsu eta zoriontsu bat izateko giltza ez digula lurretik 1400 argi urtera aurkitutako Kepler-452b planetak emango, betidanik gure aurrean izan dugunak baizik: ama naturak. XXI. mende ero honetan ere berak eskain diezazkigukeela osasun onean egoteko oinarrizko elikagaiak eta baita gaixotzen garenean sendatzeko eta minak baretzeko konposatu miragarriak ere.

“Jaten duguna gara”. Hala esan zuen Hipokrates-ek duela ia bi milurteko eta erdi. Esan bakarrik ez gainera, bere printzipioei jarraiki 107 urte luzez bizi izan zen Antzinako Greziako medikua. Gaur egun inork ez du zalantzan jartzen Hipokrates-ek arrazoi ez zuenik; aitzitik, esaldiari jarraipena eman zaio “jaten duguna gara eta izango gara” bihurtzeraino. Izan ere, jaioko den haurraren osasuna amaren haurdunaldiko elikaduraren menpe egoteaz gain [1], eboluzioa dietarekin hertsiki lotua dagoela baieztatua baitago [2]. Nahiz eta honen jakitun izan, mendebaldar herrialdeetan bizi-erritmoa azkartzeak aldaketak ekarri ditu elikadura-ohituretan eta ondorioz kontsumitzen diren elikagaietan. Hala, industrialki prozesatutako elikagaiak eta janari azkarra eta janari zaborra deiturikoak eguneroko ogi bilakatu dira gure artean; baita hauek duten nutrizio-balioaren galera konpentsatzeko gehigarrien beharra ere ordea.

Haatik, elikagai funtzionalen (EF) kontzeptua ez da atzo gaueko kontua. 80ko hamarkadan hasi ziren erabiltzen Japonian eta "erabilera dietetiko jakinetarako elikagaiak" (food for specified health uses, FOSHU) izendapenaren barruan biltzen dira. EF hauen helburua bizi-esperantza handitzearen ondorioz sortutako gastu medikuak murriztea zen hasiera batean [3]. Hamarkada bat beranduago Estatu Batuetara iritsi ziren, bereizgarri batekin ordea, EF gisa izendatu ahal izateko elikagaiak era batean edo bestean “eraldatua” izan behar zuen [4]. Baldintza hau ez da beharrezkoa Europar Batasunean, non elikagai batek EF izateko gutxienez organismoaren funtzio baterako positiboa izan behar duen [5], funtzio fisiologikoa zehatz definituz eta egiaztatuz [6].

Gaur egun, gauza jakina da elikadura egoki batek hazkuntza, garapen eta osasun egoki bat izaten laguntzen duela, baina baita elikagai batzuk gehiegizko edo gutxiegizko kopuruan hartzea gaixotasun ezberdinen iturri izan daitekeela ere. Kontuak hala, gaur egunean kontsumitzaileek ez dituzte elikagaiak bizirauteko beharrezkoa den zerbait bezala ikusten, mirarizko sendagai bailira legez baizik. Hori dela eta, behar berriei, eta batez ere, negozio-bide berriei erantzuteko, elikagai osasuntsuagoen horniketa oso bat produzitu da azken urteotan: kaltzioarekin, gantz azido asegabeekin, bitaminekin eta abarrekin aberastutako esneak, jogurtak, margarinak eta zerealak; mineralekin, bitaminekin, polifenolekin eta abarrekin aberastutako freskagarriak; azido folikoarekin eta soja proteinarekin aberastutako barra energetikoak, etab. Are gehiago, gutxienez funtzio fisiologikoren batean eragin onuragarria duten

janarietatik eratorritako konposatu bioaktibo asko pilula edo tableta moduan ere saltzen dira hainbat farmazia eta parafarmaziatan. Konposatu hauek nutrazeutiko izena hartzen dute eta denbora luze batean zehar hartuz gaixotasun ezberdin asko izatea saihestu dezakete.

Esan bezala, EF ezberdin ugari aurki genitzake merkatuan. Multzo bat probiotiko, prebiotiko eta sinbiotikoena da. Probiotikoek mikroorganismo biziak daramatzate, laktobaziloak eta bifidobakteriak kasu. Hauek immunitatea indartu, mikroflora kolonikoa orekatzen lagundu, hesteetan zeharreko mugikortasuna hobetu, proliferazio zelularra bizkortu eta hainbat produktu hartzitu onuragarri garatzen dituzte [7]. Konposatu prebiotiko bat aldiz, probiotiko baten substratu trofikoa da. Gizakiak txegos ezin ditzakeen eta elikagaien parte diren osagaiak dira. Bakterio baten edo gehiagoren hazkuntza edo aktibitatea bizkortzen dutenez hartzailearentzat onuragarriak dira. Hala, hainbat gaixotasunen aurkako eragin positiboa aurkitu zaie, esate baterako idorreria [8], osteoporosia (kaltzioaren bioeskuragarritasuna handitzearen ondorioz) [9], gaixotasun kardiobaskularrak (erresistentzia intsulinikoa hobetzen dutelako) [10], minbizia [9], etab. Prebiotiko ezagunenak inulina erako fruktanoak dira. Azkenik, sinbiotikoak, probiotiko eta prebiotiko baten elkartzetik sortzen diren konposatuak dira, adibidez, bifidobakteriekin hartzitutako zuntzetan aberatsak diren esnekiak. Elkartze honek sinergia-efektuak dakartzala uste da [9].

EFen beste multzo bat zuntzekin aberastutako elikagaiena da. Giza entzimek digeritzen ez dituzten eta bakterio kolonikoek partzialki hartzitzen dituzten landare jatorriko substantziak dira, gehienbat karbohidratoak. Hainbat produktu industrial aberasten dira zuntzekin, esaterako ogiak, edariak, hotz-egosiak, gibel-orea edo hestebeteak. Bi zuntz mota ezberdintzen dira, disolbaezinak batetik eta disolbagarriak bestetik. Zuntz disolbaezinak zelulosa, hemizelulosa eta ligninaz osatuak daude batik bat eta hainbat eragin funtzional egozten zaizkie; horien artean murtxikatzeko behar handiagoa (irenste konpultsiboari eta obesitateari aurre egiteko baliagarria) [11] eta ezaugarri antioxidatzaile eta hipokolesterolemikoak [12]. Zuntz disolbagarriak aldiz, pektinez, gomez, muzilagoz eta hemizelulosa batzuez osatuak daude gehienbat. Ura harrapatzeko eta gel likatsuak osatzeko duten gaitasuna dela eta heste-aringarriak dira. Bestalde, metabolismo lipidikoari dagokionez kolesterol eta janaldien osteko intsulina mailak jaisten dituztela uste da [12, 13].

Gantz azido asegabeek osatzen dute beste multzo bat. Gantz-azido asegabeen barruan bi talde ezberdin aurki daitezke, batetik poliasegabeak eta bestetik monoasegabeak. Gantz-azido poliasegabeek (polyunsaturated fatty acids, PUFA) eragin antiinflamatorioak eta antiarritmogenikoak [14] izateaz gain arrisku kardiobaskularrak gutxitzen dituzte [15] eta kolesterol-maila jaisteko gai dira [16]. Hori dela eta, azken urteotan elikagaien industriak gantz-azido asetuak poliasegabeengatik ordezkatu ditu hainbat produktutan; opiletan, esnekietan, hestebeteetan eta arrautzetan kasu (oiloen pentsuan arrain urdinen olioa gehituz). Gantz-azido monoasegabeak (monounsaturated fatty acids, MUFA) ere, gaitz kardiobaskularrak nozitzeko arriskua gutxitzeko eta kolesterol maila jaisteko gai dira [17]. Lipoproteina hauek "kolesterol ona" bezala ezagutzen dira, arterietatik kolesterola hartu eta gibelera eramateko gai baitira, bertan iraizketa gauzatzeko. Fitoesterolak leguminosen hazietan erruz aurkitzen diren esteroide motako molekulak dira eta kolesterolaren xurgatzea eragozten dute [18]. Ezagunena β-sitosterola da, herrialde anglosaxoietan eta Europa iparraldean gehigarri dietetiko bezala arras erabilia.

EF-en beste multzo bat bitaminen eta mineralen multzoa da. Bitaminak ezinbestekoak dira hainbat erreakzio metaboliko aurrera eramateko; gainera, gehienak giza gorputzak ezin sintetiza ditzakeenez, gure eguneroko dietaren parte izan behar dira nahitaez. Egungo dietan ez dira behar adina hartzen ordea, hori dela eta geroz eta beharrezkoagoak bihurtzen ari dira bitaminen gehigarriak. Euren artean aurkitzen dira erretinola (A bitamina; hortz osasuntsuen, hezur-ehun eta ehun bigunen, mukimintzen eta larruazalaren eraketan eta mantenuan laguntzen duena), erriboflabina (B2 bitamina, arnasketa zelularra eta gantz-azidoen sintesia burutzeko ezinbestekoa), azido folikoa (B9 bitamina, zelula berrien ekoizpenean eta mantenuan beharrezkoa), etab. [19].

Mineralak, bitaminen gisa, nahitaezkoak dira gorputzaren funtzionamendu egoki baterako. Elementu inorganikoak dira mineralak; haatik, organismoan entzimekin, hormonekin, proteinekin eta aminoazidoekin elkartuta azaldu ohi dira batik bat. Euren eskasiak eragin kaltegarriak ditu funtzio fisiologikoetan, burdinarenak anemia sor dezake esaterako, kromoarenak arteriosklerosia eta bihotzeko gaitzak eta kaltzioarenak osteoporosia [19].

Azkenik, euren gaitasun antioxidatzailea dela eta ezagunak diren polifenolen multzoa aurkitzen da [20]. Gaitasun antioxidatzaile honi esker konposatu hauek erradikal askeak neutralizatzen dituzte elektroi bat emanez eta euren burua arrisku gutxiagoko erradikal bihurtuz. Ondorioz, albo-erreakzioak gelditzen dituzte [21]. Halaber, Fe(II) gisako trantsizio metalen kelatzaile izanik, Fenton erreakzioa oztopatzearen ondorioz guztiz erreaktiboak diren hidroxilo erradikalek eragindako oxidazioak ere saihesten dituzte [22]. Bestalde, antioxidatzaile-kide legez ere jardun dezakete, bitaminen birsorkuntza bultzatuz [23]. Horregatik guztiagatik, hainbat funtzio fisiologiko garrantzitsu egozten zaizkie polifenolei; hala nola aktibitate antimikrobiala [24], antibirala [25], antimutagenikoa [26] eta antikartzinogenoa [27]. Hortaz gain, nootropiko bezala zerrendatuak daude polifenolak, garuneko hainbat funtzio hobetzen baitituzte; ezagutza, oroimena, adimena, motibazioa eta arreta kasu [28].

Dena den, ohartu behar gara nutrazeutikoek ez dituztela botikak ordezkatzen. Nutrazeutikoak gaixotasunak saihesteko erabili daitezke; hots, prebentzioan oinarritzen dira. Zoritxarrez, batzuetan ezinezkoa da gaixotasunak ekiditea eta kasu horretan nahitaezkoak dira botikak, gaixotasuna sendatu edo tratatzeko balio baitute.

Nutrazeutikoen kasuan, konposatu aktiboen erabilgarritasunaz gain, garrantzia handia ematen zaio produktu hauen iturriari. Hala, erosleak askoz nahiago du konposatu hauek landare, janari-hondakin edo algetatik ateratakoak izatea kimikoki sintetizatuak izatea baino. Botiken kasuan ere antzeko joera bat ari da nabarmentzen azken urteotan. Gauza jakina da antzina landare medizinalak medikuntzaren zutabe nagusia zirela, baina izatez oraindik ere garrantzia izugarria izaten jarraitzen dute. Horren adibide da egun oraindik Estatu Batuetako 150 botika aginduenen artean erdiek baino gehiagok landaretatik eratorritako konposaturen bat dutela, edo munduko biztanleriaren % 80ak batik bat landareetan eta hauen erauzkinetan oinarritzen duela bere osasunaren zaintza [29]. Hortaz gain, badira baita landare batzuk arrazoi ezberdinak tarteko egun medikuntzaren munduan erabilera hain hedatua ez dutenak baina etorkizun hurbil batean ziurrenik garrantzia handia izango dutenak; euren artean ezagunena eta garrantzitsuena *Cannabis sativa L.*

Cannabis sativa L. munduan gehien kontsumitzen den legez kontrako droga da, baina baita landare medizinal oso garrantzitsu bat ere. Kannabisaren erabilera medizinala 5000 urte baino gehiago luzatzen da atzera, Huang Ti enperadoreak Nei Ching medikuntza liburuan kannabisa osagai gisa zuten errezetak sartu zituen garaira hain zuzen ere [30]. Garai haietan kannabisa malariaren, idorreriaren edo hilekoaren arazoen aurka erabiltzen zen. Beranduago, bere erabilera munduan zehar barreiatu zen eta jakina da duela mende askotatik Asian, Ekialde Ertainean, Afrikan, Hego Amerikan zein Europan erabili izan dela. Antzinako Egiptoko momietan ere kannabisa sendagai moduan erabiltzen zenaren pistak aurkitu dira [31]. Are gehiago, badakigu erlijio askok harreman berezia zutela landare honekin, bere ezaugarri energetikoak medio Shiva jainkoaren janari gustukoena zen esaterako. Hala, XIX. mendeko Europan medikuen aginduz erabiltzen zen kannabisa eta hilekoaren arazoei, asmari, eztulari, loezinari, migrainei zein eztarriko infekzioei aurre egiteko, erditze-prozesuan laguntzeko eta opioa hartzeari uzteko farmazietan salgai jarri zituzten landarea bera eta landaretik eratorritako azalerako kremak edo tintura alkoholikoak [32] (1. irudia).

1. irudia: XIX. mendean Europako farmazietan aurki zitezkeen kannabisaren eratorri ezberdinak.

Nahiz eta milaka urtetan erabilia izan, azken hamarkadetako presio soziopolitikoa dela eta kannabisaren erabilera medizinala jaisten joan da. Era berean, kannabisaren inguruko ikerkuntza oztopatua izan da eta ondorioz gaixotasun bakoitzarentzako ikerketa klinikoen eta epidemiologikoen gabezia handia da. Honen ondorioz, eta landarearen konplexutasuna tarteko, kannabisaren ezaugarri farmakologikoak ez dira agian beste landare medizinal batzuenak bezain ondo ezagutzen. Bestalde, pertsona ugarik merkatu beltzera jo behar izan dute euren burua medikatzeko eta hori dela eta inongo kalitate kontrolik pasa gabeko landareak kontsumitu dituzte; kasu askotan albo-efektuak dakartzaten dosi handiegiak hartuz eta beste batzuetan eraginik ez duten dosiak hartuz gainera.

Horren jakitun, eta duela bizpahiru hamarkada egindako ikerketa zientifiko urrien emaitza esperantzatsuak tarteko, azken urteotan mundu osoko hainbat talde helburu terapeutikoetarako

kannabisaren legalizazioa eskatzen ari dira. Babes honi esker geroz eta ugariagoak diren ikerketa zientifikoek kannabisa hainbat gaixotasun tratatzeko egokia eta kasu batzuetan gaur egun errezetatzen diren botikak baino eraginkorragoa eta albo-efektu gutxiago dituena dela egiaztatu dute. Honek, legalizazioaren ikuspuntuari indar gehiago eman dio eta hala ikerkuntzek eta gizartearen alde baten bultzada sozialak elkar hartuz atzera-martxarik ez duen bide bat hasi dute urteotan. Izan ere, kannabisa botika segurua da, albo-efektu gutxi sortzen dituena, gaindosiaren erruz hiltzeko arriskurik ez duena eta gaur egungo botika gehienak baino menpekotasun gutxiago sortzen duena [33-34]. Gainera helburu terapeutikoetarako behar den dosia txikia izaten da eta botika biriketatik hartu behar den kasuetarako lurrungailuak daude erretzeak sortzen dituen konposatu toxikoak ekiditeko [35].

Kontuak hala, ez da harritzekoa azken urteotan kannabisak gizartean izan duen oihartzuna. Izan ere, aski jakinak dira jadanik kannabisak dituen hainbat ezaugarri eta erabilera; esate baterako anorexia, minbizien fase aurreratu edo hies kasuetan lagungarri den gosea pizteko baliagarria dela [36-38]; minbiziak sendatzeko erabiltzen den kimioterapia errazago jasateko efektu antiemetikoa duela [39-41]; minbiziek, hiesak eta fibromialgia edo erreuma-artritisaren gisako gaixotasunek eragindako min neuropatiko kronikoari aurre egin diezaiokeela [42-44] eta esklerosi anizkoitza tratatzeko erabili daitekeela [45-47] besteak beste.

Hala, Kannabinoideak Medikuntzan Nazioarteko Elkarteak (International Association for Cannabinoid in Medicine, IACM), horrela egiaztatzen duten hainbat eta hainbat ikerketa zientifikotan oinarrituz, kannabisa 8 talde patologiko ezberdinetan lehendabiziko terapia aukera dela baieztatzen du; hots, gaur egun medikuek agintzen duten sendagaia bezain eraginkorra ala eraginkorragoa dela. Talde hauek eta dituzten aplikazioak 1. taulan azaltzen dira.

1. taula
IACM-k kannabisa lehendabiziko terapia aukera dela baieztatzen duen talde patologikoen, eta hauei dagozkien sintomen eta aplikazioen zerrenda.

Taldea	Sintomak/Gaixotasunak	Aplikazioak
1	Goragale eta okadak	Minbiziak
2	Goserik eza	Anorexia, hiesa...
3	Muskuluen paralisia	Esklerosi anizkoitza...
4	Gaixotasun neurologikoak	Parkinsona...
5	Min kronikoak	Artrosia, traumatismoak, fibromialgia...
6	Begiko trastornoak	Glaukoma
7	Gaixotasun autoimmuneak	Ultzeradun kolitisa, Crohn, artritis erreumatoidea...
8	Bestelakoak	Insomnioa, antsietatea, depresioa...

Bestalde, oraindik guztiz frogatuak egon ez arren, geroz eta ikerketa zientifiko gehiagok babesten dute kannabisa beste gaixotasun askotarako irtenbide garrantzitsua izan daitekeela; hala nola hainbat minbizia sendatzeko [48-51], epilepsia tratatzeko [52-53] eta Alzheimerrari [54-55], Hungtintonen gaixotasunari [56-57], diabetes kasuei [58-59] zein Touretten sindromeari [60-61] aurre egiteko.

1 Polifenolak eta gantz azidoak gaixotasunen prebentzio gisa

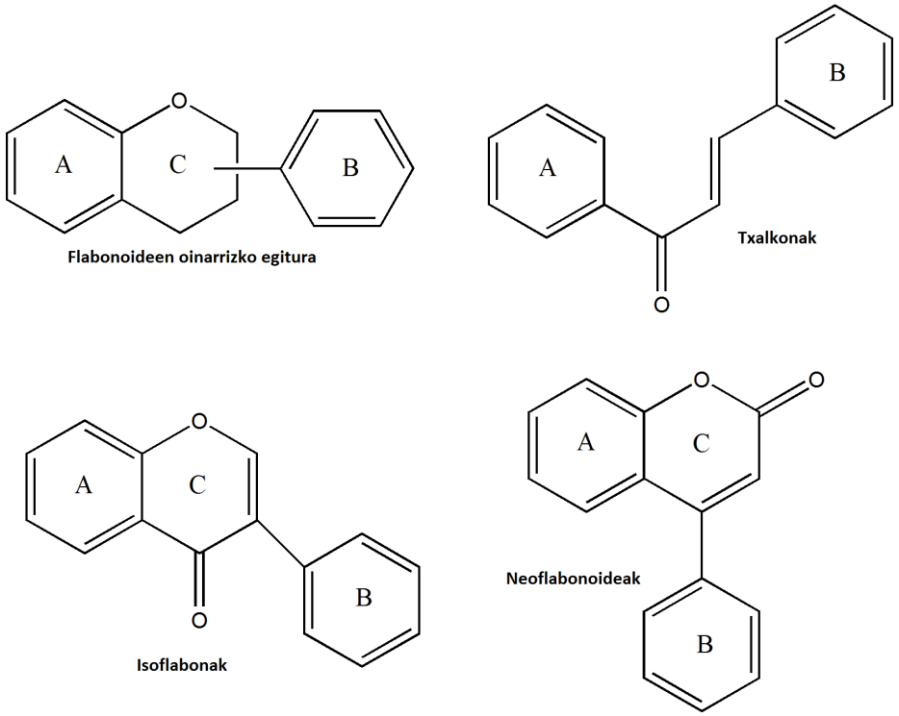
1.1 Polifenolak

Landareen erreinuan zabalduenetarikoa dagoen taldea da polifenolena. 2000. urterako 8000 egitura baino gehiago ezagutzen ziren, euren artean 4000 flabonoide inguru identifikatuak izan zirelarik [62]; kopuru hauek etengabe hazten darraie gainera. Egitura kimikoaren arabera modu honetan sailkatzen dira polifenolak [63]:

- Flabonoideak
    - Isoflabonak, neoflabonoideak eta txalkonak
    - Flabonak, flabonolak, flabanonak eta flabanonolak
    - Flabanolak eta proantozianidinak
    - Antozianidinak
- Ez-flabonoideak
    - Azido fenolikoak
    - Amida polifenolikoak
    - Bestelako polifenolak

Flabonoideen oinarrizko egitura C_6-C_3-C_6 da, non bi C_6 unitateak (A eta B eraztuna) izaera fenolikokoak diren. Hidroxilazio eta kromano eraztun (C eraztuna) ezberdinak medio, aurrez aipatutako azpitaldeak bereizten dira. Salbuespena txalkonen kasua da, nahiz eta C eraztun heteroziklikorik ez izan flabonoideen familiakotzat hartzen baitira.

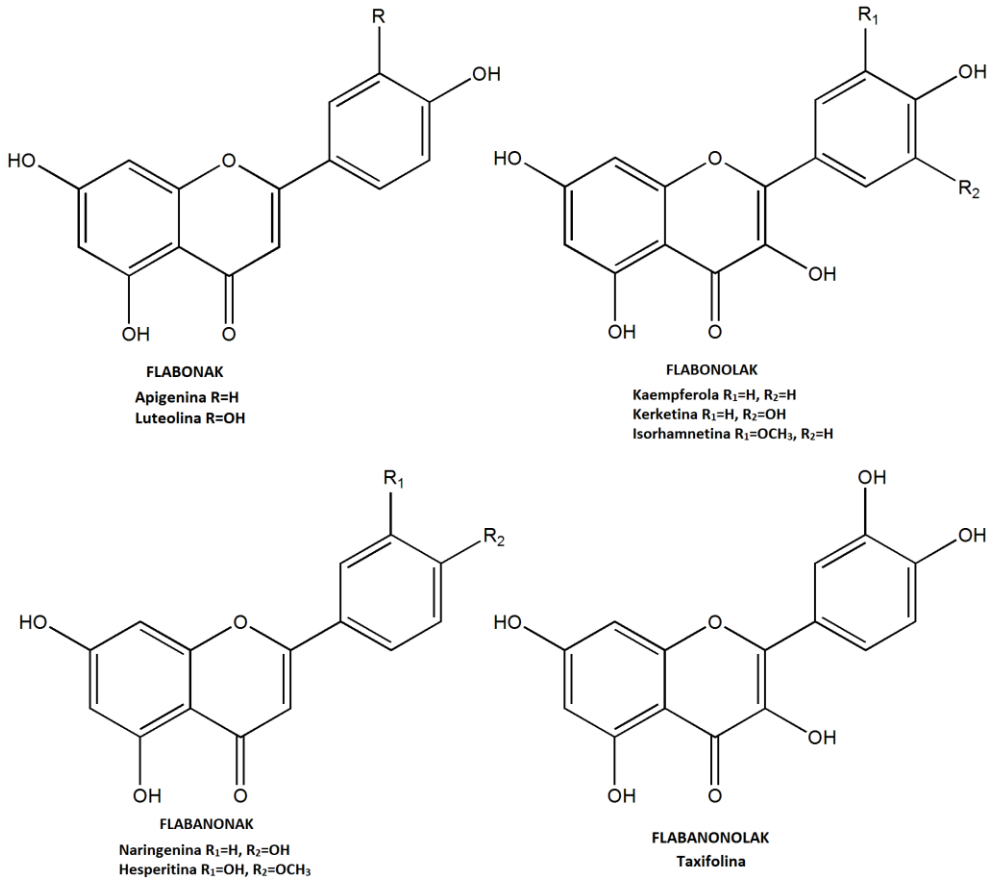
Flabonoideen lehen azpitaldea isoflabona, neoflabonoide eta txalkonena da (ikus 2. irudia). Flabonoideak lekadunen familiako landareetan aurkitzen dira batik bat. Hargatik, soja bezalako elikagai bat kultura askotan dietaren oinarri dela kontuan hartuz, isoflabonek gizakiaren osasunean eragin garrantzitsua dutela esan daiteke. Neoflobonoideek aldiz, nahiz eta eguneroko elikaduraren parte ez izan, garrantzia handia izan dute aspaldidanik, sendabelar askotan agertzen diren konposatuak baitira. Hori dela eta 2001. urterako 130 neoflabonoide ezberdin identifikatu ziren, euren artean ezagunena dalbergina [64] delarik. Txalkonak berriz, sagarra bezalako fruituetan eta garagardoan (lupuluetan) aurkitzen dira [65, 66].



2. irudia: Flabonoideen oinarrizko egitura orokorra eta hainbat azpitalderen egitura

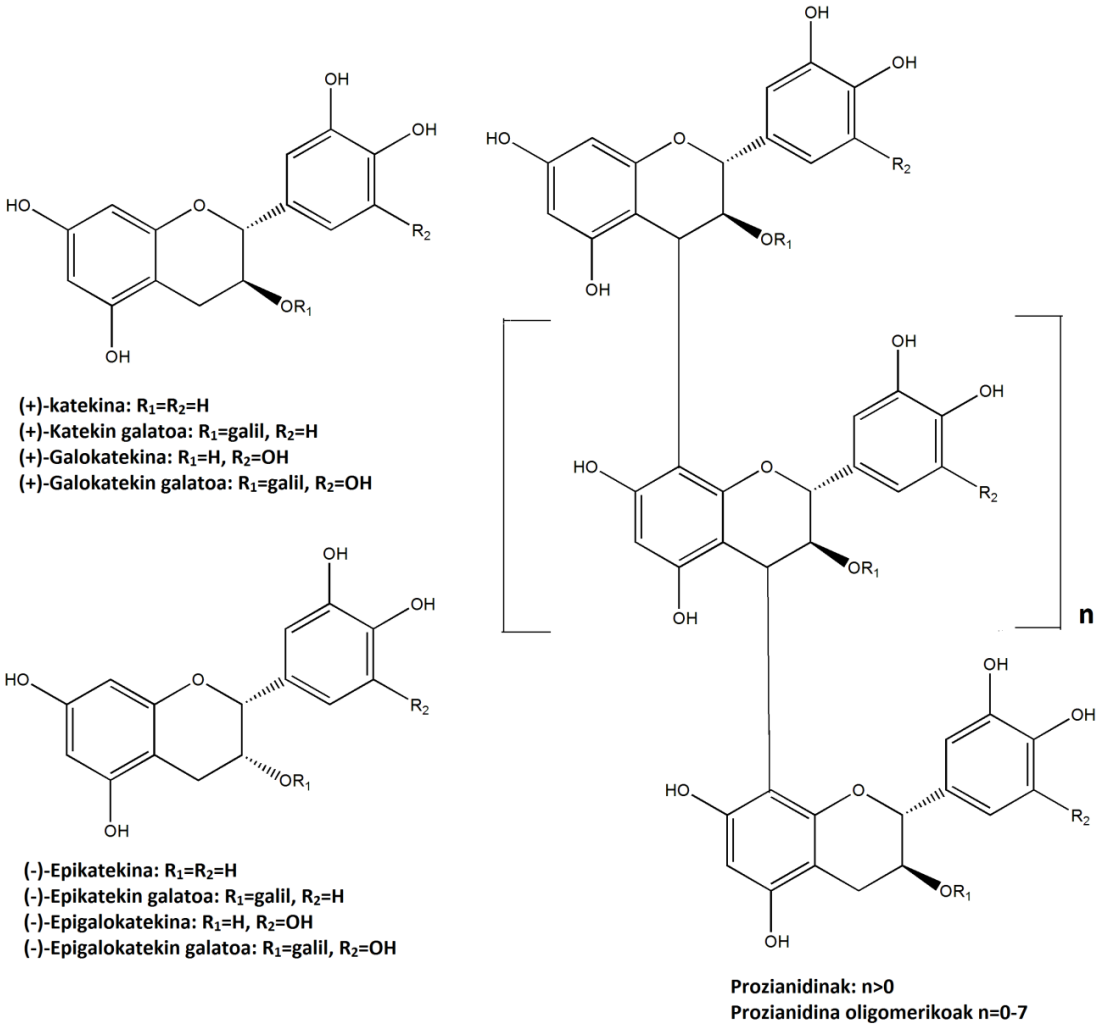
Hurrengo azpimultzoa flabona, flabonola, flabanona eta flabanonolena da, landareen erreinuan ohikoena eta nonahi aurki daitekeena. Flabonoide mota bakoitzaren adibiderik esanguratsuenak 3. irudian ikus daitezke.

Polifenol flabanoideen beste azpitalde bat flabanolek eta proantozianidinek osatzen dutena da. Flabanolei edo flaban-3-olei katekinak deitu ohi zaie eskuarki. Gainontzeko flabonoideek ez bezala, C_2 eta C_3 karbonoen artean lotura bikoitzik ez dutenez eta C_3 karbonoan hidroxilatuta daudenez, bi zentro kiral dituzte (C_2 eta C_3) eta ondorioz lau diastereoisomero ezberdin. Katekina *trans* konfigurazioa duen isomeroa da eta epikatekina *cis* konfigurazioa duena. Bi hauetako bakoitzak beste bi estereoisomero ditu (+)-katekina, (−)-katekina, (+)-epikatekina eta (−)-epikatekina, azken biak landare jakietan gehien agertzen direnak direlarik (ikus 4. irudia). Flabanolak hainbat fruituetan aurkitzen dira, hala nola mahatsen azaletan, sagarretan eta ahabietan. Bestalde, katekina, epikatekina eta euren deribatuak (galokatekinak esaterako), te hostoetan eta kakao hazietan ugarienak diren flabonoideak dira [67, 68]. Horrez gain, katekinak eta epikatekinak oligomeroak eta polimeroak osa ditzakete, proantozianidina edo tanino kondentsatu deritzenak.

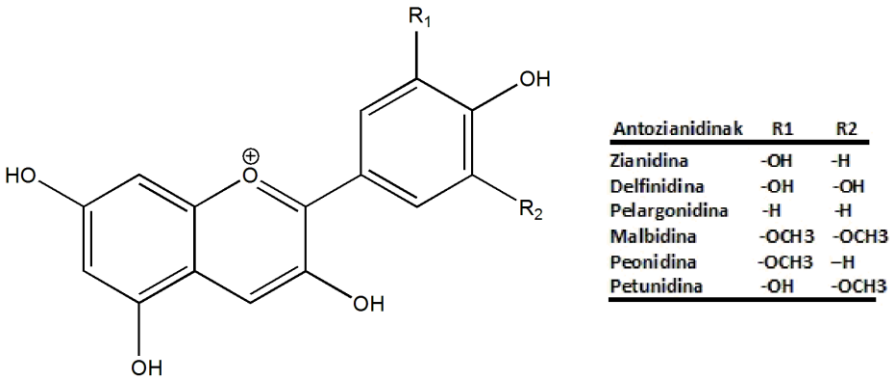


3. irudia: Flabonen, flabonolen, flabanonen eta flabanonolen egituren hainbat adibide

Polifenol flabonoideen azken azpimultzoa antozianidinena da (ikus 5. irudia). Landareen petalo gorri, urdin eta moreen pigmentuetan, fruitu eta barazkietan eta arroz beltzaren gisako hazi berezi batzuetan aurkitzen dira antozianidinak. Forma glikosidikoan aurkitzen dira batik bat eta kasu horietan antozianinak deritze. Antozianinen kolorea pH-aren araberakoa da, gorria baldintza azidoetan eta urdina basikoetan. Dena dela, hidroxilazio-mailak, eraztun aromatikoen metilazioak eta glikosilazio-mailak ere eragiten dute kolorean.



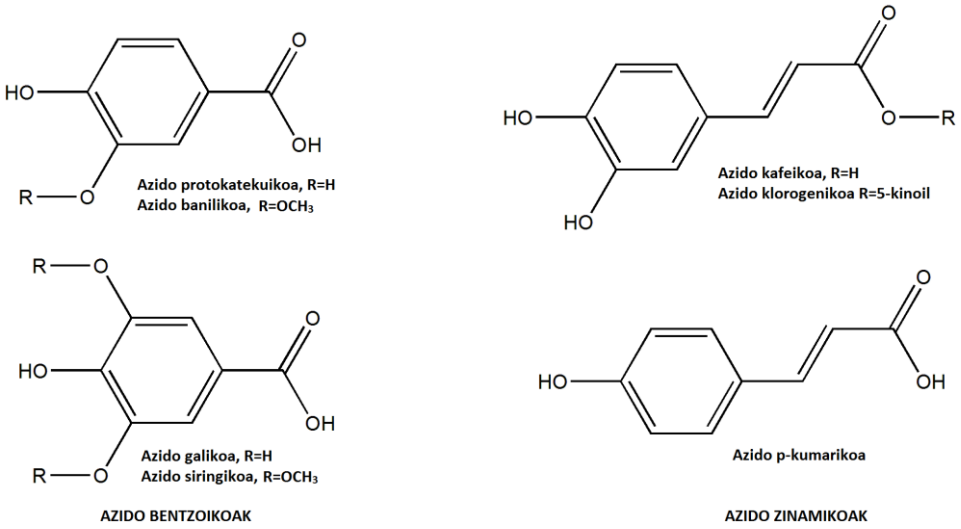
4. irudia: Flabanolen eta prozianidinen egituren hainbat adibide



Antozianidinak	R1	R2
Zianidina	-OH	-H
Delfinidina	-OH	-OH
Pelargonidina	-H	-H
Malbidina	-OCH3	-OCH3
Peonidina	-OCH3	–H
Petunidina	-OH	-OCH3

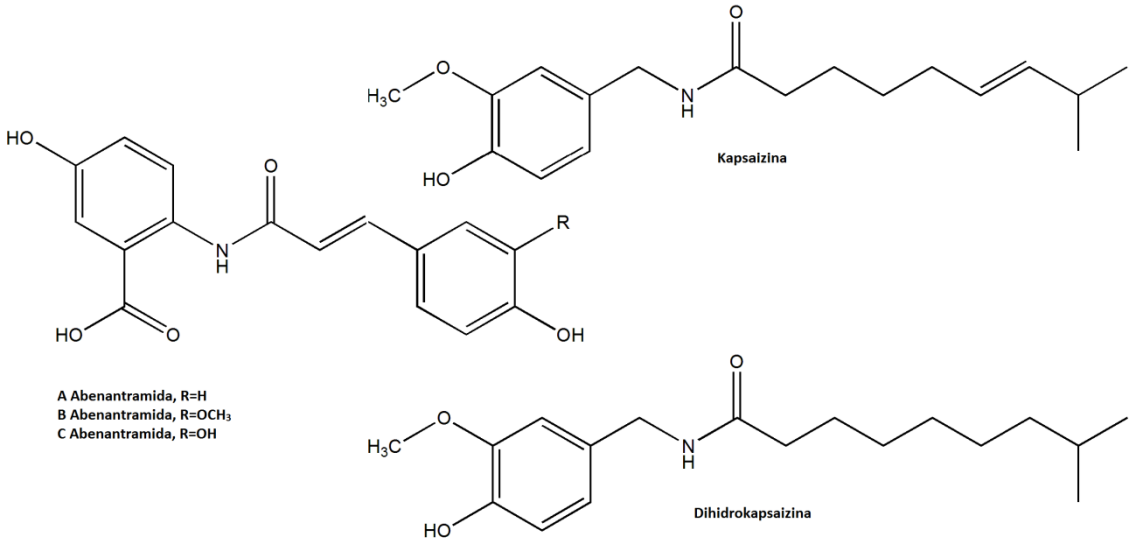
5. irudia: Antozianidinen egituren hainbat adibide

Bestalde, polifenol ez flabonoideak ditugu, euren artean azido fenolikoak, amida polifenolikoak eta bestelako polifenolak ditugularik. Azido fenolikoak (ikus 6. irudia) fruitu eta barazkien aleetan eta hazietan kausitzen dira eta bi talde orokorretan banatzen dira, azido bentzoikoa eta bere deribatuak, batetik, eta, azido zinamikoa eta bere deribatuak, bestetik.



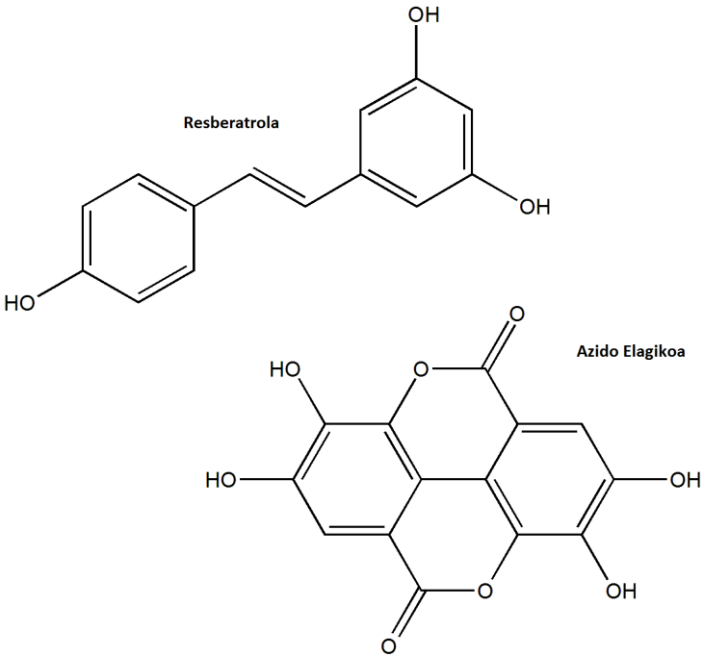
6. irudia: Azido fenolikoen egituren hainbat adibide

Amida polifenolikoek (ikus 7. irudia) nitrogenodun talde funtzionalak dituzte eta hainbat jakitan ohikoak dira, piperminetan (kapsaizinak) eta oloetan (abenantramidak) kasu [69, 70]. Piperminetako polifenol hauek, antioxidatzaileak izateaz gain, zapore minaren erantzule ere badira.



7. irudia: Amida polifenolikoen egituren hainbat adibide

Flabonoideez, azido fenolikoez eta amida polifenolikoez gain, giza-osasunarentzat garrantzitsuak diren hainbat polifenol ez flabonoide aurkitzen dira jakietan (8. irudia). Hala nola resberatrola mahatsetan; azido elagikoa eta deribatuak marrubi eta mugurdietan; lignanoak liho, sesamo eta beste hainbat hazietan; etab.



8. irudia: Bestelako polifenolen egituren bi adibide

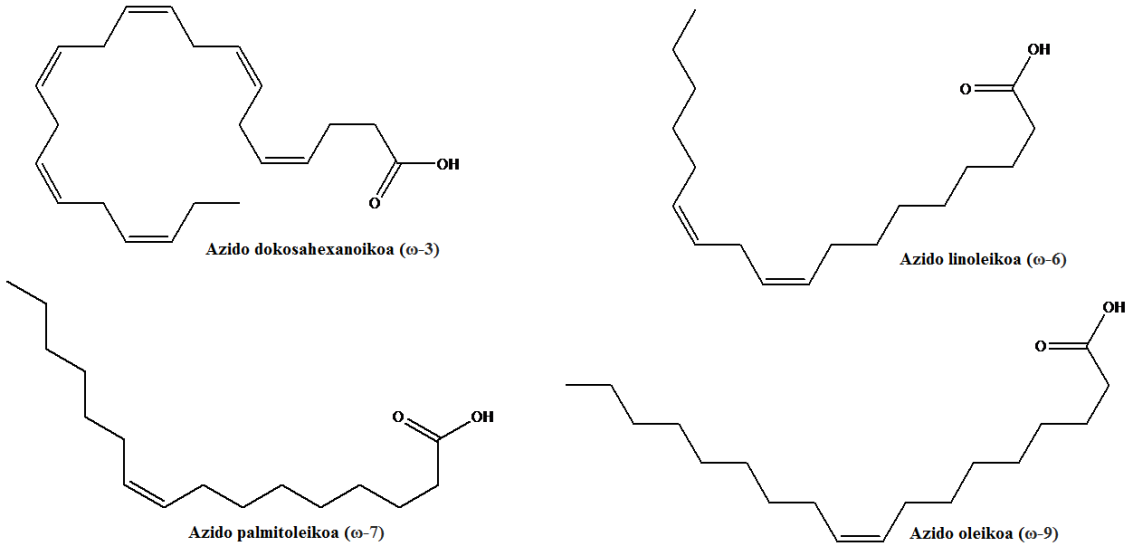
Konposatu funtzional hauek gizakiaren elikaduran duten garrantzia dela bide, elikagaien industria osagai funtzional berriak topatu beharrean aurkitu da, ahal bada jatorri naturalekoak gainera. Iturri garrantzitsu bat hondakin agroindustrialena da hain zuzen ere. Zehazki polifenolei dagokienez, ardoaren produkzioko mahats hondakinak arras erabilgarriak izan daitezke, euren polifenol kontzentrazioa handia baita [71]. Polifenol kopuru totalari eta polifenol bakoitzaren kopuruari dagokionez, ardo mota ezberdinen artean zein ardo bateko hazien eta azalen artean ezberdintasun nabariak aurkitu dira gainera [71, 72].

61 milioi tonako laborantzarekin, mahatsa laranjaren ostean munduan zehar zabalduen dagoen uzta da [73]. Bestalde, egun oraindik mahats hondakinen ehuneko txiki bat bakarrik aprobetxatzen da. Patsaren ustiatze-aukera bat bioerregaiak edo bioenergia lortzeko erabiltzea izan daiteke [74]. Beste hautu bat patsa ongarri edo animalientzako pentsu gisa erabiltzea da, polifenolen kontzentrazio altuaren ondorioz hondakinen aurretratamendu bat beharrezkoa da ordea. Kontuak hala, beste edozein erabilera eman aitzin polifenolen erauzketa burutzea hautabide zinez egokia izan daiteke [75].

1.2 Gantz azido asegabeak

Gantz azidoak kate hidrokarbonatu luzeko molekulak dira eta molekularen mutur batean buru polar bat daukate, azido karboxilikoa. Kate hidrokarbonatuek karbono kopuru ezberdina izan dezakete eta karbono hauen arteko loturak bakunak edo bikoitzak izan daitezke. Lotura bikoitzik ez dutenak gantz azido ase moduan izendatzen dira eta lotura bikoitz bat edo gehiago dituztenak gantz azido asegabe moduan. Arestian aipatu gisa, bi talde ezberdintzen dira gantz azido asegabeen barruan, poliasegabeak edo PUFA-k, lotura bikoitz bat baino gehiago dutenak, eta monoasegabeak edo MUFA-k, lotura bikoitz bakarra dutenak.

Bestalde, gure artean aski ezagunak bihurtu dira jadanik omega 3 edo omega 6 gisako izendapenak. Izen hauek gantz azidoetako lotura bikoitzek kate hidrokarbonatutan duten kokapena adierazten dute. Talde karboxiloarekiko gertuen dagoen karbonoa alfa karbonoa bezala ezagutzen da, hurrengoa beta karbonoa bezala, eta azkenengoa aldiz omega karbonoa bezala, greziar alfabetoko azken letra delako hain zuzen ere. Omega 3-k azken karbonotik zenbatzen hasita lotura bikoitza 3. karbonoan dagoela esan nahi du, omega 6-k aldiz 6. karbonoan (9. irudia).



9. irudia: Gantz azido asegabeen egituren adibide ezberdinak

Nahiz eta orain arte gantz azido asegabeez orokorrean hitz egin dugun, azpimultzo bakoitzak eta are gehiago konposatu bakoitzak eragin jakin bat du gure osasunean. Omega 3 eta omega 6 taldeko konposatuek esaterako interes handia piztu dute azken urteotan. Izan ere, gorputzak endokannabinoideak eta kannabinoide hartzaileak sintetiza ditzan beharrezkoak direla ikusi baita [76-77]. Hala, dietan hartutako omega 3 eta omega 6 kopuruaren arabera, eta bien arteko ratioaren arabera, gorputzeko endokannabinode sistema ezberdina izango da. Aurrerago azalduko den bezala, endokannabinoide sistemak gorputzeko hainbat funtzio fisiologikotan hartzen du parte, ondorioz, dietan hartutako omega (omega 3/omega 6) ratioak gaixotasun askoren prebentzioan eta kasu batzuetan sendatzean eragin zuzena duela esan genezake [78].

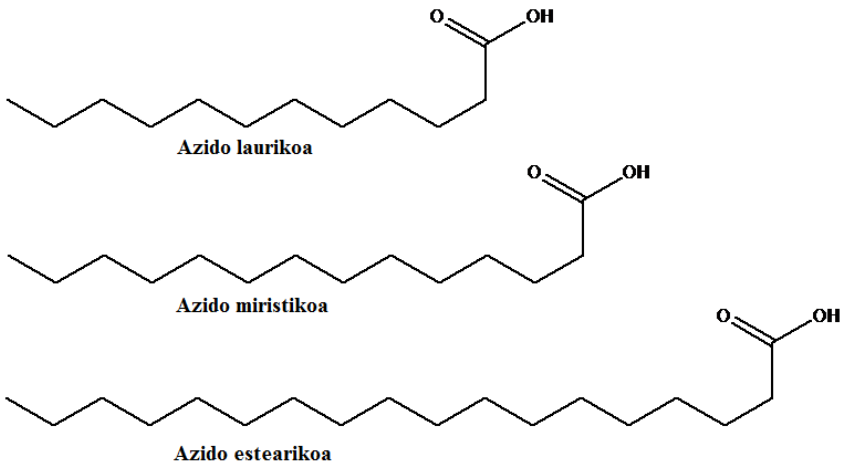
Azido dokosahexaenoikoa (docosahexaenoic acid, DHA) eta bere gisako ω-3 PUFA-k itsas-algetan eta fitoplanktonean aurkitzen dira batik bat, eta ondorioz baita arrain olioan ere [79]. Konposatu honek esaterako bere aktibitate antiarritmogenikoa dela eta bihotzekoa izateko arriskuak gutxitu ditzake [14]. Bestalde, edoskitze-esnean agertzen den konposatu bat da eta haurraren garunaren eta ikusmen funtzioen garapen egokian eragin handia duela uste da. Kontzentrazio hau ez da oso altua ordea eta ondorioz, komisio ugari edoskitzaroan emakumeek dietan gehigarri bezala har dezaten aholkatzen hasi dira, bereziki ama beganoen eta begetarianoen kasuan, euren dietaren DHA iturria ezereza eta oso urria baita hurrenez hurren [79-80].

Azido linoleikoa da omega 6 taldeko PUFA ezagunena eta fruitu eta landare-olioetan aurkitzen da gehienbat. Gizakiarentzat funtsezkoa den gantz azido bat da azido linoleikoa, gorputzak berak ezin du sintetizatu janarien beste konposatuetatik eta ondorioz dietan zuzenean hartu beharra dago. Azido linoleikoan urria den dieta batek, hazteko eta zauriak sendatzeko gaitasunak okertzen dituela ikusi da arratoietan [81-82]. Hortaz gain dentsitate handiko lipoproteinen kopurua igoarazteko gai da [16]. Lipoproteina hauek "kolesterol ona" bezala ezagutzen dira, arterietatik kolesterola hartu eta gibelera eramateko gai baitira, bertan iraizketa gauzatzeko. Bestalde, azalaren gainean emandakoan hanturaren eta aknearen aurkako efektuak aurkezteaz gain, azala ez lehortzea ere lortzen duenez, gero eta erabilera hedatuagoa hartzen ari da estetika produktuetan [83-84].

Omega 7 taldeko gantz azido ezagunenetako bat azido palmitoleikoa da. Konposatu hau gizakiaren gantz askoko ehunetako glizeridoetan oso ohikoa da, batez ere gibelean. Diabetesari aurre egiteko balio du, intsulinarekiko sentikortasuna handitzen baitu [85].

MUFA-k, azido linoleikoaren gisa, fruitu eta landare olioetan aurkitzen dira. MUFA ezagunena omega 9 taldeko azido oleikoa da eta bere eragin nagusia kolesterol maila jaistea da [17].

Alderantzizko efektua dute hain justu ere gantz azido aseek, kopuru handian hartzen badira kolesterol maila igo eta ondorioz gaixotasun kardiobaskularrak izateko aukerak handitzen baitituzte [86]. Gantz azido aseen artean efekturik handiena azido laurikoak eta azido miristikoak dute eta efekturik ahulena aldiz azido estearikoak [86] (10. irudia).



10. irudia: Gantz azido aseen egituren adibide ezberdinak

Arestian aipatu gisa, gantz azido asegabeen iturri garrantzitsuenetarikoak landare eta fruituen haziak izaten dira. Euren artean mahatsen haziak ez dira salbuespena, bertan aurkitzen den gantz azido poliasegabeen kontzentrazioa benetan altua baita, batez ere azido linoleikoarena [87]. Honenbestez, ardo produkzioko hondakinak gantz azido asegabeen iturri garrantzitsua izan daitezke.

1.3 Erauzketa eta analisia

Fruituetatik eta hazietatik polifenolak eta gantz azidoak erauzteko gehien erabili izan diren teknikak solido-likido erauzketa (solid-liquid extraction, SLE) eta likido-likido erauzketa (liquid-liquid extraction, LLE) dira; sistema klasiko hauek disolbatzaile kopuru handiak erabiltzeaz gain erauzketa-denbora luzeak behar dituzte eta horrek, gastu handiaz aparte, konposatuak degradatzeko arriskua dakar. Hori dela eta, beste hainbat teknika modernok sistema klasikoak ordezkatu dituzte, hala nola ultrasoinuen (ultrasounds US) eta ultrasoinu fokatuen (focused ultrasound extraction, FUSE) bidezkoek, mikrouhinen (microwave-assisted extraction MAE) bidezkoak, jariakin gainkritikoen (supercritical fluid extraction, SFE) bidezkoak, e.a. [75, 88].

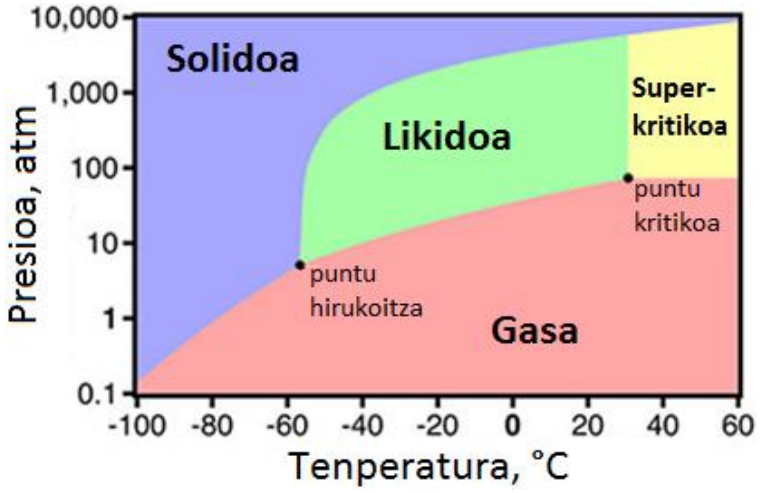
US-a jakien industrian hedatuki erabiltzen den erauzketa teknika bat da, landareetatik eta animalietatik hainbat konposatu bioaktibo denbora laburrean eta errendimendu handiarekin erauzteko baliagarria [89]. Gainera, azken urteotan garatutako FUSE teknika sistema klasikoa baino eraginkorragoa dela ikusi da, errendimenduak are gehiago hobetuz [90]. Ultrasoinu fokatuaren bidezko erauzketak kabitazioz funtzionatzen du. Intentsitate handiko ultrasoinuak likidoan zehar hedatzean presio altuko eta baxuko zikloak sortzen dira. Presio baxuko ziklo hauetan ultrasoinuek burbuila txikiak sortzen dituzte likidoan hutsuneak eratuz. Burbuilek energia gehiago xurgatu ezin duten tamainara iristean bortizki inplosionatzen dute presio altuko ziklo batean zehar, tenperatura, presio eta abiadura izugarriko likido turrustak sortuz. Ondorioz, solidoaren gainazalean erosio mekanikoa eragiten da, partikulak apurtuz eta hala erauzlearekin kontaktuan dagoen gainazala handituz. Metodo honek duen abantaila esanguratsuena erauzketa tenperatura hotzetan eginda ere eraginkorragoa dela da, konposatu hegazkorrenak ez galtzeaz gain burbuilen inplosioa bortitzagoa izatea lortzen baita horrela. Hori dela eta goi sukaldaritzan etorkizun bikaina duen teknika da ultrasoinuena, beste hainbat abantailen artean janaria berotu gabe sukaldatzea ahalbidetzen baitu [91].

Mikrouhinak janariaren berotzeko erabili izan dira eskuarki. Berrikitan MAE lurretik, sedimentuetatik, hazietatik eta janari matrizeetatik konposatu organikoak erauzteko garatua izan da ordea. Ildo horretatik, MAE jakietatik polifenolak eta gantz azidoak erauzteko sistema klasikoak baino askoz ere eraginkorragoa dela frogatu da [92].

Mikrouhinak espektro elektromagnetikoan irrati uhinen eta izpi infragorrien artean aurkitzen diren uhin elektromagnetiko ez-ionizagarriak dira. Mikrouhinek ez dute konbekzioz funtzionatzen ohiko beroketak bezala, eroankortasun ionikoz eta dipoloen errotazioz baizik. Ondorioz, berotze-energia ingurunean galtzen ez denez erauzketa denborak asko murrizten dira teknika klasikoekin alderatuz. Bestalde, teknika selektiboa da mikrouhinena, berotze-maila matrizearen eta disolbatzailearen izaera kimikoen araberakoa baita; hots, geroz eta konstante dielektriko handiagoa orduan eta absortzio eraginkorragoa [93]. Sistema honek duen abantailetako bat erauzketak argiaren absentzian egiten direla da, konposatu fenoliko batzuk argiarekiko oso sentikorrak baitira, biologikoki aktiboa den resberatrolaren trans isomeroa esaterako [94].

SFE teknika jariakin gainkritikoen ezaugarrietan oinarritzen da. Gas bat edo likido bat presiopean eta tenperatura altuetan jartzen denean bere puntu kritikora heldu eta egoera gainkritikora pasa daiteke. Gainera, puntu kritikoko presioa eta tenperatura konposatu bakoitzaren ezaugarri jakinak dira. Egoera gainkritikoan dauden jariakinek gasen eta likidoen hainbat ezaugarri ezberdin hartzen dituzte. Likidoen antzeko dentsitatea dute eta hargatik disolbatzaile onak dira; bestalde, gasen likatasuna eta mugikortasuna aurkezten dutenez eta gainazal tentsioa deuseztatzen denez disolbatzailearen garraiatzaile izaera hobetzen da eta material solidoaren poroetara hobeto heltzean erauzketak azkarrago burutzen dira [95].

Hainbat disolbatzaile erabili daitezke erauzketa gainkritikoaren gauzatzeko; dena den, ohikoena karbono dioxidoa erabiltzea da, inertea, erlatiboki ez kutsakorra, merkea, eskuragarria (hainbat industriatan albo-produktua da), segurua eta, 11. irudian ikus dezakegun bezala, parametro kritiko egokiak dituena baita.



11. irudia: CO_2-aren fase diagrama

CO_2 ez da oso disolbatzaile polarra ordea, eta ondorioz pisu molekular handiko konposatuak eta konposatu polarrak erauzteko ez da hain egokia. Hori dela eta beste disolbatzaile polarrago baten kopuru txikiak gehitzen zaizkio eskuarki; hala, disolbatzaile gainkritikoaren ezaugarriak aldatu gabe, konposatu hauen disolbagarritasuna hobetuz. Helburu honetarako etanola erabiltzen da gehienetan, merkea, ez kutsakorra eta jakien industrian erabilgarria baita [96]. Disolbatzaile honekin jokatu ahal izateak aukera aparta eskaintzen dio SFE-ri, modu horretan polartasun ezberdineko konposatuak elkarrengandik bereizita erauztea lortu baitaiteke, lehenik apolarrak, CO_2 soilik erabiliz, eta ostean, polarrak, albo-disolbatzailea gehituz [97-98].

SFE, teknologia "berdea" izateaz gain beste hainbat abantaila ditu; erauzkin puruagoak lortzea, disolbatzaile-hondakinak murriztea edo deuseztatzea, etapa bakarreko prozesua izatea, disolbatzaile kopuru txikiak erabiltzen direnez bukaeran erauzkina kontzentratu beharrik ez izatea, eta, tenperatura altuak erabiltzen ez direnez degradazio termikoen arriskua gutxitzea esaterako. Bestalde, mikrouhinen

kasuan bezala, erauzketan zeharreko argiaren eta airearen absentziak hainbat degradazio gertatzea ekiditen du [94].

Honegatik guztiagatik, teknika hau jakietatik polifenolak eta gantz azidoak erauzteko arrakastatsuki erabilia izan da, erauzketa sistema klasikoekin baino errendimendu hobeak lortuz [99-100]. Beste onura interesgarri bat, erauzketa sistema gehienekin ez bezala, jariakin gainkritikoaren kasuan eskala analitikotik eskala industrialera igaro gaitezkeela da. Gaur egun prozesu industrial askotan erabiltzen den teknika da SFE: kafearen deskafeinatzea (Kraft General Foods, AEB), kolesterol-maila baxuko arrautza likidoaren ekoizpena (Otsuka Foods Co. Japonia), bakterio-zelulen karetonoideen erauzketa (Nippon Oil Co. Japonia), zizka-mizken aerosol bidezko estaldura (Nabisco, AEB), etab.

Analisiari dagokionez, polifenolen kontzentrazio totala jakiteko metodo kolorimetrikoak erabili ohi dira, eskuarki Folin-Ciocalteu teknika. Konposatu jakinak neurtu behar direnean aldiz eraginkortasun handiko likido kromatografia (high-performance liquid chromatoraphy, HPLC) bidezko teknikak erabili ohi dira, masa espektrometro bati (mass spectrometry, MS) hala diodo-segiden detektore (diode array detector, DAD) bati lotuta normalean. Gantz azidoen kasuan berriz teknikarik ohikoenak gas kromatografiari akoplaturiko masa espektrometria (gas chromatography-mass spectrometry, GC-MS) eta garraren bidezko ionizazioa (gas chromatography-flame ionization detector, GC-FID) izan ohi dira.

Nahiz eta hasiera batean proteinen analisirako garatua izan, Folin-Ciocalteu metodoak aztertu nahi den lagineko fenol edukia determinatzeko balio du, hori dela eta jakien industrian hedatuki erabili izan da [101-103]. Horretarako komertzialki prestatutako erreaktibo bat erabiltzen da, Folin-Ciocalteu (FC) deritzona hain zuzen ere.

FC erreaktiboaren izaera kimikoa ez da ezaguna, baina heteropolifosfotungsteno-molibdatoak dituela uste da. Hori dela eta, molibdenoa konplexuan aisa erreduzitzen dela uste da [101]:

$$Mo(VI)\ (horia) + e^- \rightarrow Mo(V)\ (urdina)$$

FC erreaktiboa ez da konposatu fenolikoentzat espezifikoa, beste konposatu batzuk ere erreduzi dezakete, hala nola C bitaminak edo Cu(II)-ak. Konposatu fenolikoei dagokienez, baldintza basikoetan (pH ~ 10) soilik erreakzionatzen dute FC erreaktiboarekin. Baldintza hauetan protoi fenolikoaren disoziazioak FC erreaktiboa erreduzitzeko gai den fenolato anionikoa sortzen du [104]. Ingurune basiko hau lortzeko sodio bikarbonatoa erabiltzen da.

Polifenol zehatzak neurtzeko HPLC-MS/MS eta HPLC-DAD teknikak erabiltzen dira eskuarki [90, 96]. Bertan, fase mugikorrean doazen polifenolak modu ezberdinean banatzen dira zutabean zehar eta hala denbora bereiztuetan iristen dira detektorera. Lehenengoaren kasuan kuadrupolo hirukoitza erabili ohi da detektore gisa. Lehen detektorean intereseko masak aukeratzen dira, bigarrenean masa horien fragmentazioa ematen da eta hirugarrenean apurketa bakoitzaren fragmentu jakinak aukeratzen dira, hala, sentikortasun eta selektibitate benetan handiak lortuz. DAD detektorearen kasuan aldiz konposatuen absorbantzia neurtzen da ultramore-ikuskor uhin luzera tartean.

Gantz azidoak HPLC bidez ere neurtu daitezkeen arren [105] ohikoena gas kromatografia erabiltzea izan ohi da. Gantz azidoak neurri handi batean triglizerido moduan aurkitzen dira ordea. Beraz, lehenik beharrezkoa da glizerido loturak hautsi eta gantz azidoak aske uztea. Horretarako saponifikazio-erreakzio bat gauzatzen da ingurune alkalino gogor baten bidez. Behin gantz azido askeak dituguneanbeste urrats bat eman beharra dago, deribatizazioa. Gas kromatografian konposatuak neurtu ahal izateko hegazkorrak izan behar dute, gantz azidoak ez dira ordea. Ondorioz, hegazkorrak diren beste konposatu batzuetara deribatizatzen dira, kasu gehienetan esterifikazio erreakzio baten bidez [106].

2 Kannabisa sendagai gisa

2.1 Kannabisa

Cannabis sativa L. Cannabaceae familiako espezie belarkara bat da, urteroko landare dioikoa, lore eme eta arrak landare ezberdinetan hazten direna, baina helburu sendagarrietarako zein ludikoetarako emeak soilik erabiltzen direna. Gaur egungo sailkapen zientifikoa hurrengoa da:

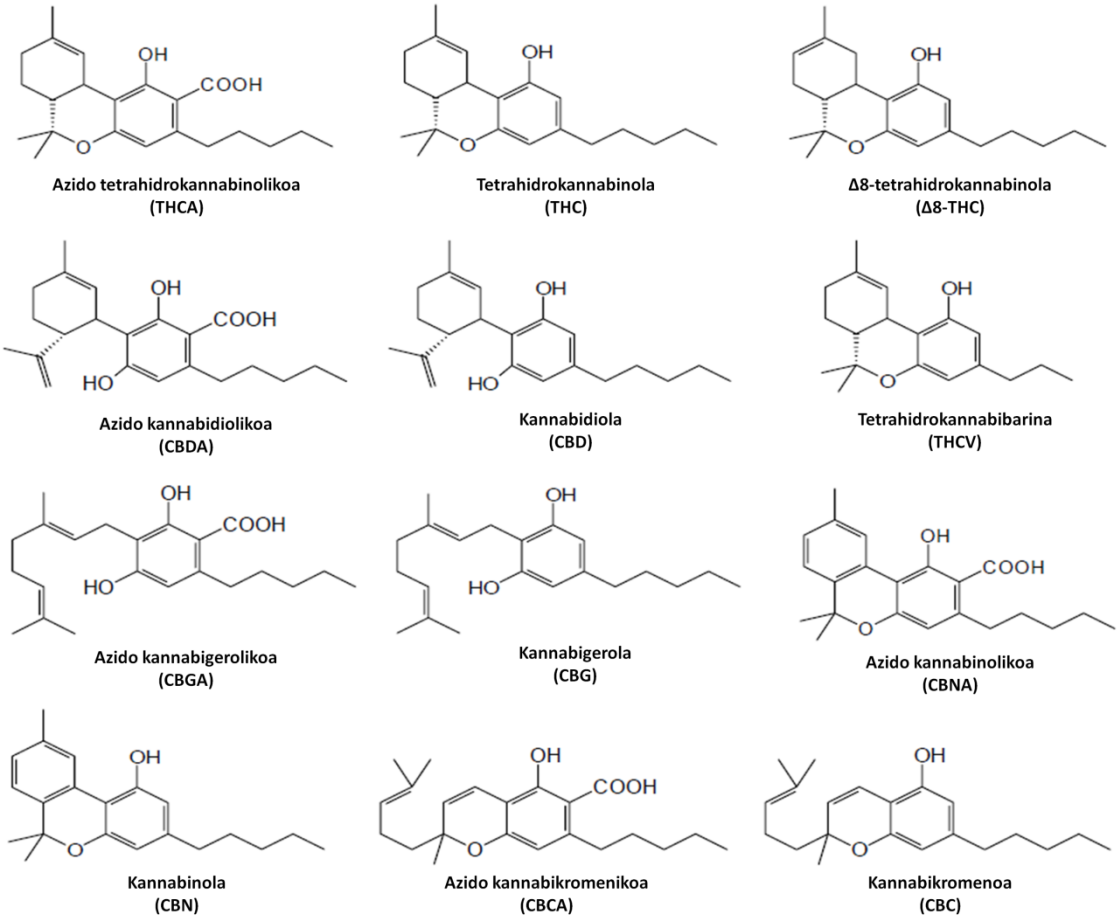
Erreinua: Plantae
Kladoa: Angiosperms
Klasea: Dicotyledon
Subclase: Archichlamyeae
Ordena: Urticales
Familia: Cannabinaceae
Generoa: Cannabis
Especie: sativa L.

Milaka kannabis barietate ezagutzen dira gaur egun, eta kopurua handituz doa egunetik egunera, efektu ezberdinak lortu nahian barietate ezberdinak elkarren artean gurutzatzen baitira berriak sortzeko. Dena den, nahiz eta *rudelaris* izeneko hirugarren azpiespezie bat egon badagoen, ia barietate guzti hauek kannabisaren bi azpiespezie nagusien gurutzaketetatik datoz; hots, *sativa*-tik eta *indica*-tik.

2. taulan ikus daitekeen bezala 500 osagaitik gora identifikatu dira kannabisean [107-108]. Nahiz eta polifenolen eta gantz azidoen gisako konposatuek ere eragin esanguratsuak izan osasunean, kannabisaren konposatu interesgarrienak kannabinoideak eta terpenoak dira. Kannabis landarean agertzen diren kannabinoide eta terpeno ohikoenak 12. eta 13. irudietan irudikatu dira hurrenez hurren.

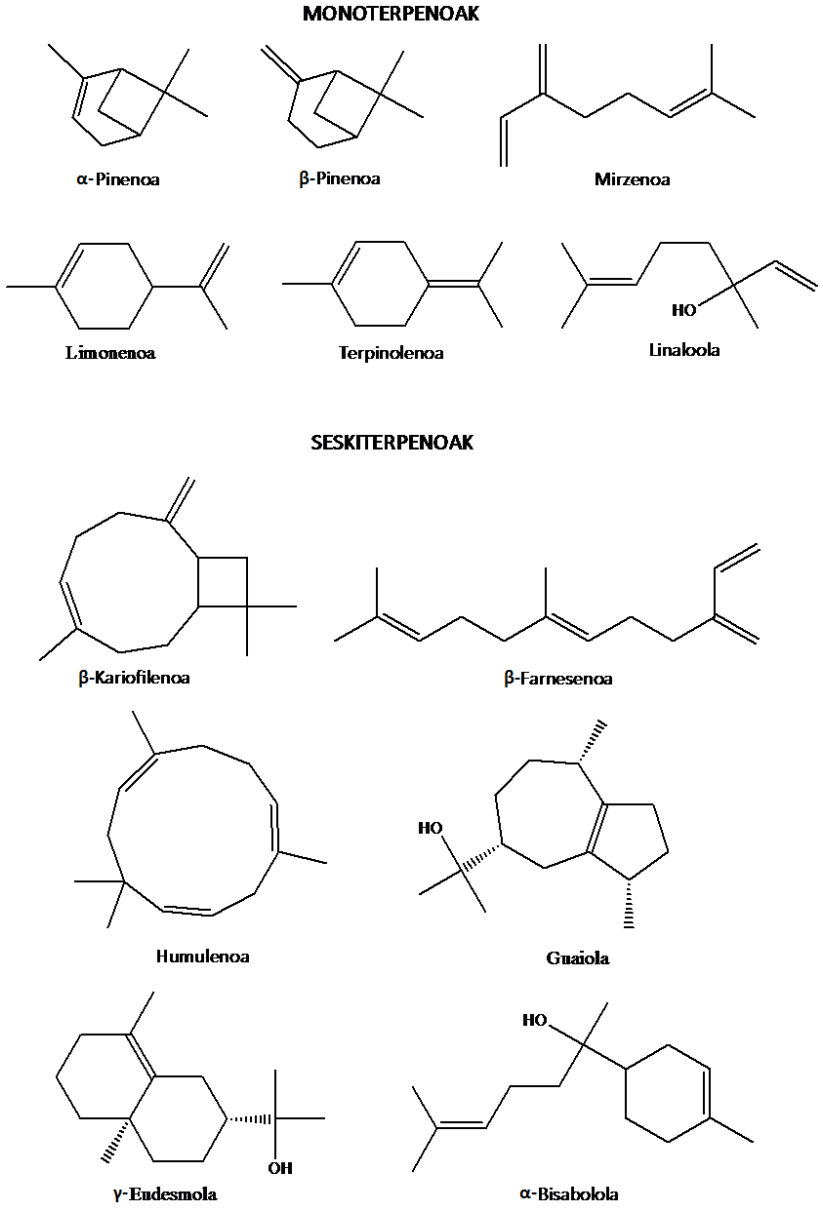
2. taula
Kannabis landarean identifikatutako konposatu talde ezberdinak eta bakoitzeko osagai kopurua.

Osagai taldea	Identifikatutako osagai kopurua
Terpenoak	120
Kannabinoideak	113
Hidrokarburoak	50
Polifenolak	48
Azukreak eta antzekoak	34
Osagai nitrogenodunak	27
Gantz azidoak	22
Azido sinpleak	21
Aminoazidoak	18
Zetona sinpleak	13
Ester sinpleak eta laktonak	13
Aldehido sinpleak	12
Proteinak eta entzimak	11
Esteroideak	11
Elementuak	9
Alkohol sinpleak	7
Pigmentuak	2
Bitaminak	1



12. irudia: *Cannabis sativa L.* landarean aurkitzen diren kannabinoide ohikoenak.

Kannabinoideak forma azidoan biosintetizatzen dira landarean, baina berotzean edo argiaren presentzian dagokien karboxilo taldea galdu eta forma neutrora deskonposatzen dira. Bestalde, degradazioak ere jasan ditzakete eta beste kannabinoide batean bihurtu. Modu honetan, 14. irudian islatzen den bezala, kannabinoideak 3 multzotan sailka daitezke: zuzenean landarean biosintetizatzen direnak, dekarboxilazio bat jasanda sortzen direnak eta degradazio baten ondorioz eratzen direnak. Hala, nahiz eta landare freskoan ia kannabinoide neutrorik ez aurkitu, argiarekin, beroarekin eta denboraren poderioz konposatu azidoak dagokien konposatu neutroetara igarotzen dira [109]. Berdina pasatzen da degradazio produktuekin, luzaroan gordetako kannabis laginetan edo baldintza gogorragoak jasan dituztenetan CBN kontzentrazio altuagoak ikus daitezke, bestalde, tenperatura altuetan THC isomerizatu egiten da eta Δ^8-THC-ra pasa [110].



13. irudia: *Cannabis sativa L.* landarean aurkitzen diren terpeno ohikoenak.

Biosintetizatuak	THCA	CBDA	CBGA	THCVA	CBCA
Dekarboxilatuak	THC	CBD	CBG	THCV	CBC
Degradatuak	*CBNA* → *CBN* ← *Delta-8-THC*				BESTE BATZUK

14. irudia: kannabinoide ohikoenen jatorriaren araberako sailkapena.

Legislazioari dagokionez kannabisa bi multzo nagusitan sailkatzen da: droga motakoak eta zuntz motakoak (kalamua). Bien arteko ezberdintasuna THC konposatuaren kontzentrazioan oinarritzen da. Droga motako kannabisetan THC-ren kontzentrazioa altua izaten da, zuntz motakoetan ordea ez da % 0.2-0.3 baino altuagoa izaten. Zuntz motakoak ehungintzarako edo haziak kontsumitzeko hazi ohi dira eskuarki; dena den, legislazioan arautua ez dagoen CBD-ren balio altu xamarrak izan ohi dituzte [111].

Kimikoki modu ezberdin batean sailkatzen dira. Sailkapena egiteko THC/CBD erlazioa hartzen da kontuan. Hala, erlazio hau >> 1.0 duten landareak I. taldekoak dira. Talde honetan kalean aurkitzen diren droga motako ia landare guztiak aurkitzen dira. II. taldeko landareek THC/CBD erlazioa 0.5-2 artekoa izaten dute. Landare hauek helburu medizinala dute batik bat, CBD THC-ren efektu psikotropikoa modulatzeko gai baita [112]. III. taldea THC/CBD erlazioa << 1.0 dutenek osatzen dute. Talde honetan aurkitzen dira legislazioak zuntz motakoak bezala sailkatzen dituenak, baina baita 2014. urtean sortu eta erabilera medizinaletarako hedatzen ari diren CBD oso altuko eta THC % 1 baino baxuagoa duten droga motako landareak ere [113]. Hain ezagunak ez diren beste bi talde ere badaude, IV. taldean kannabinoide nagusia CBG duten zuntz motako landareak aurkitzen dira eta V. taldean aldiz ia kannabinoiderik ez duten zuntz motako landareak.

Kaleko kulturan jarraitzen den sailkapena beste bat da, sailkapen morfologikoa hain zuzen ere. Hala, gaur egun aurkitzen diren barietateak *sativa* modukoak, batez ere *sativa* modukoak, hibridoak, batez ere *indica* modukoak ala guztiz *indica* modukoak izan daitezke bi hauen arteko nahastearen ehunekoaren arabera. Orain arte, azpiespezie bakoitzari justu elkarren aurkakoa den efektu jakin bat egotzi izan zaie gainera; sativa-k THC/CBD ratio altuekin lotu izan direnez estimulatzaileak eta bizigarriak direla uste izan da eta indica-k aldiz ratio baxuagoekin lotu izan direnez erlaxagarriak. Hala, teorikoki azpiespezie bakoitzari aplikazio jakinak aurkitzeko modua egongo litzateke. Dena den, nahiz eta morfologikoki ezberdinak izan, oraindik eztabaida dago ia kimikoki eta, ondorioz, efektu aldetik ezberdintasun agerikorik duten ala ez [114]. Izan ere, hamaika gurutzaketen ondorioz bi azpiespezieak banatzen dituen lerroa gero eta ilunagoa da, eta nahiz eta bakoitzaren ezaugarri diren terpeno jakin batzuk aurkitu diren, kimikoki elkarrengandik bereiztea zaila da [114-115].

Kannabisa sendagai gisa erabiltzen dutenek, medikuen komunitatearen babes falta dela eta kaleko kulturako aholkuak jarraitu ohi dituzte euren burua tratatzeko. Kannabisa oso landare konplexua da ordea. Ondorioz, landare jakin batek izan ditzakeen efektuak jakin ahal izateko beharrezkoa da ahalik eta konposatu aktibo gehienen identifikazio zehatza egitea eta horien farmakologia hobeto ulertzea. Izan ere, lehen aipatu dugunaren arabera aktibitate ezberdina aurkezten duten ehunetik gora kannabinoide aurkitzen dira kannabisean. Bestalde, CBD THC-ren efektu psikotropikoa modulatzeko gai den gisa, gainerako kannabinoideek ere elkarren artean efektu sinergikoak aurkezten dituzte. Azkenik, terpenoek ere aktibitate ezberdinak dituzte eta hauek ere kannabinoideekin sinergian joka dezakete [116].

THC da kannabiseko eragile psikoaktibo nagusia eta hanturaren eta minaren aurkako efektuak izateaz gain, gosea pizteko gaitasuna eta kimioterapiaren eraginak hobeto jasateko efektu antiemetikoa ditu eta dosi txikietan garuna defizit kognitiboetatik babesteko gai da [117-118]. Dena den, THC-ren eguneroko kontsumo handiak defizit kognitiboak eragin ditzake oraindik garuna garatze-prozesuan duten

gazteetan [119-120]. Bestalde, helduetan eguneroko kontsumoak eragin ditzakeen efektu neurokognitiboak funts gehiegirik gabeak dira oraindik [119]. Aipatu bezala, THC ez da efektuak dituen kannabinoide bakarra ordea. CBD-k esaterako THC-ren efektu psikoaktiboa modulatzeaz gain psikoasiaren, epilepsiaren, antsietatearen, minbiziaren, aknearen eta diabetesaren aurkako efektuak aurkezten ditu eta beldurra kontrolatzen eta tabakoa erretzeari uzten laguntzen du [112, 121-125]. Bere aldetik, CBG-k bakterioen eta proliferazioaren aurkako efektuak ditu eta baita hezurrak indartzekoa ere; CBN-k aldiz efektu lasaigarriak ditu, eta, THC-rekin eta CBD-rekin sinergian jokatzen du insomnioaren aurka; THCV-k bestalde gizentasunaren eta epilepsiaren aurkako efektuak ditu [112, 122].

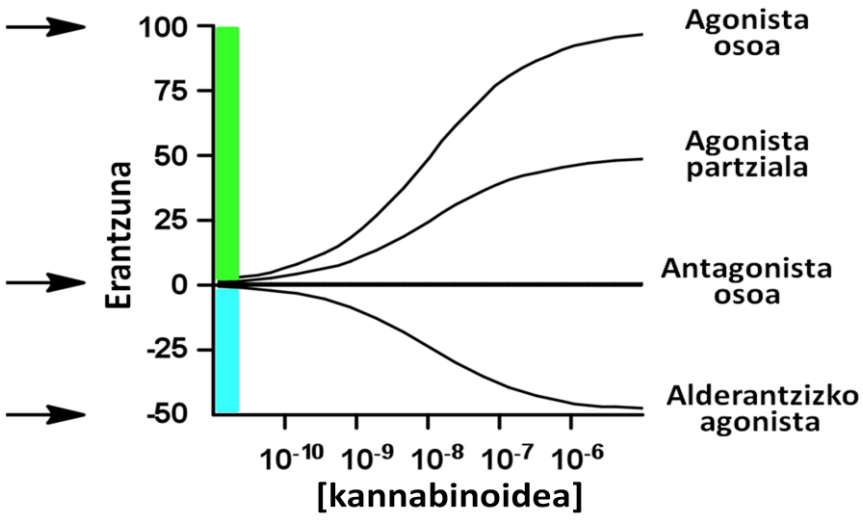
Lehenago aipatu bezala, kannabinoideez gain terpenoek ere, landareari lurrina emateaz gain, aktibitate ezberdinak aurkezten dituzte eta kannabinoideekin sinergian joka dezakete gainera. Hala, nahiz eta bi landarek kannabinoideen kontzentrazio bera eduki, terpeno ezberdinak baldin badituzte efektu ezberdina sortuko dute. α-Pinenoak esaterako, bronkioak dilatatzeko eta hanturaren aurka egiteko gaitasuna izateaz gain, THC-k iraupen laburreko oroimenean eragiten dituen gabeziak ahultzen ditu eta CBG-ren eragin antiseptikoa eta CBD-k akneari aurre egiteko duen gaitasuna handitzen ditu [126-130]. Limonenoak antsietatearen aurkako efektuak ditu eta CBD-rekin sinergian joka dezake lan horretan, baita kantzerraren aurka egiterako orduan ere [131-132]. Bestalde, aknearen aurka egiteko gaitasun oso handia du eta kasu honetan ere CBD-rekin eta pinenoarekin sinergian joka dezake [133]. Linaloolak ere antsietatearen aurkako efektuak ditu baina baita efektu lasaigarria eta konpultsioei aurre egitekoa ere [134]. Ondorioz esan daiteke CBD-rekin batera epilepsia tratatzeko egokia izan daitekeela. β-kariofilenoa aldiz hanturaren aurkako agente biziki indartsua da eta CBD-rekin batera sinergian joka dezake tabakoa edo kokaina gisako adikzioei aurre egiteko [135-137].

Agerikoa da beraz kannabisaren konplexutasuna, eta ezinezkoa dela eragindako efektuak konposatu bati edo biri soilik egoztea. Ildo horretatik, nahiz eta oraindik sinergia guzti hauek hobeto ulertzeko hainbat ikerketa egin beharko diren, badirudi etorkizunean kannabisetik etorriko diren botikak, aurrez estandarizatutako eta konposatu jakinen kontzentrazio jakin bat duten landareak, edo, terpeno zein kannabinoide konposatu puru bat baino gehiagoren nahasteak, izango direla.

Landare miragarri honek hainbeste gaixotasunerako balio izateak badu bere azalpen zientifikoa: sistema endokannabinoidea. Kannabinoideen sistema endogenoa kannabinoide hartzailez (CB), ligando endogenoz (endokannabinoideoak) eta euren biosintesiaz eta degradazioaz arduratzen diren entzimez osatzen da eta gorputzeko hainbat prozesu fisiologikoetan hartzen du parte; hala nola gosean, min sentsazioan, umorean eta oroimenean. Endokannabinoideak CB hartzaileekiko guztiz selektiboak dira; hots, giltza batek sarraila batekin duen funtzio bera betetzen dute hartzaileekin. Kontua da, kannabisean aurkitzen diren kanabinoide ezberdinek giltza hauen kopiak balira legez jokatzen dutela, hartzaileekin lotzeko gaitasuna dutela alegia, modu honetan prozesu fisiologiko guzti horietan eraginez [138-140].

Endokannabinoideek zein kannabinoideek, hartzaileei lotzean izaera agonista, antagonista zein alderantzizko-agonista izan dezakete (15. irudia). Izaera agonista dutenek maila batean edo bestean baina endokannabinoide agonisten funtzio bera betetzen dute; hots, hartzaile jakin bati lotuz erantzun biologiko bat sortzea (minarekiko sentikortasuna gutxitzea esaterako). Antagonistek hartzaileak blokeatzen dituzte,

hala beste edozein konposatu lotzea eta, ondorioz, erantzun bat sortzea saihestuz. Alderantzizko-agonista bat hartzaile jakin bati lotzean aldiz, endokannabinoide agonista batek sortzen duen erantzunaren alderantzizko erantzuna sorrarazten du. Kasu honetan, beharrezkoa da hartzaileak aktibitate konstitutiboa izatea; hau da, hartzailea inongo estekatzailerekin lotu gabe maila jakin bateko erantzun biologikoa sortzeko gauza izatea.



15.irudia. Kannabinoide eta endokannabinoide ezberdinek hartzaileekiko izan ditzaketen izaera ezberdinak islatzen dituen irudikapena.

Kannabinoide hartzaileei dagokionez bi dira gehien aztertu izan direnak, CB1 eta CB2. Biak G proteinei loturiko hartzaileen superfamiliakoak dira [141] eta glikosilazio guneak dituen zelula, kanpoko N-terminal domeinu batez, G proteina konplexuari loturiko C-terminal domeinu batez eta zelula kanpoko eta barneko euskarriei txandaka loturiko 7 transmintz segmentu hidrofoboz osatuak daude [142-143]. CB1 eta CB2 hartzaileen proteinen sekuentziak % 44ean berdinak dira [144], eta transmintzeko inguruak soilik hartzen badira kontuan aldiz, bien arteko aminoazidoen berdintasuna % 68ra iristen da [145].

CB1 hartzailea nerbio sistema zentralean aurkitzen da batik bat, CB2 aldiz sistema immunologikoarekin erlazionatutako ehun eta zelula periferikoetan [146]. Dena den, hainbat ehun periferikoetan ere aurkitu da CB1, kardiobaskularretan, ugalketa sistemetan eta gastrointestinaletan kasu [147-150]. Bestalde, nerbio sistema zentralean CB2 hartzailea aurkitzen dela ere ikusi da [151].

Hartzailea endokannabinoide batek, kannabiseko konposatuek edo kannabinoide sintetikoren batek aktibatzen duenean zelula barneko hainbat seinale transdukzio aktibatzen dira. Hala, osagai aurre-sinaptikoan hartzailea seinale transdukzioko G proteinari lotzen zaio MAP kinasa kitzikatuz eta adenilil ziklasa entzima inhibituz. Modu honetan bigarren mezularia den AMP ziklikoaren produkzioaren ahultzea gertatzen da, bide batez bere menpe dagoen proteina kinasa (PKA) inhibituz baita. Kannabinoideen absentzian PKA-k potasio kanalen proteina fosforilatzen du kanporanzko potasio korrontea gutxituz. Kannabinoideen presentzian fosforilazio hau indargabetu egiten da ordea, hala kanporanzko potasio korrontea handituz. Ondorioz, kannabinoideek neurotransmisoreak jaregiterako orduan garrantzia berezia

dutela esan daiteke. Esate baterako, aztertua dago nola gai diren glutamatoa, azetilkolina eta noradrenalina neurotransmisoreak inhibitzeko [152-154]. Bestalde, hartzaileak Gi/o proteinen bidez ioi-kanalei loturik daude baita, positiboki A motak barrurantz zuzendutako potasio kanalei, eta negatiboki N motako eta P/Q motako kaltzio kanalei eta D motako potasio kanalei [148, 155].

CB1 eta CB2 kannabinoide hartzaile klasikoez gain, badira hartzaile gehiago ere, heuren artean ezagunena vanilloid hartzailea (transient receptor potential cation channel subfamily V member 1, TRPV1) deritzana. Gorputzeko tenperatura detektatzeaz eta erregulatzeaz arduratzen da hartzaile hau, baita minaren eta bero jasangaitzaren estimuluaz ere. Batez ere nerbio sistema periferikoan agertzen den eta konposatu exogenoek zein endogenoek (anandamida endokannabinoideak esaterako) aktibatu dezaketen katioi kanal ez-selektiboa da TRPV1.

Halaber, nahiz eta gehien aztertu diren kannabinoide hartzaileak CB1, CB2 eta TRPV1 izan, hauen bidez azaldu ezineko farmakologiak hartzaile ezberdin gehiago egon daitezkeela pentsatzera eraman zituen ikertzaileak. Hala, azken urteetan funtzio ezberdinen erantzule diren hainbat hartzaile berri aurkitu dira: GPR18, GPR55, GPR119, etab. [155-157].

Arestian aipatu bezala, kannabisean aktibitate ezberdina duten ehunetik gora kannabinoide aktibo ageri dira eta gainera sinergian joka dezakete. Sinergia hori hein handi batean hartzaile ezberdinekin zer modutan jokatzen dutenaren araberakoa da. Hala, THC esaterako agonista partziala da CB1-arekiko eta CB2-arekiko, lehenengoarekiko sentikortasun handiagoa du ordea [158-160]. Bestalde, CB1-arekin eta CB2-arekin zerikusirik ez duten eta ondorioz beste kannabinoide hartzaileekin erlazionatuta dauden, hainbat efektu ere aurkitu zaizkio Δ9-THC-ri, esaterako noradrenalina bidezko melatonina hormonaren biosintesian eta epidermiseko keratinozitoen proliferazioan eragina [161-162]. CBD aldiz, maila mikromolar batean CB1-arekiko eta CB2-arekiko afinitate txikiko agonista izan arren [163-164], maila nanomolar batean CB1-en alderantzizko-agonista dela ikusi da eta CB2-ren kasuan ere berdin joka dezakeela uste da [165]. Hortik dator THC-ren efektu psikotropikoak modulatzeko gaitasuna hain zuzen ere. Elkarrekintza horiez gain, CBD-k ez-ohiko hartzaileen bidez ere hainbat efektu sortzen dituela uste da, tartean zelula mikroglialen migrazioa modulatzea [166]. THCV ere kontzentrazio altuetan CB1 hartzailearen agonista izan arren kontzentrazio baxuetan CB1-aren zein CB2-aren antagonista dela ikusi da [167]. CBN hartzaile biekiko agonista da, Δ9-THC-rekin alderatu ezkero CB1-ekiko modu ahulean eta CB2-rekiko modu sentikorragoan [168]. CBG aldiz CB1 hartzailearekiko antagonista da modu ahulean eta CB2-rekin ere lotzen da baina bere jokaera ez da oso argia [169]. Bestalde, β-kariofileno terpenoak ere kannabinoidea balitz gisan jokatzen du eta CB2 hartzaileak aktibatzeko gai da [170].

Kannabisean konposatu aktibo asko eta euren arteko sinergiak izateaz gain, hauek hartzeko moduak ere eragin handia izan dezake sortuko dituzten efektuetan. Hala, saiakera klinikoetan gehien aztertu izan diren administrazio moduak birika-bidezkoak eta ahotik hartzekoak izan dira. Kannabisa zigarro modura errez edo lurrunketa bidez hartzen denean birikek osagai aktiboak zuzenean xurgatzen dituzte, kearen edo lurrunaren gainerako konposatuekin batera. Modu honetan, gai aktiboak odolean sartu eta ehunetan zehar banatzea izugarri azkar ematen den prozesua da, benabarnetiko administrazioaren

antzeko zinetika agertzen duelarik. Hain da azkarra garraioa non THC eta honek gorputzean osatzen dituen metabolito nagusien odoleko kontzentrazio altuena zigarroa amaitu aurretik erdiesten den [171].

Lehenago aipatu dugun bezala, landareetan kannabinoideak forma azidoan ageri dira batik bat, aktibitate gehien aurkezten dutenak konposatu neutroak dira ordea. Hori dela eta, administrazio modu bakoitzean erabiltzen den berotze mailaren arabera dekarboxilazio gehiago edo gutxiago emango da, eta honek hartzailearengan eragingo dituen efektuetan eragin zuzena izango du.

Bestalde, administrazio-modu bakoitzean landarea ezberdin gordetzen eta erabiltzen denez, kannabinoide batzuk degradatu egin daitezke prozesuan zehar, baita terpeno batzuk lurrundu ere. Ondorioz, nahiz eta landare-material berdina erabili, administrazio mota bakoitzaren arabera, efektu eta iraupen ezberdinak ikusi ahal izango dira [108].

Kannabinoideen hartzea ahotik zertzen denean lortzen diren THC-ren maila plasmatikoak birika-bidezko administrazio moduekin eskuratzen direnak baino baxuagoak izaten dira. Izatez, urin gastrikoaren azidotasunarekiko duen sentikortasunaren ondorioz eta gibeleko eta hesteetako metabolismoa eta euren arteko zirkulazioa dela bide bere bioeskuragarritasuna txikiagoa baita [172]. Honenbestez, kannabisaren produkturen bat ahotik hartzen denean arnasketa bidez lortutakoaren pareko eragin fisiologikoa jadesteko behar den konposatu aktiboen kopurua handiagoa da.

Kannabisa ahotik hartzeko erabiltzen den modu ezagun bat infusio moduan hartzea da. Erabilera hau arras zabaldua dago landarea sendagai gisa kontsumitzen dutenen artean. Herbehereetan esaterako, 2003an helburu medizinaletarako marihuana legeztatu ostean gauzaturiko galdeketa batean ia lautik hiruk landarea era honetan hartzen zutela ikusi zen [173]. Jamaikan bestalde, ez-erretzaileen artean oso ohikoa da marihuana infusioa hartzea, baita adinekoen zein haurren artean ere. Izan ere, infusioari hainbat gaitasun terapeutiko egozten zaizkio eta hargatik bertan ohikoa da sukarraren, hotzeriaren edo estresaren aurkako sendagai gisa erabiltzea [174]. Dena den, uraren irakite-puntua ez da nahikoa eta ondorioz dekarboxilazioa ez da guztiz ematen. Horrela izanik, gorputzeratzen diren kannabinoideak forma azidoan daude batez ere [175]. Bestalde, teknika honen eraginkortasuna kanabinoideen disolbagarritasun baxuak baldintzatzen du (THC 1-2 µg/mL % 0.9 den NaCl disoluzioan) [176]. Hori dela eta, konposatu aktiboen disolbagarritasuna handitzeko gantzak dituen beste osagairen bat gehitzen zaio teari eskuarki, esne-gaina kasu. Edonola ere, duela gutxi THCA-k ezaugarri immunomodulatzaileak dituela ikusi zen [177], beraz infusioaren efektuetan eragin zuzena izan dezakeela aurresan daitekeen zerbait da. Bestalde, beste hainbat kannabinoide azido ere aurkitzen dira infusioan, CBGA eta THVA kasu. Nahiz eta euren aktibitate biologikoak oraindik ez diren aztertuak izan, euren presentziak infusioa THC-aren garraiatzaile soil izateaz gain, kannabisa administratzeko modu berezi bat izatera darama.

Bestalde, aisiarako droga bezala hedatuen dagoen birika-bidezko administrazio modua tabakoarekin nahastuta erretzea da. Bertan, nahiz eta mekanismoa oraindik ezezaguna izan, ikusia dago tabakoaren presentzian THC gehiago askatzen dela kannabis purua erretzearekin alderatu ezkero [178]. Dena den, erretzean, kanabinoideak arnasteaz gain erreketan sortzen diren albo-produktu pirolitikoak ere arnasten direnez, osasuna are gehiago kaltetzen da, hori dela eta mugatua dago erabilera hau gaixoen tratamenduetarako.

Ildo honetatik sortu zen kannabisa lurrunketa bidez hartzearen aukera. Hala, landarea konposatu aktiboak lurruntzeko beharrezkoa den tenperaturara berotzen da, baina erreketa tenperaturatik behera beti ere. Modu honetan, birika bidezko administrazioaren abantailak mantentzen dira aldi berean konposatu pirolitiko kaltegarriak sortzea saihestuz. Azken urteotan eginiko ikerketek lurrunketa segurua izateaz gain kannabisa zein kannabinoide puruak administratzeko modu eraginkorra dela egiaztatu dute, lortzen den THC-aren bioeskuragarritasuna erreketan lortzen denaren antzekoa baita [35, 179-180].

2.2 Kannabinoideen eta terpenoen analisia

Kannabinoideen analisia gauzatzeko teknika arruntenak gas kromatografia eta likido kromatografia dira. Gas kromatografian, kannabinoide nagusiak neurtzeko GC-MS eta GC-FID erabili ohi dira eskuarki. Bestalde, dimentsio bateko GC banaketa konposatu guztiak banatzeko aski ez denean GC x GC moduan ezagutzen den bi dimentsiotako gas kromatografia erabili daiteke. Hala, hegaldi-denbora bidezko masa espektrometro bati akoplatutako gas kromatografia bikoitza GC x GC-TOF-MS kannabisaren eta heroinaren lagin ezberdinen profil kimikoak zehazteko erabilia izan da esaterako [181]. GC-ak badu muga bat ordea, kannabinoide azidoak neurtu ahal izateko deribatizazio-prozesu bat burutzea ezinbestekoa da, kromatografoan injekzio-portuan dekarboxilatu egiten dira eta bestela. Deribatizatzeko erabiltzen diren konposatu ohikoena bis(trimetilsilil)trifluoroazetamida (BSTFA) izan ohi da [182].

Ondorioz, konposatu azidoak neurtu nahi direnean, deribatizazio-urrats hau saihesteko HPLC teknikak erabili ohi dira. Hala, HPLC-DAD eta HPLC-MS/MS erabili ohi dira eskuarki [183-184]. Azken honetan analisi selektiboagoak lortzeaz gain detektatutako konposatuen egituraren berri izan dezakegu, baita gainezartzen diren analisi-gailurretako konposatuak ezberdindu ere [185]. Izan ere, bi konposatuk erretentzio-denbora berdina izan arren, masa ezberdina izan baitezakete edo masa bera izanda ere modu ezberdinean hautsi eta ioi ezberdinak eman baititzakete.

Ohiko teknika hauez gain beste metodo batzuk ere ikertu izan dira kannabinoideak neurtzeko. Kromatografia kapilarra kannabinoide garrantzitsuenak analizatzeko gai dela ikusi da esaterako [186]. Arrakasta mugatuarekin baina jariakin superkritikoen kromatografiak ere balio duela ikusi da, GC-ak eta LC-ak baino analisi denbora laburragoekin gainera [187]. Beste hainbat kasutan teknika ez-instrumentalak erabiltzea aski izan daiteke ordea. Kanabinoideen profil kualitatibo bat erdiesteko edo kannabisaren konposatuen presentzia baieztatzeko geruza meheko kromatografia (TLC) eta erreakzio kolorimetrikoak erabili daitezke esaterako [188]. Bestalde, sentikortasun baxua izan arren, analisi selektiboak eta MS bati akoplaturiko kromatografiekin alderatuz analisi oso azkarrak, errepikakorrak eta ez-suntsitzaileak gauzatzeko balio duen tresna apropos bat erresonantzia magnetiko nuklearrezko espektroskopia (Nuclear Magnetive Resonance, NMR) da. Teknika honek hartzen duen metabolitoen tarte dinamikoa handiagoa da gainera eta laginen prestaketa errazagoa eta azkarragoa [189-190].

Kannabinoideak gorputzeratzean metabolizatu egiten dira neurri handi batean. Kannabinoideen eta metabolitoen gorputzeko kontzentrazioak landarean aurkitzen direnak baino askoz baxuagoak izan ohi dira eta ondorioz beharrezkoa da HPLC-MS/MS edo GC-EI-MS bezalako teknika sentikorrak erabiltzea konposatu hauek neurtzeko [182, 185]. Gainera, kannabinoideek zein hauetatik osatutako metabolitoek, glukuronido loturak osatu ohi dituzte. Glukuronido lotura hauek ezberdinak izan daitezke, eter eta ester

modukoak hain zuzen ere [182]. Kannabinoideen eta metabolitoen kontzentrazio totala jakiteko beharrezkoa da, beraz, analisiaren aurretik lotura glukuronido hauek haustea. Hidrolisi basikoa zein hidrolisi entzimatikoa erabili ohi dira eskuarki horretarako [182, 191].

Terpenoei dagokienez, konposatu hegazkorrak izanik, gas kromatografia bidez neurtu ohi dira normalean. Landare bakoitzak bere usain berezia dauka eta usain hori terpeno guztien baturak osatzen du. Hala, landareak dituen terpeno guztiak neurtuko bagenitu bere lurrinaren hatz marka deiturikoa lortuko genuke. Kannabinoideen kontzentrazioa landare bakoitzaren genetikaren adierazle den bezala, terpenoen kontzentrazioak landarea hazi den inguruaren araberakoak dira neurri batean. Hala, posible da terpenoen hatz markaren bidez landarea zer lurraldetan hazia izan den jakitea [192]. GC-MS eta GC-FID bidez lortutako datuen tratamendu kimiometrikoa da landarea ezaugarritzen duten lurrinak eraginkortasunez interpretatzeko lagungarriena den teknika [114, 193]. Ohiko lurrinaren laginketan buru-guneko erauzketa dinamikoa, lurrun-destilazioa, aldibereko destilazio bidezko erauzketa eta purga eta tranpa ere erabiltzen dira datuak lortzeko [193].

3 Konposatu aktiboen mikrokapsularatzea

Konposatu aktiboek, gaixotasunen prebentziorako gehigarri bezala erabiltzen direnek, zein sendagai bezala erabiltzen direnek, arazo ezberdinak aurkezten dituzte euren aktibitatea gorputzean eraginkor bihurtzerako garaian. Alde batetik, polifenolen kasuan gertatzen den bezala, konposatuak inguruneko baldintza ezberdinekiko ezegonkorrak dira eta ondorioz bizitza baliagarri oso laburra dute [194]. Neurri txikiagoan, baina antzekoa gertatzen da gantz azido asegabeekin ere, euren lotura bikoitzak oxidatzen joaten baitira denborarekin eta beraz aktibitatea galtzen [195]. Ondorioz, konposatu puruak edo erauzkin aberatsak lortzen direnetik kontsumitzailearengana iritsi eta honek hartu arte konposatuen eraginkortasuna izugarri txikitzeko arriskua egoten da.

Beste muga garrantzitsu bat konposatu hauek ahotik hartzen ditugunean aurkezten duten bioeskuragarritasun baxua da. Polifenolak esaterako gehienetan ez dira oso bioeskuragarriak, uretan disolbagarritasun baxua baitute eta jariakin biologikoetan dagoen baldintza alkalinoetan ezegonkorrak baitira gainera [196]. Kannabinoideen kasuan ere antzera gertatzen da, arestian aipatu bezala urin gastrikoaren azidotasunarekiko duen sentikortasunaren ondorioz, eta, gibeleko eta hesteetako metabolismoa eta euren arteko zirkulazioa dela bide, bioeskuragarritasuna birika bidez hartzen denean baino dezente txikiagoa baita [172].

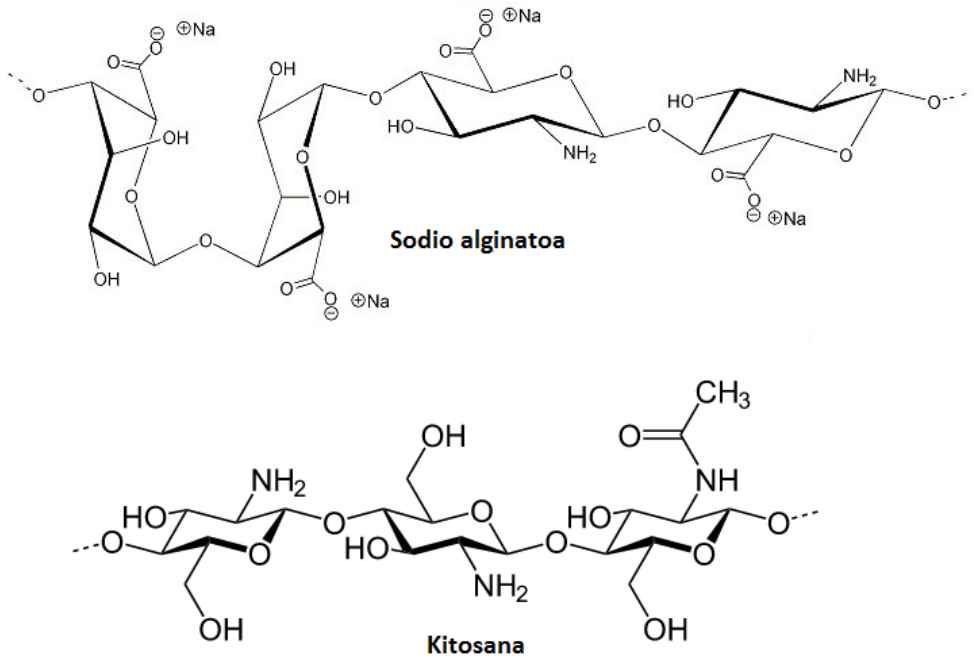
Bestalde, gantz azidoen kasuan bezala konposatu asko olio moduan aurki daitezke, edo kannabioideen kasuan gertatzen den moduan konposatu bat uretan disolbagarria ez denean oliotan disolbatu ohi da ahotik hartzeko prestakin bat erdietsi ahal izateko. Olio moduan dagoen botika bat hartzerakoan kopurua kontrolatzea askoz zailagoa da ordea. Sendagaia tableta solido moduan dagoenean konposatu aktibotik zenbat miligramo hartzen diren zehatza izaten da, olioaren 3 tantatxo hartzen direnean, esaterako, ez.

Azkenik, hirugarren muga garrantzitsu bat hartzen den dosiak eragiten duen efektuaren abiaduraren kontrol falta izan ohi da. Prebentziorako erabili zein sendagai gisa erabili, kasu batzuetan hartzen den konposatu aktiboaren efektua berehalakoa eta iraupen laburrekoa izatea komeni da, beste batzuetan ordea efektu lausoago bat denbora luzean zehar mantentzea komenigarriagoa da. Bestalde, kasu askotan konposatu aktiboa organo edo ehun jakin batzuetara iristea izaten da helburua. Konposatuak besterik gabe ahotik hartzerakoan ez dago ordea efektuen abiadura eta eragina egingo duten lekua kontrolatzerik [194].

Zorionez, bada oztopo guzti hauei aurre egiteko bide bat: mikrokapsularatzea. Mikrokapsulek 1 µm eta 2000 µm arteko diametroa izan ohi dute eskuarki eta bi mota ezberdinetakoak daude, nukleodunak eta nukleo gabeak. Nukleodunek geruza babesle baten erdian konposatu aktiboak gordetzen dituen eta material ezberdinez eginda dagoen nukleo bat izan ohi dute; nukleo gabeek aldiz ez dituzte bi geruza izaten eta konposatu aktiboak mikrokapsula osoan zehar banatzen dira.

Hainbat material ezberdin erabili daitezke konposatu aktiboak kapsularatzeko. Gehienetan polimero inerteak eta karboxilo- eta amino-deribatuen gisa pH-arekiko sentikorrak diren materialak

erabiltzen dira, etil zelulosa, polibinil alkohola, gelatina, ziklodextrinak eta sodio alginatoa esaterako [197]. Hauetatik erabilienetako bat sodio alginatoa da hain zuzen ere (ikus 16. irudia). Material hau alga marroietatik lortzen da eta kaltzio katioiekin erreakzionatu dezaketen talde karboxiliko glukuroniko eta mannuroniko ugari ditu. Hala, konposatu aktiboak babesteko gai den gel egonkorrak osatzeko gai da [198]. Hori dela eta, agente gisa alginatoa erabiltzen denean kaltzio klorurorekin elkartu ohi da mikrokapsulak osatzeko. Kapsulen egonkortasuna are gehiago hobetzeko aukera gehigarri bat bada gainera alginatoa erabiltzen denean, kitosana hain zuzen ere [199]. Kitosana karramarroen exoeskeletoetatik erdiesten da eta elektroi emaile gisa diharduten –OH eta $–NH_2$ talde ugari ditu. Ondorioz, alginatoak eta kitosanak bateragarritasun bikaina dute, aurkako kargak dituztenez elkarrekin erreakzionatu baitezakete [200].



16. irudia: Mikrokapsularatzean sarri erabiltzen diren sodio alginatoaren eta kitosanaren irudikapenak

Mikrokapsulen ezaugarri fisiko-kimikoak hautatutako kapsulatze-agentearen araberakoak izango dira batik bat. Ondorioz, hau hautatzerako orduan hainbat gauza hartu behar dira kontuan. Lehenik eta behin kasu bakoitzeko kapsulatzearen helburuak betetzea, esaterako konposatuak erraz degradatzen badira hori saihestea edo konposatuak gorputzean zer abiaduratan askatuko diren kontrolatzea. Bestalde, mikrokapsula nukleodunen kasuan nukleoaren materialarekin kimikoki bateragarria izan behar du, eta nukleo gabekoen kasuan aldiz konposatu aktiboekin bateragarria. Azkenik, kasuan kasu, beharrezkoak diren ezaugarri fisiko jakin batzuk izatea; hala nola, malgutasuna, gogortasuna, iragazgaiztasuna, ezaugarri optiko jakinak edota egonkortasuna [201].

Hainbat teknika ezberdin aurkitzen dira konposatu aktiboak kapsularatzeko. Teknika guzti hauek 4 multzo nagusitan bana daitezke: kimikoak, fisiko-kimikoak, elektrostatikoak eta mekanikoak. Teknika

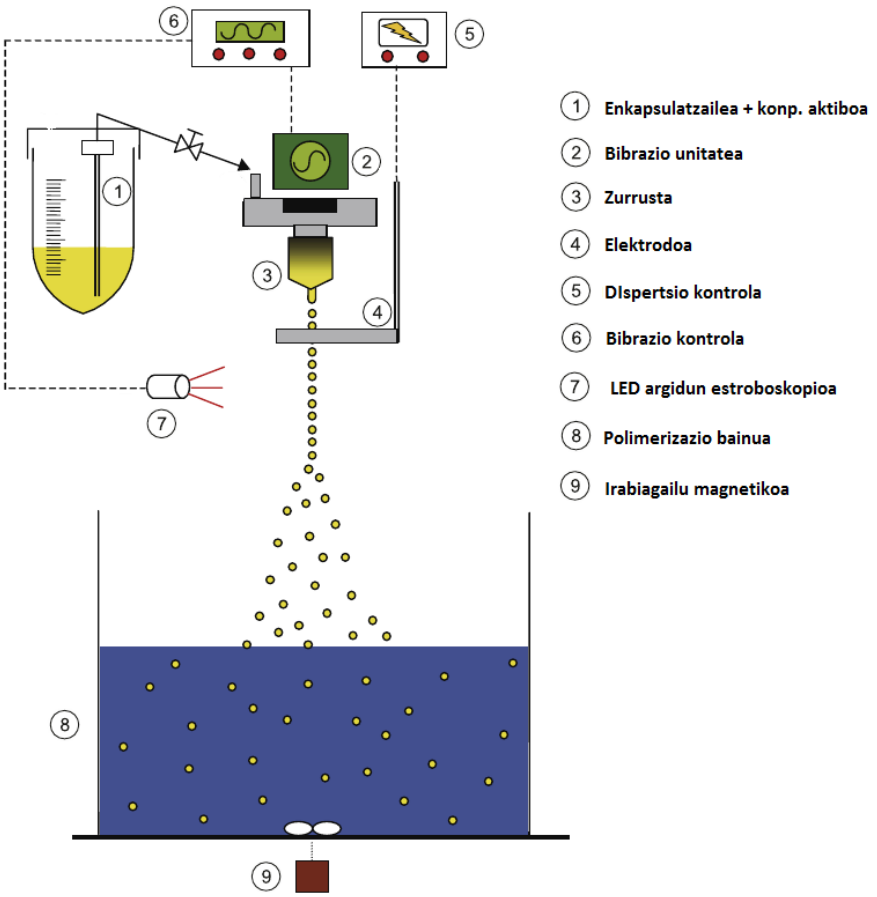
kimikoen artean ezagunena faseen arteko polimerizazioa da. Teknika honetan bi monomero fase nahastezinen artean polimerizatzen dira. Barneko fasea likidoa bada, monomero bat bertan disolbatu ohi da eta hala kanpoko fasean nahastea desio den partikula tamaina lortu arte emultsifikatzen da. Momentu horretan kanpoko fasean nahasgarria den monomero ezberdin bat gehitzen da. Monomero hau barneko faseko monomeroarekin elkartzen da faseen artean, hala intereseko konposatu aktiboak harrapatuz [202-203].

Teknika fisiko-kimikoen artean garrantzitsuena koazerbazio bidezkoa da. Bi koazerbazio-mota daude, sinplea eta konplexua deiturikoak. Lehenengoan koloide nahaste bati konposatu oso hidrofilo bat gehitzen zaio eta horrela bi fase osatzen dira. Bigarrengoak berriz pH-aren arabera funtzionatzen du. Sistemaren izaera azidoaren edo basikoaren aldaketan oinarritzen da mikrokapsularatzea [204-205].

Teknika elektrostatiko erabiliena aerosolarena da. Bertan, kapsulatze-agentea eta kapsulatu beharreko konposatuak bi aerosoleko sistemaren bidez elkartzen dira. Horretarako, kapsulatze agenteak aerosolean likidoa izan behar du kapsularatu nahi dena inguratu ahal izateko eta bi aerosolek aurkako karga izan behar dute. Aurkako kargadun ioiak osatzen dira modu honetan eta hauek tantatxo likidoetan jalkitzen dira atomizatzen diren bitartean [206-207].

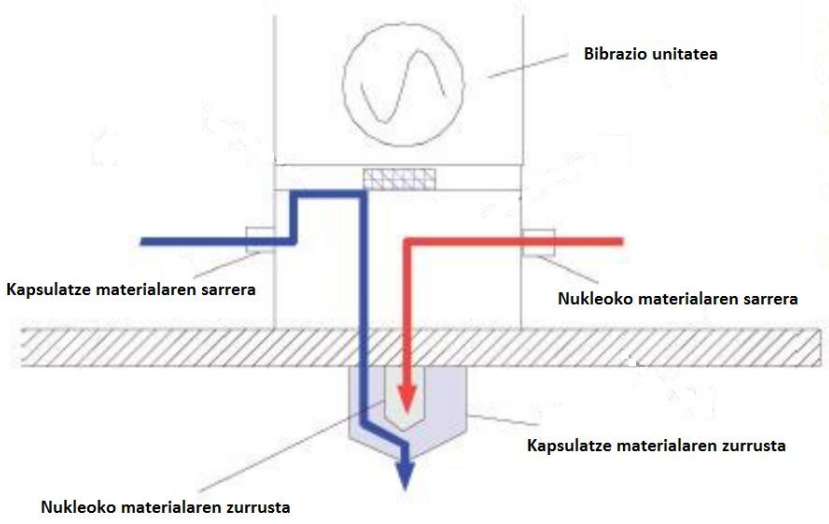
Teknika mekanikoetan ongi definitutako gertaera kimiko edo fisiko batean oinarritzen diren prozedura mekaniko ezberdinak erabiltzen dira. Euren artean erabilienak aerosol bidezko lehorketa metodoa eta zurrusta-bibrazio bidezkoa (Vibration Nozzle Microencapsulation VNM) aurkitzen dira. Aerosol bidezko lehorketan kapsularatu nahi den konposatua enkapsulazio-agentearekin nahasten da uretan suspentsio bat osatuz. Suspentsio hau, atomizagailu batetik pasarazten da eta osatzen diren tantatxo txikiak tenperatura altuan egoten den gas batekin lehortzen dira, hala mikrokapsulak eratuz [208-209].

VNM-n aldiz, nukleo gabeko mikrokapsulak osatzeko kapsulatu nahi diren konposatuak agente kapsulatzailearekin nahastu eta nahastearen fluxu laminar bat zulo txiki batetik pasarazten da, aplikaturiko bibrazioari esker tanta nimiñoak osatuz. Tentsio bat ezartzean tanta horiek dispertsatu egiten dira eta gogortze-disoluziora erortzean mikrokapsulak osatzen dira [210] (17. irudia). Hala, osatzen diren kapsulen ezaugarriak, erabilitako zurrustaren tamainaren, agente kapsulatzailearen eta kapsulatu nahi diren konposatuen nahastearen fluxu laminarra osatzeko ezarririko presioaren, fluxua banakako tanta nimiñoetan bihurtzen duen bibrazioaren maiztasunaren, eta, tantatxo hauek banatzeko aplikaturiko tentsioaren, araberakoak izango dira [211].



17. irudia: VNM bidez nukleo gabeko mikrokapsulak eratzeko sistemaren azalpen eskematikoa.

Bestalde, nukleodun mikrokapsulak osatu nahi direnean zurrusta baten barruan zurrusta txikiago bat kokatu ohi da; hala, nukleoko materiala barneko zurrusta txikitik pasatzen den bitartean kapsulatze agentea kanpoko zurrustatik pasatzen da, tantatxoa osatzean nukleoko materiala erdian harrapatuz [211] (18. irudia).



18. irudia: VNM bidez nukleodun mikrokapsulak eratzeko sistemaren azalpen eskematikoa.

Laburbilduz, mikrokapsularatzea azken urte hauetan industria farmazeutikoan indar handia hartzen ari den bidea dela esan daiteke. Izan ere, hainbat eta hainbat aplikazio ezberdinetarako erabili daiteke:

- Konposatu aktiboa denbora luzean zehar astiro askatzen doan dosi formak egiteko [212].
- Konposatu aktiboa gorputzeko leku jakin batean askatzea lortzeko; esaterako, hesteetan askatzea sabelean beharrean [213].
- Konposatu mingotsen zaporea estaltzeko [214].
- Olio gisako konposatuak errazago erabili daitekeen tableta solido formara pasatzeko [215].
- Neurri handi batean konposatuak hezetasunetik, argitik, oxigenotik edo berotik babesteko [216].
- Elkarrekin bateragarriak ez diren bi konposatu elkartzeko. Bakoitza bere aldetik kapsularatzen da horretarako eta ostean elkartu. Aspirina eta klorfeniramina maleatoa elkartzeko erabili izan da teknika hau esaterako [217].
- Konposatu aktiboen hegazkortasuna txikitzeko eta hala ez lurruntzeko [217].
- Substantzia toxikoak modu seguruago batean maneiatzeko [218].
- Konposatuen izaera higroskopikoa txikitzeko [219].
- Urdaila narritatzen duten konposatu aktiboen albo-efektuak murrizteko [219].

4 **Bibliografia**

[1] Mayer-Davis EJ, Rifas-Shiman SL, Zhou L, Hu FB, Colditz GA, Gillman MW. 2006. Breast-feeding and risk for childhood obesity: does maternal diabetes or obesity status matter? Diabetes Care, 10, 2231-2237.

[2] Hunter P. 2008. We are what we eat, The link between diet, evolution and non-genetic inheritance. EMBO Rep, 9, 413-415.

[3] Arai S. 2000. Functional food science in Japan: state of the art. Biofactors, 12, 13-16.

[4] Milner JA. 2002. Functional foods: the US perspective. Brit J Nutr, 88, S151–S158.

[5] FUFOSE, International Life Sciences Institute, ILSI Europe. 1999. Scientific concepts of functional foods in Europe. Consensus Document. Brit J Nutr, 81, S1-S27.

[6] Shahidi F. 2009. Nutraceuticals and functional foods: Whole versus processed foods. Trends Food Sci Tech, 20, 376-387.

[7] Kaila M, Isolauri E, Soppi E, Virtanen E, Lainen S, Avirlommi H. 1992. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain. Pediatr Res, 32, 141-144.

[8] Van Loo J, Cummings J, Delzene N, Englyst H, Franck A, Hopkins M, Kok N, Macfarlane G, Newton D, Quigley M, Roberfroid M, Van Vliet T, Van den Heuvel E, Van den Heuvel E. 1999. Functional Food properties of non-digestible oligosaccharides: a consensus report from the ENDO projects (DGXII IRII-CT94-1095). Brit J Nut, 81, 21-32.

[9] Reddy BS. Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. 1999. J Nutr, 129, S1478-S1482.

[10] Takase S, Goda T, Watanabe M. 1994. Monostearylglycerol-starch complex: its digestability and effects on glycemic and lipogenic responses. J Nutr, 40, 23-36.

[11] Howarth NC, Saltzman E, Roberts SB. 2001. Dietary fiber and weight regulation. Nutr Rev, 59, 129-139.

[12] Kay RM, Truswell AS. 1980. Dietary fiber: effects on plasma and biliary acids in man. In: Spiler GA, Kay RM ed. Medical aspects of dietary fiber. New York, Plenum Press, 153-173.

[13] Jenkins DJ, Axelsen M, Kendall CW, Agustín LS, Vuksan V, Smith U. 2000. Dietary fiber, lente carbohydrates and the insulin-resistant diseases. Brit J Nutr, 83, S157-S163.

[14] Kang JX, Leaf A. 2000. Prevention of fatal cardiac arrythmias by polyunsaturated fatty acids. Am J Clin Nutr, 71, S202-S207.

[15] GISI-Prevenzione Investigators. 1999. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISI-Prevenzione trial. Lancet, 99, 779-785.

[16] Rassias G, Kestin M, Nestel PJ. 1991. Linoleic acid lowers LDL cholesterol without a proportionate displacement of saturated fatty acid. Eur J Clin Nutr, 45, 315-320.

[17] Due A, Larsen TM, Mu H, Hermansen K, Stender S, Astrup A. 2008. Comparison of 3 ad libitum diets for weight-loss maintenance, risk of cardiovascular disease, and diabetes: a 6-mo randomized, controlled trial. Am J Clin Nutr, 88, 1232-1241.

[18] Plaza I. 2001. Los fitosteroles, el colesterol y la prevención de las enfermedades cardio-vasculares. Clín Inv Arterioscl, 13, 209-218.

[19] Sardesai V. 2012. Introduction to Clinical Nutrition, Third Edition. Boca Raton, CRC Press, 103-275.

[20] Kim DO, Lee CY. 2004. Comprehensive study on vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship. Crit Rev Food Sci, 44, 253-273.

[21] Tikhonov I. Roginsky V. Pliss E. 2009. The chain-breaking antioxidant activity of phenolic compounds with different numbers of O-H groups as determined during the oxidation of styrene. Int J Chem Kinet, 41, 92-100.

[22] Perron NR. Brumaghim JL. 2009. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochem Biophys, 53, 75-100.

[23] Zhou B, Wu LM, Yang L, Liu ZL. 2005. Evidence for alpha-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. Free Radical Bio Med, 38, 78-84.

[24] Taguri T, Tanaka T, Kouno I. 2006. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. Biol Pharm Bull, 29, 2226–2235.

[25] Perez RM. 2003. Antiviral activity of compounds isolated from plants. Pharm Biol, 41, 107–157.

[26] Lazarou D, Grougnet R, Papadopoulos A. 2007. Antimutagenic properties of a polyphenol-enriched extract derived from sesame-seed perisperm. Mutat Res-Gen Tox En, 634, 163–171.

[27] Lambert JD, Hong J, Yang G, Liao J, Yang CS. 2005. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am J Clin Nutr, 81, S284-S291.

[28] Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, Lamari FN. 2009. Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. Behav Brain Res, 198, 352–358.

[29] Ellis J. 2013. Medicinal plants: past, present and future. Plant Cell, 25, 112-113.

[30] Zuardi AW. 2006. History of cannabis as a medicine: a review. Rev Bras Psiquiatr, 28, 153-157.

[31] Balabanova S, Parsche F, Pirsig W. 1993. First identification of drugs in Egyptian mummies. Naturwissenschaften, 79, 358-361.

[32] Russo E. 2002. Cannabis and cannabinoids: pharmacology, toxicology, and therapeutic potential. Binghamton, Haworth Press.

[33] Hollister LE. 1986. Health aspects of cannabis. Pharmacol Rev, 38, 1-20.

[34] Bureau of Mortality Statistics. 1988. U.S. Public Health Service statistics. National Institute of Drug Abuse statistics. U.S. Surgeon General's Report.

[35] Fischedick J, Van Der Kooy F, Verpoorte R. 2010. Cannabinoid receptor 1 binding activity an quantitative analysis of *Cannabis sativa L.* Smoke and Vapor. Chem Pharm Bull, 58, 201-207.

[36] Abrams DI, Hilton JF, Leiser RJ, Shade SB, Elbeik TA, Aweeka FT, Benowitz NL, Bredt BM, Kosel B, Aberg JA, Deeks SG, Mitchell TF, Mulligan K, Bacchetti P, McCune JM, Schambelan M. 2003. Short-term effects of cannabinoids in patients with HIV-1 infection. A randomized, placebo-controlled clinical trial. Ann Intern Med, 139, 258–266.

[37] Beal JE, Olson R, Laubenstein L, Morales JO, Bellman P, Yangco B, Lefkowitz L, Plasse TF, Shepard KV. 1995. Dronabinol as a treatment for anorexia associated with weight loss in patients with AIDS. J Pain Symptom Manage, 10, 89–97.

[38] Jatoi A, Windschitl HE, Loprinzi CL, Sloan JA, Dakhil SR, Mailliard JA, Pundaleeka S, Kardinal CG, Fitch TR, Krook JE, Novotny PJ, Christensen B. 2002. Dronabinol *versus* megestrol acetate *versus* combination therapy for cancer-associated anorexia: a North central cancer treatment group study. J Clin Oncol, 20, 567–573.

[39] Herman TS, Einhorn LH, Jones SE, Nagy C, Chester MB, Dean J, Furnas B, Williams SD, Leigh SA, Dorr RT, Moon T.E. 1979. Superiority of nabilone over prochlorperazine as an antiemetic in patients receiving cancer chemotherapy. N Engl J Med, 300, 1295–1297.

[40] Hutcheon AW, Palmer JBD, Soukop M, Cunningham D, Mcardle C, Welsh J, Stuart F, Sangster G, Kaye S, Charlton D. 1983. A randomised multicentre single blind comparison of a cannabinoid antiemetic (levonantradol) with chlorpromazine in patients receiving their first cytotoxic chemotherapy. Eur J Cancer Clin Oncol, 19, 1087–1090.

[41] Ungerleider JT, Andrysiak T, Fairbanks L, Goodnight J, Sarna G, Jamison K. 1982. Cannabis and cancer chemotherapy. A comparison of oral delta-9-THC and prochlorperazine. Cancer, 50, 636–645.

[42] Lynch ME, Campbell F. 2011. Cannabinoids for treatment of chronic non-cancer pain; a systematic review of randomized trials. Br J Clin Pharmacol, 72, 735-744.

[43] Wilsey B, Marcotte T, Deutsch R, Gouaux B, Sakai S, Donaghe H. 2013. Low-dose vaporized cannabis significantly improves neuropathic pain. J Pain, 14, 136-148.

[44] Wilsey B, Marcotte T, Tsodikov A, Millman J, Bentley H, Gouaux B, Fishman S. 2008. A randomized, placebo-controlled, crossover trial of cannabis cigarettes in neuropathic pain. J. Pain, 9, 506-521.

[45] Corey-Bloom J, Wolfson T, Gamst A, Jin S, Marcotte TD, Bentley H, Gouaux B. 2012. Smoked cannabis for spasticity in multiple sclerosis: a randomized, placebocontrolled trial. CMAJ, 10, 1143-1150.

[46] Rog DJ, Nurmikko TJ, Friede T, Young CA. 2005. Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. Neurology, 65, 812-819.

[47] Wade DT, Makela P, Robson P, House H, Bateman C. 2004. Do cannabis-based medicinal extracts have general or specific effects on symptoms in multiple sclerosis? A double-blind, randomized, placebo-controlled study on 160 patients. Mult Scler, 10, 434-441.

[48] De Petrocellis L, Vellani V, Schiano-Moriello A, Marini P, Magherini PC, Orlando P, Di Marzo V. 2008. Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. J Pharmacol Exp Ther, 325, 1007-1015.

[49] Galve-Roperh I, Sánchez C, Cortés ML, Gómez Del Pulgar T, Izquierdo M, Guzmán M. 2000. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med, 6, 313-319.

[50] Parolaro D, Massi P. 2008. Cannabinoids as a potential new drug therapy for the treatment of gliomas. Expert Rev Neurother, 8, 37-49.

[51] Velasco G, Sánchez C, Guzmán M. 2012. Towards the use of cannabinoids as antitumour agents. Nat Rev Cancer, 12, 436-444.

[52] Cunha JM, Carlini EA, Pereira AE, Ramos OL, Pimentel C, Gagliardi R, Sanvito WL, Lander N, Mechoulam R. 1980. Chronic administration of cannabidiol to healthy volunteers and epileptic patients. Pharmacol, 21, 175–185.

[53] Porter BE, Jacobson C. 2013. Report of a parnet survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy. Epilepsy Behav, 29, 574-577.

[54] Campbell VA, Gowran A. 2007. Alzheimer′s disease; taking the edge off with cannabinoids? Br J Pharmacol, 152, 655-662.

[55] Eubanks LM, Rogers CJ, Beuscher AE, Koob GF, Olson AJ, Dickerson TJ, Janda KD. 2006. A molecular link between the active component of marijuana and Alzheimer′s disease pathology. Mol Pharm, 3, 773-777.

[56] Luvone T, Esposito G, De Filippis D, Scuderi C, Steardo L. 2009. Cannabidiol: a promising drug for neurodegenerative disorders? CNS Neurosci Ther, 15, 65-75.

[57] Sagredo O, Pazos MR, Valdeolivas S, Fernandez-Ruiz J. 2012. Cannabinoids: Novel Medicines for the Treatment of Huntington′s Disease. Recent Pat CNS Drug Discover, 7, 41-48.

[58] Penner EA, Buettner H, Mittleman MA. 2013. Marijuana use on glucose, insulin, and insulin resistance among US adults. Am J Med, 126, 583-589.

[59] Weiss L, Zeira M, Reich S, Har-Noy M, Mechoulam R, Slavin S, Gallily R. 2006. Cannabidiol lowers incidence of diabetes in non-obese diabetic mice. Autoimmunity, 39, 143-151.

[60] Müller-Vahl KR, Schneider U, Prevedel H, Theloe K, Kolbe H, Daldrup T, Emrich H.M. 2003. Δ9-tetrahydrocannabinol (THC) is effective in the treatment of tics in Tourette syndrome: a 6-week randomized trial. J Clin Psychiatry, 64, 459–465.

[61] Müller-Vahl KR, Schneider U, Koblenz A, Jobges M, Kolbe H, Daldrup T, Emrich H.M. 2002. Treatment of Tourette's syndrome with Δ9-tetrahydrocannabinol (THC): a randomized crossover trial. Pharmacopsychiatry, 35, 57–61.

[62] Harborne JB, Williams CA. 2000. Advances in flavonoid research since 1992. Phytochemistry, 55, 481–504.

[63] Tsao R. 2010. Chemistry and Biochemistry of Dietary Polyphenols. Nutrients, 2, 1231-1246.

[64] Garazd M, Garazd YL, Khilya VP. 2003. Neoflavones. 1. Natural Distribution and Spectral and Biological Properties. Chem Nat Compd, 39, 44-121.

[65] Tsao R, Yang R, Young JC, Zhu H. 2003. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). J Agr Food Chem, 51, 6347-6353.

[66] Zhao F, Watanabe Y, Nozawa H, Daikonnya A, Kondo K, Kitanaka S. 2005. Prenylflavonoids and phloroglucinol derivatives from Hops (*Humulus lupulus*). J Nat Prod, 68, 43-49.

[67] Si W, Gong J, Tsao R, Kalab M, Yang R, Yin Y. 2006. Bioassay-guided purification and identification of antimicrobial components in Chinese green tea extract. J Chromatogr A, 1125, 204-210.

[68] Kwik-Uribe C, Bektash RM. 2008. Cocoa flavanols: measurement, bioavailability and bioactivity. Asia Pac J Clin Nutr, 17, S280-S283.

[69] Davis CB, Markey CE, Busch MA, Busch KW. 2007. Determination of capsaicinoids in habanero peppers by chemometric analysis of UV spectral data. J Agr Food Chem, 55, 5925-5933.

[70] Bratt K, Sunnerheim K, Bryngelsson S, Fagerlund A, Engman L, Andersson RE, Dimberg LH. 2003. Avenanthramides in oats (*Avena sativa L.*) and structure-antioxidant activity relationships. J Agr Food Chem, 51, 594-600.

[71] Makris DP, Boskou G, Andrikopoulos NK. 2007. Polyphenolic content and *in vitro* antioxidant characteristics of wine industry and other agri-food solid waste extracts; J Food Compos Anal, 20, 125-132.

[72] Casazza AA, Aliakbarian B, Mantegna S, Cravotto G, Perego P. 2010. Extraction of phenolics from Vitis vinífera wastes using non-conventional techniques. J Food Eng, 100, 50-55.

[73] FAO (Food and Agriculture Organization of the United Nations). http://www.fao.org/

[74] Fernández J. 2006. Los residuos de las agroindustrias como biocombustibles sólidos. Vida Rural, 233, 14–18.

[75] Arvanitoyannis IS, Varzakas TH. 2008. Fruit/Fruit Juice Waste Management: Treatment Methods and Potential Uses of Treated Waste. In: Arvanitoyannis IS ed. Waste Management for the Food Industries. Massachusetts, Academic Press, 569-628.

[76] Lafourcade M, Larrieu T, Mato S, Duffaud A, Sepers M, Matias I, De Smedt-Peyrusse V, Labrousse VF, Bretillon L, Matute C, Rodríguez-Puertas R, Layé S, Manzoni OJ. 2011. Nutritional omega-3 deficiency abolishes endocannabinoid-mediated neuronal functions. Nat Neurosci, 14, 345-350.

[77] Hutchins-Wiese HL, Li Y, Hannon K, Watkins BA. 2012. Hind limb suspension and long-chain omega-3 PUFA increase mRNA endocannabinoid system levels in skeletal muscle. J Nutr Biochem, 23, 986-993.

[78] Murru E, Banni S, Carta G. 2013. Nutritional Properties of Dietary Omega-3-Enriched Phospholipids. Biomed Res Int, DOI: 10.1155/2013/965417.

[79] Guesnet P, Alessandri JM. 2011. Docosahexaenoic acid (DHA) and the developing central nervous system (CNS) – Implications for dietary recommendations. Biochimie, 93, 7-12.

[80] Sanders TB. 2009. DHA status of vegetarians. PLEFA, 81, 137-141.

[81] Cunnane SC, Anderson MJ. 1997. Pure linoleate deficiency in the rat: influence on growth, accumulation of n-6 polyunsaturates, and [1-14C] linoleate oxidation. J Lipid Res, 38, 805-812.

[82] Ruthig DJ, Meckling-Gill KA. 1999. Both (n-3) and (n-6) fatty acids stimulate wound healing in the rat intestinal epithelial cell line, IEC-6. J Nutr, 129, 1791-1798.

[83] Pereira LM, Hatanaka E, Martins EF, Oliveira F, Liberti EA, Farsky SH, Curi R, Pithon-Curi TC. 2008. Effect of oleic and linoleic acids on the inflammatory phase of wound healing in rats. Cell Biochem Funct, 26, 197-204.

[84] Letawe C, Boone M, Piérard GE. 1998. Digital image analysis of the effect of topically applied linoleic acid on acne microcomedones. Clin Exp Dermatol, 23, 56-58.

[85] Yang ZH, Miyahara H, Hatanaka A. 2011. Chronic administration of palmitoleic acid reduces insulin resistance and hepatic lipid accumulation in KK-Ay Mice with genetic type 2 diabetes. Lipids Health Dis, 10, 120-125.

[86] Mensink RP. 2013. Fatty Acids: health effects of saturated fatty acids. In Allen LH, Prentice A, Caballero B (Eds.). Encyclopedia of Human Nutrition. Massachusetts, Academic Press, 215-219.

[87] Kamel BS, Dawson H, Kakuda Y. 1985. Characteristics and composition of melon and grape seed oils and cakes. J Am Oil Chem Soc, 62, 881-883.

[88] Ridgway K. 2012. Food For Throught, the latest trends in sample preparation for food analysis. LC-GC Eur, 25, 66-70.

[89] Vilkhu K, Mawson R, Simons L, Bates D. 2008. Applications and opportunities for ultrasound assisted extraction in the food industry- A review. Inno Food Sci Emerg, 9, 161-169.

[90] Jerman T, Trebše P, Vodopivec BM. 2010. Ultrasound-assisted solid liquid extraction (USLE) of olive fruit (*Olea europaea*) phenolic compounds. Food Chem, 123, 175–182.

[91] Chemat F, Huma Z, Khan MK. 2011. Applications of ultrasound in food technology: Processing, preservation and extraction. Ultrason Sonochem, 18, 813-835.

[92] Sutivisedsak N, Cheng HN, Willett JL, Lesch WC, Tangsrud RR, Biswas A. 2010. Microwave-assisted extraction of phenolics from bean (*Phaseolus vulgaris L.*). Food Res Int, 43, 516-519.

[93] Letellier M, Budzinski H. 1999. Microwave assisted extraction of organic compounds. Analysis, 27, 259-271.

[94] Garcia P, Morales A, Segura A, Fernández A. 2010. Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples. Molecules, 15, 8813-8826.

[95] Karale K, Dere J, Honde S, Sachin K, Kote P. 2011. A overview of supercritical fluid extraction for herbal drugs. Pharmacologyonline, 2, 575-596.

[96] Murga R, Ruiz R, Beltrán S, Cabezas JL. 2000. Extraction of natural phenols and tannins from grape seeds by using supercritical mixtures of carbon dioxide and alcohol. J Agr Food Chem, 48, 3408-3412.

[97] Chaudot X, Tambuté A, Caude M. 2000. Selective extraction of hydrocarbons, phosphonates and phosphonic acids from soils by successive supercritical fluid and pressurized liquid extractions. J Chromatogr A, 866, 231–240.

[98] Omar J, Alonso I, Garaikoetxea A, Etxebarria N. 2013. Optimization of focused ultrasound extraction (FUSE) and supercritical fluid extraction (SFE) of citrus peel volatile oils and antioxidants. Food Anal Meth, 6, 1244-1252.

[99] Canela APRF, Rosa PTV, Marques MOM, Meireles MAA. 2002. Supercritical fluid extraction of fatty acids and carotenoids from the microalgae *spirulina maxima*. Ind Eng Chem Res, 41, 3012-3018.

[100] Pinelo M, Ruiz A, Sineiro J, Señoráns FJ, Reglero G, Núñez MJ. 2007. Supercritical fluid and solid–liquid extraction of phenolic antioxidants from grape pomace: a comparative study. Eur Food Res Tech, 226, 199-205.

[101] Ivanova V, Stefova M, Chinnici F. 2010. Determination of the polyphenol contents in Macedonian grapes and wines by standardized spectrophotometric methods. J Serb Chem Soc, 75, 45–59.

[102] Vinson JA, Cai Y. 2012. Nuts, especially walnuts, have both antioxidant quantity and efficacy and exhibit significant potential health benefits. Food Funct, 3, 134-40.

[103] Zieliński H, Zielińska D, Kostyra H. 2012. Antioxidant capacity of a new crispy type food products determined by updated analytical strategies. Food Chem, 130, 1098-1104.

[104] Huang D, Ou B, Prior RL. 2005. The chemistry behind antioxidant capacity assays. J Agr Food Chem, 53, 1841-1856.

[105] Wang H, Suo Y, Wang X, Li Y, You J, Zhao X. 2007. Extraction of *Nitraria tangutorum* seed oil by supercritical carbon dioxide and determination of free fatty acids by HPLC/APCI/MS with fluorescence detection. Sep Purif Technol, 56, 371–377.

[106] Dayhuff L, Wells MJM. 2005. Identification of fatty acids in fishes collected from the Ohio River using gas chromatography–mass spectrometry in chemical ionization and electron impact modes. J Chromatogr A, 1098, 144–149.

[107] Ahmed SA, Ross SA, Slade D, Radwan MM, Khan IA, ElSohly MA. 2015. Minor oxygenated cannabinoids from high potency *Cannabis sativa L*. Phytochem, 117, 194-199.

[108] ElSohly M, Slade D. 2005. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. Life Sci, 78, 539–548.

[109] Russo EB. 2007. History of cannabis and its preparations in saga, science, and sobriquet. Chem Biodiv, 4, 1614-1648.

[110] Mechoulam R. 1970. Marihuana chemistry. Science, 168,1159-1166.

[111] Rustichelli C, Ferioli V, Baraldi M, Zanoli P, Gamberini G. 1998. Analysis of cannabinoids in fiber hemp plant varieties (*Cannabis sativa L.*) by high-performance liquid chromatography. Chromatographia, 48, 215-222.

[112] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci, 30, 515-527.

[113] Cohen Y. 2014. Cannabis plant named 'avidekel'. US patent 20140259228 A1.

[114] Hazekamp A, Fischedick JT. 2012. Cannabis - from cultivar to chemovar. Drug Test Anal, 4, 660-667.

[115] Elzinga S, Fischedick J, Podkolinski R, Raber JC. 2015. Cannabinoids and terpenes as chemotaxonomic markers in cannabis. Nat Prod Chem Res, 3: 181. doi:10.4172/2329-6836.1000181

[116] Russo EB. 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol, 163, 1344-1364.

[117] Grotenhermen F. 2002. Effects of Cannabis and the cannabinoids, in Grotenhermen F, Russo E (ed). Cannabis and Cannabinoids-Pharmacology, Toxicology, and Therapeutic Potential. New York, Haworth Press, 55-65.

[118] Fishbein M, Gov S, Assaf F, Gafni M, Keren O, Sarne Y. 2012. Long-term behavioral and biochemical effects of an ultra-low dose of Δ9-tetrahydrocannabinol (THC): neuroprotection and ERK signaling. Exp Brain Res, 221, 437-448.

[119] Schweinsburg AD, Brown SA, Tapert SF. 2008. The influence of marijuana use on neurocognitive functioning in adolescents. Curr Drug Abuse Rev, 1, 99-111.

[120] Lisdahl KM, Wright NE, Medina-Kirchner C, Maple KE, Shollenbarger S. 2014. Considering the cannabis: the effects of regular cannabis use on neurocognition in adolescents and young adults. Curr Addict Rep, 1, 144-156.

[121] Consroe P. 1998. Brain cannabinoid systems as targets for the therapy of neurological disorders. Neurobiol Dis, 5, 534-551.

[122] McPartland JM, Russo EB. 2011. Cannabis and cannabis extracts: greater than the sum of their parts? J Cann Therap, 1, 103-132.

[123] Mechoulam R, Parker LA, Gallity R. 2002. Cannabidiol: an overview of some pharmacological aspects. J Clin Pharmacol, 42, S11-S19.

[124] Bergamaschi MM, Queiroz RH, Chagas MH, De Oliveira DC, De Martinis BS, Kapczinski F, Quevedo J, Roesler R, Schröder N, Nardi AE, Martín-Santos R, Hallak JE, Zuardi AW, Crippa JA. 2011. Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. Neuropsychopharmacol, 36, 1219-1926.

[125] Morgan CJ, Das RK, Joye A, Vurran HV, Kamboj SK. 2011. Cannabidiol reduces cigarette consumption in tobacco smokers: preliminary findings. Addict Behav, 38, 2433-2436.

[126] Gil ML, Jimenez J, Ocete MA, Zarzuelo A, Cabo MM. 1989. Comparative study of different essential oils of *Bupleurum gibraltaricum Lamarck*. Pharmazie, 44, 284-287.

[127] Falk AA, Hagberg MT, Löf AE, Wigaeus-Hjelm EM, Wang ZP. 1990. Uptake, distribution and elimination of alpha-pinene in man after exposure by inhalation. Scand J Work Environ Health, 16, 372-378.

[128] Miyazawa M, Yamafuji C. 2005. Inhibition of acetylcholinesterase activity by bicyclic monoterpenoids, J Agric Food Chem, 53, 1765-1768.

[129] Köse EO, Deniz IG, Sarıkürkçü C, Aktaş O, Yavuz M. 2010. Chemical composition, antimicrobial and antioxidant activities of the essential oils of *Sideritis erythrantha Boiss*. and *Heldr*. (*var. erythrantha* and *var. cedretorum* P.H. Davis) endemic in Turkey. Food Chem Toxicol, 48, 2960-2965.

[130] Raman A, Weir U, Bloomfield SF. 1995. Antimicrobial effects of tea-tree oil and its major components on *Staphylococcus aureus*, *Staph. epidermidis* and Propionibacterium acnes. Lett Appl Microbiol, 21, 242-245.

[131] Carvalho-Freitas MI, Costa M. 2002. Anxiolytic and sedative effects of extracts and essential oil from *Citrus aurantium* L. Biol Pharm Bull, 25, 1629-1633.

[132] Vigushin DM, Poon GK, Boddy A, English J, Halbert GW, Pagonis C, Jarman M, Coombes RC. 1998. Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer. Cancer Research Campaign Phase I/II Clinical Trials Committee. Cancer Chemother Pharmacol, 42, 111-117.

[133] Kim SS, Baik JS, Oh TH, Yoon WJ, Lee NH, Hyun CG. 2008. Biological activities of Korean *Citrus obovoides* and *Citrus natsudaidai* essential oils against acne-inducing bacteria. Biosci Biotechnol Biochem, 72, 2507-2513.

[134] Nunes DS, Linck VM, da Silva AL, Figueiro M, Elisabetsky E. 2010. Psychopharmacology of essential oils. In: Baser KHC, Buchbauer G, editors. Handbook of Essential Oils: Science, Technology, and Applications. Boca Raton, CRC Press, 297–314.

[135] Basile AC, Sertié JA, Freitas PC, Zanini AC. 1988. Anti-inflammatory activity of oleoresin from Brazilian *Copaifera*. J Ethnopharmacol, 22, 101-109.

[136] Xi ZX, Peng XQ, Li X, Song R, Zhang HY, Liu QR, Yang HJ, Bi GH, Li J, Gardner EL. 2011. Brain cannabinoid CB_2 receptors modulate cocaine's actions in mice. Nat Neurosci, 14, 1160-1166.

[137] Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ, Altmann KH, Karsak M, Zimmer A. 2008. Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci USA, 105, 9099-9104.

[138] Salzet M. 2000. Invertebrate molecular neuroimmune processes. Brain Res Rev, 34, 69-79.

[139] Maccarrone M, Bab I, Bíró T, Cabral GA, Dey SK, Di Marzo V, Konje JC, Kunos G, Mechoulam R, Pacher P, Sharkey KA, Zimmer A. 2015. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci, 36, 277-296.

[140] Di Marzo V, Bifulco M, De Petrocellis L. 2004. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov, 3, 771-784.

[141] Howlett, AC, Qualy JM, Khachatrian LL. 1986. Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol Pharmacol, 29, 307-313.

[142] Bramblett RD, Panu AM, Ballesteros JA, Reggio PH. 1995. Construction of a 3D model of the cannabinoids CB1 receptor: determination of the helix ends and helix orientation. Life Sci, 56, 1971-1982.

[143] Onaivi ES, Chakrabarti A, Chaudhuri G. 1996. Cannabinoid receptor genes. Progr Neurobiol, 48, 275-305.

[144] Munro S, Thomas KL, Abu-Shaar M. 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature, 365, 61-65.

[145] Sylvaine G, Sophie M, Marchand J, Dussossoy D, Carriere D, Carayon P, Monsif B, Shire D, Le Fur G, Casellas P. 1995. Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. Eur J Biochem, 232, 54-61.

[146] Ameri A. 1999. The effects of cannabinoids on the brain. Progr Neurobiol, 58, 315-348.

[147] Croci T, Manara L, Aureggi G, Guagnini F, Rinaldi-Carmona M, Maffrand JP, Le Fur G, Mukenge S, Ferla G. 1998. *In vitro* functional evidence of neuronal cannabinoid CB1 receptors in human ileum. Brit J Pharmacol, 125, 1393-1395.

[148] Pertwee RG. 1997. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Therapeut, 74, 129-180.

[149] Pertwee RG. 2001. Cannabinoid receptors and pain. Progr Neurobiol, 63, 569-611.

[150] Szabo B, Norheim U, Niederhoffer N. 2001. Effects of cannabinoids on sympathetic and parasympathetic neuroeffector transmission in the rabbit heart. J Pharmacol Exp Therapeut, 397, 819-826.

[151] Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, Uhl GL. 2006. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res, 1071, 10-23.

[152] Shen M, Piser TM, Seybold VS, Thayer SA. 1996. Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. J Neurosci, 278, 989-999.

[153] Gifford AN, Samiian L, Gatley SJ, Ashby CR. 1997. Examination of the effect of the cannabinoid receptor agonists, CP55, 940, on electrically evoked transmitter release from rat brain slices. Eur J Pharmacol, 324, 187-192.

[154] Shlicker E, Timm J, Zentner J, Göthert M. 1997. Cannabinoid CB1 receptor-mediated inhibition of noradrenaline release in the human and guinea-pig hippocampus. Naunyn Schmiedebergs Arch Pharmacol, 356, 583-589.

[155] McHugh D, Sherry SJ, Rimmerman N, Juknat A, Vogel Z, Walker M, Bradshaw H. 2010. N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. BMC Neurosci, 11:44.

[156] Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ. 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. Brit J Pharmacol, 152, 1092-1101.

[157] Overton HA, Babbs AJ, Doel SM, Fyfe MCT, Gardner LS, Griffin G, Jackson HC, Procter MJ, Rasamison CM, Tang-Christensen M, Widdowson PS, Williams GM, Reynet C. 2006. Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. Cell Metab, 3, 167-175.

[158] Breivogel CS, Selley DE, Childers SR. 1998. Cannabinoid receptor agonist efficacy for stimulating [35S]GTP γS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J Biol Chem, 273, 16865-16873.

[159] Griffin G, Atkinson PJ, Showalter VM, Martin BR, Abood ME. 1998. Evaluation of cannabinoid receptor agonists and antagonists using the guanosine-5´-O-(3-[35S]thio)-triphosphate binding assay in rat cerebellar membranes. J Pharmacol Exp Therapeut, 285, 553-560.

[160] Pertwee RG. 1999. Pharmacology of cannabinoid receptor ligands. Curr Med Chem, 6, 635-664.

[161] Koch M, Dehgnani F, Habazettl I, Schomerus C, Korf HW. 2006. Cannabinoids attenuate norepinephrine-induced melatonin biosynthesis in the rat pineal gland by reducing arylalkylamine N-acetyltransferase activity without involvement of cannabinoid receptors. J Neurochem, 98, 267-278.

[162] Wilkinson JD, Williamson EM. 2007. Cannabinoids inhibit human keratinocyte proliferation through a non-CB1/CB2 mechanism and have a potential therapeutic value in the treatment of psoriasis. J Dermatol Sci, 45, 87-92.

[163] Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R, Di Marzo V. 2001. Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Brit J Pharmacol, 134, 845-852.

[164] Showalter VM, Compton DR, Martin BR, Abood ME. 1996. Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. J Pharmacol Exp Therapeut, 278, 989-999.

[165] Thomas A, Baillie GL, Phillips AM, Razdan RK, Ross RA, Pertwee RG. 2007. Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists *in vitro*. Brit J Pharmacol, 150, 613-623.

[166] Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, Mackie K, Stella N. 2003. Nonpsychotropic cannabinoid receptors regulate microglial cell migration. J Neurosci, 23, 1398-1405.

[167] Thomas A, Stevenson LA, Wease KN, Price MR, Baillie G, Ross RA, Pertwee RG. 2005. Evidence that the plant cannabinoid D9-tetrahydrocannabivarin is a cannabinoid CB1 and CB2 receptor antagonist. Brit J Pharmacol, 146, 917-926.

[168] Rhee M, Vogel Z, Barg J, Bayewitch M, Levy R, Hanus L, Breuer A, Mechoulam R. 1997. Cannabinol Derivatives: Binding to Cannabinoid Receptors and Inhibition of Adenylylcyclase. J Med Chem, 40, 3228-3233.

[169] Cascio MG, Gauson LA, Stevenson LA, Ross RA, Pertwee R. 2009. Evidence that the plant cannabinoid cannabigerol is a highly potent alpha(2)-adrenoceptor agonist and moderately potent 5HT receptor antagonist. Brit J Pharmacol 159, 129-141.

[170] Bento AF, Marcon R, Dutra RC, Claudino RF, Cola M, Leite DF, Calixto JB. 2011. β-Caryophyllene inhibits dextran sulfate sodium-induced colitis in mice through CB2 receptor activation and PPARγ pathway. Am J Pathol, 178, 1153-1166.

[171] Wall ME, Perez-Reyes M. 1981. The metabolism of delta-9-tetrahydrocannabinol and related cannabinoids in man. J Clin Pharmacol, 21, 178-189.

[172] Agurell S, Halldin M, Lindgren J, Ohlsson A, Widman M, Gillespie H, Hollister L. 1986. Pharmacokinetics and metabolism of delta-9-THC and other cannabinoids with emphasis on man. Pharmacol Rev, 38, 21-42.

[173] Janse AFC, Breekveldt-Postma NS, Erkens JA, Herings RMC. 2007. Pharmo rapport: medicinal gebruik van cannabis. Pharmo Institute for drug outcome research, Utrecht, The Nederlands.

[174] Van Solinge, BT. 1996. Ganja in Jamaica. Amsterdams Drug Tijdschrift, 2, 11-14.

[175] Hazekamp A, Bastola K, Rashidi H, Bender J, Verpoorte R. 2007. Cannabis tea revisited: a systematic evaluation of the cannabinoid composition of cannabis tea. J Ethnopharmacol, 113, 85-90.

[176] Garrett ER, Hunt CA. 1974. Physiochemical properties, solubility, and protein binding of delta 9-tetrahydrocannabinol. J Pharmacol Sci, 63, 1056-1064.

[177] Verhoeckx KC, Korthout HA, Van Meeteren-Kreikamp AP, Ehlert KA, Wang M, Van der Greef J, Rodenburg RJ, Witkamp RF. 2006. Unheated Cannabis sativa extracts and its major compounds THC-acid have potential immuno-modulating properties not mediated by CB1 and CB2 receptor coupled pathways. Int Immunopharmacol, 6, 656-665.

[178] Van der Kooy F, Pomahacova B, Verpoorte R. 2009. Cannabis smoke condensate II: influence of tobacco on tetrahydrocannabinol levels. Inhal Toxicol, 21, 87-90.

[179] Hazekamp A, Ruhaak R, Zuurman L, Gerven J, Verpoorte R. 2006. Evaluation of a vaporizing device (Volcano®) for the pulmonary administration of tetrahydrocannabinol. J Pharmaceut Sci, 95, 1308-1317.

[180] Abrams DI, Vizoso HP, Shade SB, Jay C, Kelly ME, Benowitz NL. 2007. Vaporization as a smokeless cannabis delivery system: a pilot study. Clin Pharmacol Therapeut 82, 572-578.

[181] Gröger T, Schäffer M, Pütz M, Ahrens B, Drew K, Eschner M, Zimmermman R. 2008. Application of two-dimensional gas chromatography combined with pixel-based chemometric processing for the chemical profiling of illicit drug samples. J Chromatogr A, 1200, 8-16.

[182] Abraham TT, Lowe RH, Pirnay SO, Darwin WD, Huesti MA. 2007. Simultaneous GC–EI-MS Determination of Δ9-Tetrahydrocannabinol, 11-Hydroxy-Δ9-Tetrahydrocannabinol, and 11-nor-9-Carboxy-Δ9-Tetrahydrocannabinol in Human Urine Following Tandem Enzyme-Alkaline Hydrolysis. J Anal Toxicol, 31, 477-485.

[183] De Backer B, Debrus B, Lebrun P, Theunis L, Dubois N, Decock L, Verstraete A, Hubert P, Charlier C. 2009. Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material. J Chromatogr B, 877, 4115-4124.

[184] Roth N, Moosmann B, Auwärter V. 2013. Development and validation of an LC-MS/MS method for quantification of Δ9-tetrahydrocannabinolic acid A (THCA-A), THC, CBN and CBD in hair. J Mass Spectrom, 48, 227-233.

[185] Kneisel S, Auwärter V. 2012. Analysis of 30 synthetic cannabinoids in serum by liquid chromatography-electrospray ionization tandem mass spectrometry after liquid-liquid extraction. J Mass Spectrom 47, 825-35.

[186] Lurie IS, Meyers RP, Conver TS. 1998. Capillary electrochomatography of cannabinoids. Anal Chem, 70, 3255-3260.

[187] Bäckström B, Cole MD, Carrott MJ, Jones DC, Davidson G, Coleman K. 1997. A preliminary study of the analysis of Cannabis by supercritical fluid chromatography with atmospheric pressure chemical ionisation mass spectroscopic detection. Sci Justice, 37, 91-97.

[188] Sharma P, Bharath SMM, Murthi P. 2010. Qualitative high performance thin layer chromatography (HPTLC) analysis of cannabinoids in urine samples of Cannabis abusers. Ind J Med Res, 132, 201-208.

[189] Heyman HM, Meyer JJM. 2012, NMR-based metabolomics as a quality control tool for herbal products. S Afr J Bot, 82, 21-32.

[190] Politi M, Peschel W, Wilson N, Zloh M, Prieto JM, Heinrich M. 2008. Direct NMR analysis of cannabis water extracts and tinctures and semi-quantitative data on D9-THC and D9-THC-acid. Phytochemistry, 69, 562-570.

[191] Kemp PM, Abukhalaf IK, Manno JE, Manno BR, Alford DD, McWilliams ME, Nixon FE, Fitzgerald MJ, Reeves RR, Wood MJ. 1995. Cannabinoids in humans. II. The influence of three methods of hydrolysis on the concentration of THC and two metabolites in urine. J Anal Toxicol, 19, 292-298.

[192] Mahmoud A, ElSohly MA, Donald F. Stanford, Timothy P. Murphy. 2007. Chemical Fingerprinting of Cannabis as a Means of Source Identification. In Mahmoud A, ElSohly MA, Ed. Marijuana and the Cannabinoids. Humana Press, Totowa, 51-66.

[193] Zhang Z, Li G. 2007. A preliminary study of plant aroma profile characteristics by a combination sampling method coupled with GC–MS. Microchem J, 86, 29-36.

[194] Bakowska AM, Kucharska AZ, Oszmianski J. 2003. The effects of heating, UV irradiation and storage on stability of anthocyanin-polyphenol copigment complex. Food Chem, 81, 349-355.

[195] Ruiz JC, Ortiz Vazquez EL, Campos MR. 2015. Encapsulation of Vegetable Oils as Source of Omega-3 Fatty Acids for Enriched Functional Foods. Crit Rev Food Sci Nutr, DOI:10.1080/10408398.2014.1002906.

[196] Munin A, Edwards-Lévy F. 2011. Encapsulation of Natural Polyphenolic Compounds; a Review. Pharmaceutics, 3, 793-829.

[197] Mainardes RM, Silva LP. 2004. Drug delivery systems: past, present, and future. Curr Drug Targets, 5, 449-455.

[198] King AH. 1983. Brown Seaweed Extracts (Alginates). In M. Glicksman (Ed). Food Hydrocolloids. Boca Raton, CRC Press, 115.

[199] Krasaekoopt W, Bhandari B, Deeth H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. Int Dairy J, 13, 3-13.

[200] Xu Y, Zhan C, Fan L, Wang L, Zheng H. 2007. Preparation of dual crosslinked alginate-chitosan blend gel beads and *in vitro* controlled release in oral site-specific drug delivery system. Int J Pharmaceut, 336, 329-337.

[201] Birnbaum DT, Brannon-Peppas L. 2003. Microparticle drug delivery systems. In: Brown DM, (ed). Drug delivery systems in cancer therapy. Totowa, Humana Press, 117-136.

[202] Wang J Chua KM, Wang CH. 2004. Stabilization and encapsulation of human immunoglobulin G into biodegradable microspheres. J Colloid Interface Sci, 271, 92-101.

[203] Kissel T, Li Y, Unger F. 2002. ABA-triblock copolymers from biodegradable polyester A- blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins. Adv Drug Deliv Rev, 54, 99-134.

[204] Kiyoyama S, Shiomori K, Kawano Y, Hatate Y. 2003. Preparation of microcapsules and control of their morphology. J Microencapsulation, 20, 497-508.

[205] Sinha VR, Trehan A. 2003. Biodegradable microspheres for protein delivery. J Control Rel, 90, 261-280.

[206] Tabata Y, Gutta S, Langer R. 1993. Controlled delivery systems for proteins using polyanhydride microspheres. Pharm Res, 10, 487–496.

[207] Itoi K, Tabata CY, Ike O, Shimizu Y, Kuwabara M, Kyo M, Hyon SH, Ikada Y. 1996. *In vivo* suppressive effects of copoly(glycolic/l-lactic acid) microspheres containing CDDP on murine tumor cells. J Control Rel, 42, 175-184.

[208] Fanga Z, Bhandari B. 2011. Effect of spray drying and storage on the stability of bayberry polyphenols. Food Chem, 129, 1139-1147.

[209] Teixeira MI, Andrade LR, Farina M, Rocha-Leão MHM. 2004. Characterization of short chain fatty acid microcapsules produced by spray drying. Mater Sci Eng C, 24, 653-658.

[210] Dorati R, Genta I, Modena T, Conti B. 2013. Microencapsulation of a hydrophilic model molecule through vibration nozzle and emulsion phase inversion technologies. J Microencapsul, 30, 559-570.

[211] Whelehan M, Marison IW. 2011. Microencapsulation using vibrating technology. J Microencapsul, 28, 669-688.

[212] Benita S, Donbrow M. 1982. Effect of polyisobutylene on ethylcellulose-walled microcapsules: wall structure and thickness of salicylamide and theophylline microcapsules. J Pharm Sci, 71, 205-210.

[213] Felt O, Buri P, Gurny R. 1998. Chitosan: a unique polysaccharide for drug delivery. Drug Dev Ind Pharm, 24, 979-993.

[214] Fukushima S, Kishimoto S, Takeuchi Y, Fukushima M. 2000. Preparation and evaluation of o/w type emulsions containing antitumor prostaglandin. Adv Drug Deliv Rev, 45, 65–75.

[215] Hombreiro PM, Zinutti C, Lamprecht A, Ubrich N, Astier A, Hoffman M, Bodmeier R, Maincent P. 2000. The preparation and evaluation of poly(epsilon-caprolactone) microparticles containing both a lipophilic and a hydrophilic drug. J Control Rel, 65, 429-438.

[216] Sun-Waterhouse D, Zhou J, Miskelly GM, Wibisono R, Wadhwa SS. 2011. Stability of encapsulated olive oil in the presence of caffeic acid. Food chem, 126, 1049-1056.

[217] Chaumeil JC, Chemtob C, Ndongo M. 1986. Tablets of metronidazole microcapsules: release characterization. Int J Pharm Sci, 29, 83-92.

[218] Carrasquillo KG, Stanley AM, Aponte-Carro JC, De Jesus P, Costantino HR, Bosques CJ. 2001. Non-aqueous encapsulation of excipient-stabilized spray-freeze dried BSA into poly(lactide-co-glycolide) microspheres results in release of native protein. J Control Rel, 76, 199-208.

[219] Jiang W, Schwendeman SP. 2001. Stabilization of a model formalinized protein antigen encapsulated in poly(lactide-co-glycolide)-based microspheres. J Pharm Sci, 90, 1558-1569.

Helburuak

2. ATALA

Azken bi hamarkadetan emandako bizi-erritmoaren aldaketak guztiz aldatu ditu mendebaldar herrialdeen elikadura ohiturak, janari azkarra eta janari zaborra deiturikoak eguneroko ogi bihurtuz. Honen ondorioz, eta dietak osasunean duen eragin zuzenaren inguruko kontzientziazio zabalago bat dela tarteko, gaur egungo elikadurak dituen hutsuneak betetzeko nutrazeutiko gehigarrien beharra agerian geratu da. Kontsumitzaileek nahiago izaten dute gainera konposatu hauek iturri naturaletatik ateratakoak izatea kimikoki sintetizatuak izatea baino. Iturri natural garrantzitsuenetarikoak hondakin agroindustrialak dira. Euren artean interes berezia hartzen dute ardo produkzioko hondakinek, sortzen den mundu mailako bolumena izugarria izateaz gain, duten polifenolen eta gantz azido asegabeen kontzentrazioa benetan altua baita.

Hori kontuan hartuz, tesi honetako bi helburu nagusietako bat ardo hondakinen balioa handitzeko bertatik polifenolak eta gantz azidoak erauztea eta erauzkin hauek ondoren gehigarri bezala erabili ahal izateko prestatzea izan da. Helburu nagusi hau bete ahal izateko bi azpi-helburu zehaztu dira:

(1) *Optimization of supercritical fluid consecutive extractions of fatty acids and polyphenols from vitis vinifera grape wastes*. Gantz azido asegabeak eta polifenolak gehigarri bezala erabili ahal izateko bakoitza bere aldetik erauztea beharrezkoa da. Bestalde, erauzkinek garbiak eta seguruak izan behar dute. Azpi-helburu honek hori gauzatzea ahalbidetzen du.

(2) *Microencapsulation and storage stability of polyphenols from Vitis vinifera grape wastes.* Polifenolak oso erraz degradatzen diren konposatuak dira. Ondorioz, gehigarri bezala erabiltzean euren eraginkortasuna ziurtatzeko beharrezkoa da degradazio hau ekiditea. Hau mikrokapsularatzearen bidez lortu da.

Tesi honetako bigarren helburu nagusia, gaixotasunen tratamenduan oinarritzen da hauen prebentzioan beharrean. Nutrazeutikoekin gertatzen den gisan, garai batean izandako indarra berreskuratzen hasi dira landare medizinalak. Euren artean adibiderik esanguratsuena kannabisa da. Kannabisa, edo kasuan kasu bere konposaturen bat ala euren nahasteren bat, esklerosi anizkoitzean, glaukoman eta beste hainbat gaixotasunetan gaur egun medikuak agintzen duen droga bezain eraginkorra ala eraginkorragoa dela baieztatuta dago jada. Bestalde, hainbat minbizien aurka, epilepsiaren aurka eta beste hainbat gaixotasunen aurka ere eraginkorra izan daitekeela uste da. Oraindik bidearen zati handi bat egiteke dago ordea. Ildo horretatik, bide hori gauzatzen lagunduko duten hiru azpi-helburu zehaztu dira tesi honetan:

(3) *Identification and quantification of cannabinoids in cannabis sativa L. plants by high performance liquid chromatography-mass spectrometry*. Kannabisak ehun kannabinoidetik gora ditu, guztiak aktiboak. Landaretik landarera euren kontzentrazioak asko aldatzen dira ordea, eta ondorioz baita sortuko dituen efektuak ere. Azpi-helburu honi esker landareen kannabinoideen hatz-marka gauzatzea lortu da. Hatz-marka hau landareak sortuko duen efektu terapeutikoa aurreikusteko lagungarria izan daiteke.

(4) *Simultaneous quantification of major cannabinoids and metabolites in human urine and plasma by HPLC-MS/MS and tandem enzyme-alkaline hydrolisis*. Hainbat aplikaziotarako kannabisak

duen eraginkortasuna ikertu ahal izateko saiakera kliniko ugari gauzatuko dira datozen urteotan. Saiakera hauetan kontzentrazio/efektu erlazioak aztertu ahal izateko, kannabinoideak eta euren metabolitoak gernuan eta plasman kuantifikatzeko metodo sentikor bat garatzea izan da lan honen helburua. Horretarako, metodo analitikoa garatzeaz gain lotura glukuronidoak hausteko hidrolisi-prozesua ere optimizatu da.

(5) *Evolution of the content of cannabinoids and terpenes during the growth of cannabis plants from different chemotypes*. Saiakera klinikoetarako beharrezkoa da kontzentrazio jakineko landareak eta hauetatik erauzitako konposatu puruak kopuru handian eskuragarri izatea. Azpi-helburu honetan kannabis landare-mota ezberdinetatik kannabinoideak eta terpenoak eskuratzeko prozesua optimizatzeko konposatu hauek landareen hazkuntza-prozesuan zehar duten garapena aztertu da. Bestalde, kannabinoideen eta terpenoen sinergia-efektuak hobetu ulertu ahal izateko landare-mota bakoitzaren ezaugarri diren terpenoak ere identifikatu dira.

Optimization of supercritical fluid consecutive extractions of fatty acids and polyphenols from *Vitis vinifera* grape wastes

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CHAPTER 3

Abstract

In this study supercritical fluid extraction (SFE) has been successfully applied to a sequential fractionation of fatty acids and polyphenols from wine wastes (two different *Vitis vinifera* grapes). To this aim, in a first step just fatty acids were extracted and in a second one the polyphenols. The variables which affected to the extraction efficiency were separately optimized in both steps following an experimental design approach. The effect of extraction temperature flow, pressure and time were thoroughly evaluated for the extraction of fatty acids whereas the addition of methanol was also considered in the case of the polyphenols extraction. A quantitative extraction with high efficiency was achieved at very short time and low temperatures. Concerning quantification, fatty acids were determined by means of Gas Chromatography coupled to Mass Spectrometry (GC-MS) after a derivatization step whereas the polyphenols were analyzed by means of High Performance Liquid Chromatography coupled to tandem Mass Spectrometry (HPLC-MS/MS) and the Folin-Ciocalteu method.

Keywords: Supercritical Fluid Extraction; Fatty acids; Polyphenols; Optimization; Wine wastes.

1 Introduction

Due to the hectic pace of life where we live in, during last decades, there have been changes in food consumption habits as well as in food itself. Hence, industrially processed food has turned indispensable into our daily life. As a consequence of the loss of nutritional value of many processed foods, the addition of nutrients which compensate this loss such as antioxidants and a large variety of dietary supplements is gaining interest. Among others, polyphenols and the polyunsaturated fatty acids are included in those compounds with high nutritional interest [1].

Polyphenols has a high antioxidant capacity, since they can neutralize free radicals turning them less dangerous and thereby, stopping side reactions [2-4]. Therefore, this property makes them interesting for many different applications such as the treatment of inflammations [5] or human degenerative diseases as cancer [6-7], anti-ageing aims in cosmetics [8-9] or nutraceutical purposes [10]. Moreover, polyphenols are known to be nootropics, owing to be helpful in several brain functions, such as learning, memory, attention or motivation [11].

On the other hand, fatty acids are commonly classified in three different families: polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs) and saturated fatty acids (SAFAs). PUFAs, such as linoleic acid, have anti-inflammatory, antiarrhythmic, lipid lowering and antithrombotic effects and thus, they may play an important role in cardiovascular prevention [12-13]. The main MUFA in vegetal oils is the oleic acid; it reduces blood total triacylglycerol and cholesterol levels and it is essential for cancer prevention [14-15]. Furthermore, it presents positive effects on coronary heart disease and hypertension prevention [15]. On the contrary, in general, SAFAs increase serum cholesterol levels [16]. Among them, lauric and myristic acids have the strongest effects and stearic acid the weakest [16].

Agro-industrial wastes have gained the interest of several industrial areas since many of them may become an interesting source of by-products of high added-value, including antioxidants and fatty acids. In this sense, wine production is one of the most outstanding industrial activity, due to the high concentration of polyphenols and PUFAs present in wine products and the high volume of wastes produced yearly (according to the International Organization of Vine and Wine the worldwide production of wine requires 7.575.000 hectares). Currently, however, only a small percentage of the wine wastes are reused in Europe. The production of biofuel and bioenergy, and the preparation of manure or feed for animals are the most used alternatives to avoid costs overrun of waste management. Owing to these drawbacks, grape pomace may become a promising and economically profitable source of polyphenols and PUFAs. However, their quantitative extraction with suitable analytical techniques is still a challenge [17].

Carbon dioxide based supercritical fluid extraction (SFE) offers remarkable advantages over traditional methods in the extraction of bioactive compounds and in food processing [18-20]. On the one side, CO_2 is an inert, non-toxic, environmentally safe solvent and allows extraction at low temperatures and pressures, avoiding thereby the degradation of thermosensitive compounds. On the other side, these

high purity extracts are recognized as safe to be used in food products. In addition, SFE technique is scalable up to industrial scale. Moreover, in spite of the fact that supercritical CO_2 is not a polar solvent, the addition of a cosolvent such as ethanol or methanol improves the extraction of polar compounds and may allow the fractionation of the extracts that contain compounds of different polarities [21-23].

Taking into account the rise in value of agroindustrial wastes, the aim of this study is a complete optimization of SFE procedure in order to obtain two different extracts: one rich in fatty acids and the other one rich in polyphenols, using the same grape pomace. The enhanced value of performing a full optimization of each step is getting extracts with high concentration of bioactive compounds with the least reagent cost and time wasting. The polarity of the solvent mixture used during the SFE is the key parameter to separate these two groups of compounds. In the case of the fatty acids the extraction based on pure CO_2 is foreseen, and in the case of polyphenols, the addition of methanol is required.

2 Materials and methods

2.1 Chemicals and materials

A standard solution mixture containing 19 fatty acid methyl esters (Grain FAME mix 10 mg/mL in dichlorometane; methyl arachidate, methyl behenate, methyl caprylate, methyl decanoate, methyl cis-11-eicosenoate, methyl elaidate, methyl erucate, methyl heptadecanoate, methyl laurate, methyl linoleate, methyl linolenate, methyl myristate, methyl myristoleate, methyl oleate, methyl palmitate, methyl palmitoleate, methyl pentadecanoate, methyl stearate and methyl tridecanoate), sodium hydroxide (98 %), Folin-Ciocalteu reagent, gallic acid (97.5-102.5 %), catechin (≥ 96 %), epicatechin (≥ 90 %) and coumaric acid (≥ 98 %) were purchased from Sigma-Aldrich (Steinheim, Germany) and propyl gallate from Supelco (Bellefonte, Pennsylvania, USA).

HPLC quality methanol (MeOH) used as co-solvent in SFE extraction and mobile phase in HPLC-MS/MS analysis and isopropanol used for collecting SFE extracts, ethanol (EtOH, 96 %) used for Folin-Ciocalteu analysis and sodium chloride (99 %) were obtained from Panreac (Barcelona, Spain). HPLC quality isooctane used in fatty acids derivatization was purchased from Lab-Scan (Gliwice, Poland); sodium carbonate (99.5 %) and sulfuric acid (97 %) were obtained from Merck (Madrid, Spain); and formic acid (LC/MS quality) was purchased from Thermo Fisher Scientific (Erembodegem, Belgium).

Ultra-pure water (< 0.057 S/cm) was generated with a Milli-Q water purification system Element 10 from Millipore (Billerica, Massachusetts, USA). The CO_2 (99.5 %) used as mobile phase in SFE system was obtained from Air Liquide (Madrid, Spain) and washed thin sea sand from Scharlab (Barcelona, Spain).

2.2 Sample preparation

Wine wastes were selected from two different Basque designations of origin (D.O.): D.O. Rioja and D.O. Txakoli. Regarding the first one, the wastes were collected from Samaniego (Araba, *Ostatu* winery and *Tempranillo* red grapes) and the second wastes were collected from Getaria (Getaria, *Mokoroaga* winery and *Hondarribi zuri* white grapes), both in the Basque Country (North of Spain).

First of all, the wine wastes were air dried for two days. A representative fraction of seeds per each type of wine wastes was separated and used for the optimization of the extraction of fatty acids. The optimization of the extraction of polyphenols was performed using a mixture of seeds and skins. These fractions were further analyzed under optimized conditions. Separated sample fractions were ground in a cryogenic mill (SPEX SamplePrep 6770 Freezer/Mill, Stanmore, UK) under liquid nitrogen for 10 minutes at 11 cps, and stored in the refrigerator at 4 ℃ until extraction.

2.3 Supercritical fluid extraction

SFE was performed on a Thar SFC, (Waters company, Saint-Quentin, France) Method Station SFC system, consisting of a Fluid Delivery Module (CO_2 pump and solvent pump), an Automated Back Pressure Regulator (ABPR) and a high pressure extraction vessel of 1 mL (EV-1 Jasco).

Under optimized conditions, approximately 0.25 g of sample and 0.75 g of sea sand were accurately placed in a "sandwich" way (sand-sample-sand) into an extraction vessel with frits placed at the ends to maintain the sample and prevent particulate matter entering the fluid transfer lines. Samples were extracted in dynamic mode with supercritical CO_2 for 5 min. Supercritical fluid pressure was maintained at 250 bar at a constant flow of 2 mL/min for the extraction of fatty acids and the extraction temperature was fixed at 35 °C. Regarding the second fraction, where polyphenol compounds were obtained, the supercritical fluid pressure was set at 103 bar, at a constant flow of 2 mL/min and at 35 °C, and in this case, the use of 40 % of MeOH as cosolvent was required. The use of MeOH is justified because the supercritical conditions of CO_2-MeOH are milder than those obtained with CO_2-EtOH, which ease to test the performance of the extraction and then, it can be modified if necessary for industrial applications. Finally, the extracts were collected in glass vials containing 4 mL of isopropanol as solvent for fatty acids and 3 mL of MeOH for polyphenols. The sample extracts were kept at -20 °C in complete darkness until the analysis.

2.4 Derivatization of fatty acids and analysis by gas chromatography mass spectrometry

All the extracts were evaporated by a gentle stream of N_2 (Caliper Life Sciences TurboVap® LV) and the fatty acids were transformed to methyl ester (FAME) derivatives in order to be determined by gas chromatography mass spectrometry (GC-MS). For this purpose, a derivatization process described by Dayhuff and Wells [24] was carried out with minor modifications. Briefly, 1.5 mL of sodium hydroxide (0.5 M) in MeOH were added to evaporated fatty acid extracts and maintained in an oven at 100 °C for 5 minutes. Afterwards, 2 mL of 15 % sulfuric acid in MeOH were added and maintained at 100 °C for one hour. Subsequently, 5 mL of saturated sodium chloride solution and 1 mL of isooctane

were added. The two phases were thoroughly mixed and the organic phase was collected. Finally, 1 mL of isooctane was added to ensure a quantitative recovery of the compounds and the organic phase was collected again. The two organic fractions were mixed and diluted for quantification.

GC-MS analysis was performed in a 7890A gas chromatograph coupled to an Agilent 5975C inert triple-axis mass detector and an Agilent autosampler 7693 (Agilent Technologies, Avondale, PA, USA). The analysis was performed in the splitless mode for 35 min at 275 °C. A capillary column DB-Wax (30 m × 0.25 mm, 0.25 µm, Agilent Technologies, Avondale, PA, USA) was used for the separation of FAMEs. The temperature program used was as follows: started at 80 to 250 °C (at 8 °C/min) where it was held for 13.75 min. Helium (99.999 %, Air Liquide) was used as carrier gas at a constant pressure of 9.38 psi. The transfer line temperature was maintained at 300 °C, and the ion source and quadrupole at 230 and 150 °C, respectively. The measurements were performed both in SCAN (30–550 amu) and selected ion monitoring (SIM) modes. Peak identification of the FAMEs was carried out by the comparison with retention times and mass spectra of Grain FAME mix standards. In real samples only four FAMEs were identified among all standards; methyl palmitate (t_R: 14.77 min), methyl stearate (t_R: 17.02 min), methyl oleate (t_R: 17.25 min) and methyl linoleate (t_R: 17.76 min).

2.5 Folin-Ciocalteu method for total phenolic content

The total amount of phenolic compounds was determined by using the Folin–Ciocalteu colorimetric method according to Singleton and Rossi [25]. Briefly, all the extracts were evaporated by a gentle stream of N_2, dissolved in 1 mL of EtOH and diluted 10 times. 1 mL of diluted samples was mixed with 5 mL 1:10 (v/v) of diluted Folin-Ciocalteu reagent and 4 mL of sodium carbonate (0.7 M). Absorbance was measured at 765 nm in a spectrophotometer Multispec-1501 Shimadtzu UV/VIS after 2 h of incubation in darkness at room temperature. Total phenolics were expressed as mg of GAE (gallic acid equivalents)/g by comparison to the gallic acid standard calibration curve.

2.6 Liquid Chromatography tandem mass spectrometry method for polyphenol analysis

Liquid chromatographic system was an Agilent Technologies (Santa Clara, USA) Infinity Liquid Chromatograph 1260 consisted of an autosampler, a column thermostat and a binary solvent management system coupled to a triple quadrupole mass spectrometer equipped with an electrospray ionization ion source (ESI) (Agilent Technologies 6430).

The chromatographic separation was achieved using a Kinetex C_{18} column (2.6 µm, 150 mm x 3 mm i.d.) with a guard column (0.5 µm Depth Filter x 0.1 mm) both purchased from Phenomenex (Aschaffenburg, Germany) and a binary A/B gradient (Solvent A was water with 0.5 % formic acid and solvent B was methanol with 0.5 % formic acid). The gradient program was as follows: 0 min: 20 % B; 0-4 min: 20-50 % B, 4-10 min: 50-100 % B, 10-14 min: 100 % B, 14-16 min: 95-20 % B, 16-33 min: 20 % B. A flow rate of 0.20 mL/min was used, the column temperature was maintained at 35 °C and the injection volume was 10 µL.

MS acquisition was carried out in the ESI positive and negative ionization mode using the following conditions: capillary voltage of 4000 V, a nebulizer gas to spray the sample of 15 psi and heated nitrogen flow of 6 L/min at 300 °C. High purity nitrogen gas (99.999 %, Air Liquide) was used as nebulizer, drying and collision gas. Under these conditions, the quantitative analysis of the target compounds was performed in multiple reaction monitoring (MRM) mode. System variations were corrected with an internal standard (propyl gallate). The method included quantitation and confirmation transitions for each analyte and for each transition, the fragmentor potential and the collision energy were optimized. Table 1 summarizes the LC-MS/MS parameters used for the four main target analytes (available standards) including quantitation and confirmation transitions, optimized energies and retention times.

Table 1
MRM transitions, optimized potentials and applied voltage polarity and retention times of the analytes and the internal standard for HPLC-MS/MS analysis. Mass/charge ratio of the precursor ion (Q1), mass/charge ratio of the fragment ion (Q2), fragmentation potential (FP), collision energy (CE), retention time (t_R).

Analyte	Q1(amu)	Q2(amu)	FP (V)	CE(eV)	t_R(min)	Polarity
Gallic acid	169	125	100	12	1.8	Negative
		79	100	21		
Catechin	291	139	100	10	3.3	Positive
		123	100	5		
Epicatechin	291	139	100	10	5.4	Positive
		123	100	20		
Coumaric acid	163	119	100	10	8.8	Negative
		93	100	35		
Propyl gallate	213	153	60	10	10.1	Positive
		127	60	10		

2.7 Fatty acids and polyphenols SFE optimization by response surface methodology

To extract fatty acid compounds, the effect of temperature (°C), CO_2 flow (mL/min), pressure (bar) and the extraction time by supercritical fluid was assessed by means of a Central Composite Design (CCD). The design matrix consisted of 27 randomized assays allowing the study of the effects of the variables within the ranges described in Table 2. Those ranges were established taking into account the equipment restrictions and the limits of CO_2 to be a supercritical fluid. Since fatty acids are essentially non-polar compounds the extraction was carried out with pure CO_2 (i.e., the addition of modifier was not considered). All the experiments were performed with a representative mixture of the two types of seeds.

Table 2
Studied variable ranges in FAMEs and polyphenol extraction optimizations and optimum values.

Variable	Studied range in FAMEs	Optimum values	Studied range in polyphenols	Optimum values
Temperature (°C)	35-55	35	35-55	35
Flow (mL/min)	1-2	2	1-2	2
Pressure (bar)	103-253	253	103-253	103
Extraction time (min)	5-79	5	-	-
Methanol as a modifier (%)	-	-	0-40	40

All the extracts were analyzed by means of GC-MS after the derivatization step and the responses were defined as the chromatographic peak area of the main four FAMEs (methyl palmitate,

methyl linoleate, methyl oleate and methyl stereate). The optimum conditions were established by the analysis of the response surfaces, which were build by multiple linear regression (MLR) analysis including linear and squared terms and the interactions of any two variables. The precision was estimated from the 3 replicates of the central point and the data was treated with the statistics software The Unscrambler® (9.7 Camo Asa, Oslo, Norway). Model fitness was assured by the analysis of variance (ANOVA) of the residuals (lack of fit significantly lower than pure experimental uncertainty) and the uncertainties of each parameter.

The same strategy was performed in order to find the optimum extraction conditions of polyphenol compounds by means of SFE. In this case, the studied variables were extraction temperature, pressure and CO_2 flow; and due to the chemical properties of the target compounds, the addition of a polar cosolvent (MeOH) was also considered. All these parameters were simultaneously studied by means of a CCD in the ranges summarized in Table 2. The extractions were accomplished using a mixture of both ground seeds and skins of grapes. The extraction time was initially fixed at 10 min and once the optimum conditions were attained this variable was fine-tuned (5, 10, 15 and 20 min). CCD responses were obtained by means of Folin-Ciocalteu method and the results were defined as total phenolic content (mg GAE/g).

3 Results and discussion

3.1 Fatty acid SFE optimization

MLR data obtained with the experimental design assays revealed that the significant variables affecting the extraction yield of fatty acids were the pressure and the flow (p-level < 0.10, no factors were significant with a higher confidence level, i.e. a p-level < 0.05, probably due to the high deviations introduced by the derivatization process). As it is illustrated for linoleic acid in Figure 1, the response surface was flat and a local maximum was found at the highest flow and pressure values within the factor space. The same result was observed for the rest of the analytes for all the evaluated variables. Hence, high pressure and flow values were established as optimum conditions whereas temperature and extraction time were set at the minimum values because they were not significant and the method throughput was guaranteed with short extraction times. In this way, the fatty acids optimal extraction conditions were established as follows: 35 °C of extraction temperature, 2 mL/min of CO_2 flow, 253 bar of pressure and 5 minutes of extraction (Table 2).

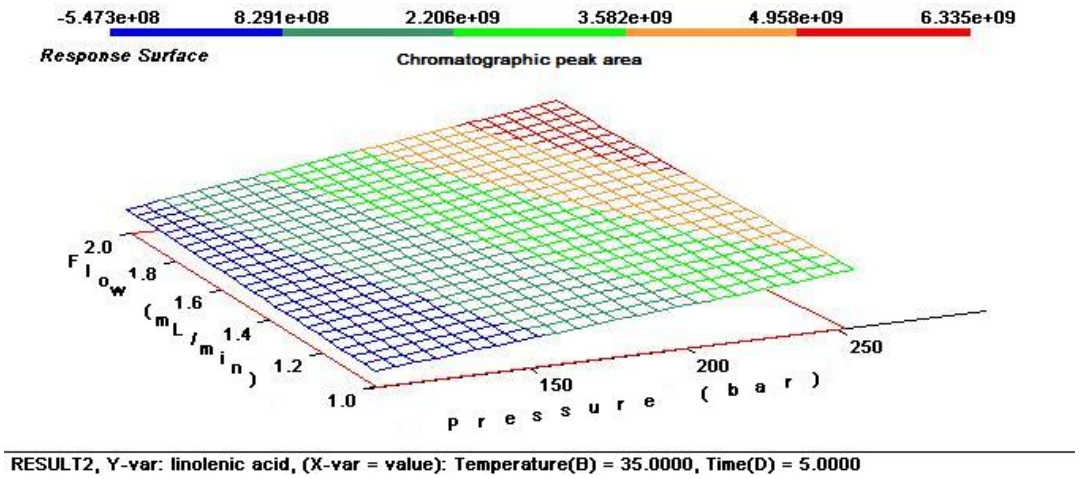

Figure 1: Response surface of the pressure (bar) and flow (mL/min) factors for the extraction of linoleic acid.

3.2 Polyphenol SFE optimization

Regarding the second fraction, in which more polar compounds must be recovered, besides the parameters studied for the first extraction step the effect of the addition of MeOH as co-solvent was evaluated. In this case, MLR results showed that besides the flow of CO_2 used during the extraction the percentage of meOH was also significant parameter at 95 % of confidence level (p-level < 0.05) to obtain the best extraction yields. Figure 2 illustrates the response surface in which both significant parameters are varied; a local maximum was found when both the CO_2 flow and the MeOH % were fixed at the highest values. As it occurred for the isolation of fatty acids extraction temperature and pressure were not significant parameters. Consequently, optimal extraction conditions for polyphenols were established as follows: low extraction temperature (35 °C), high flow (2 mL/min), low pressure (103 bar) and high presence of co-solvent (40 % of MeOH) (Table 2).

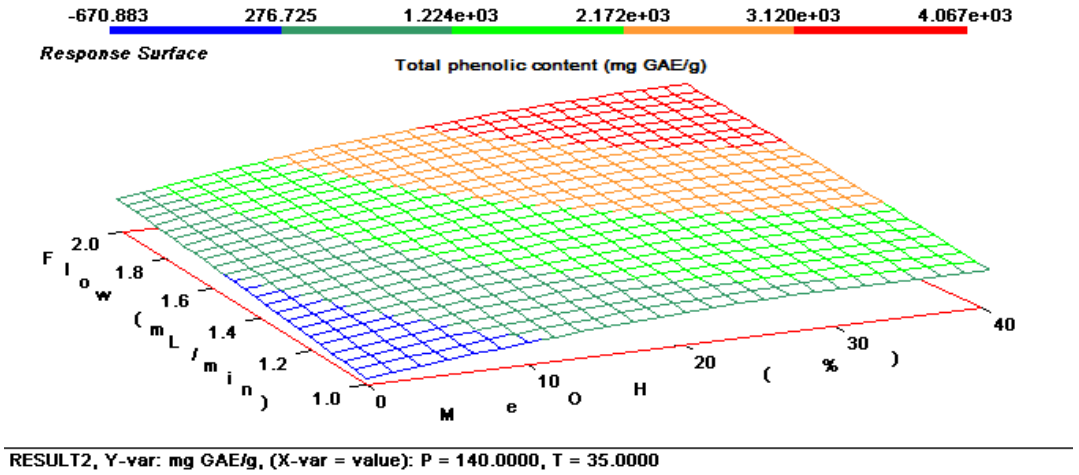

Figure 2: Response surface of flow (mL/min) and percentage of methanol (%) as modifier factors for the extraction of total content of polyphenols.

Regarding the extraction time, it was fine-tuned between 5–20 minutes once the other parameters affecting the extraction procedure were fixed. According to the results, it was concluded that the

extraction time was non-significant (p-level > 0.10) in the studied range and, therefore, the minimum extraction time (5 min) was chosen. In fact, three successive extractions of 5 min were carried out in order to check the effectiveness of the extracted polyphenols and the results were as follows: 88.2 ± 0.9 %, 7 ± 1 % and 4.4 ± 0.4 %. According to these results, extraction time was established in 5 minutes, since it was considered that it is not worth the expense of reagent that would be realized in case of doing longer extractions in order to improve a small per cent.

3.3 Consecutive extraction of fatty acids and polyphenols using SFE

Once the SFE variables were optimized for the extraction of fatty acids and polyphenols, consecutive extraction from the same sample were carried out in order to isolate fatty acids and polyphenols, and hence, estimate the efficiency of the fractionation. For this purpose, each fraction was independently collected (i.e., first step: isolation of fatty acids and second step: isolation of polyphenols) at previously established conditions and further analyzed. In addition, in order to check the percentage of fatty acids remaining in the second fraction (i.e., fatty acids co-eluting with polyphenols) and to assess if there is any loss during consecutive extraction, samples were extracted directly using the experimental conditions set up for each fraction. In this case, the analysis of fatty acid and polyphenols were performed by GC-MS and HPLC-MS/MS respectively.

As it is illustrated in Figure 3, only traces of the fatty acids were extracted in the polyphenol fraction and no polyphenols were observed in the fatty acid fraction so the differentiation of both fractions can be easily conducted in the same extraction run at the established conditions. Moreover, the extraction yields carried out sequentially or directly did not show significant differences (p-level > 0.05) so no losses occur during the consecutive extraction.

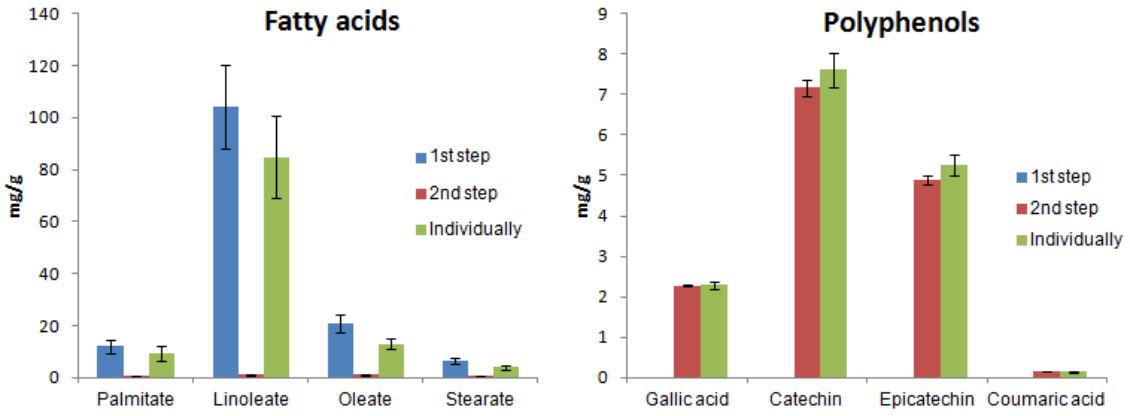

Figure 3: Fatty acid and polyphenol content in the 1st fraction (fatty acid step), the 2nd fraction (polyphenol step) and the individual extractions (extractions performed directly) with the corresponding standard deviations (n=3).

3.4 Figures of merit

The reproducibility of the optimized SFE extraction and analysis was checked performing the optimized fractionate extractions of fatty acids and polyphenols in different days. A mixture of both grounded seeds was used as sample. As it can be observed in Table 3, there were no statistical differences

between results obtained in different days (p-level > 0.05). The repeatability of the analytes in terms of relative standard deviations (RSD %) was calculated carrying out 5 extractions from the same samplein the same day. The RSD values were in the 5 and 20 % range for fatty acids and 3 and 10 % range for polyphenols.

Table 3
Fatty acid and polyphenol content of consecutive extractions (n=3) in different days.

Extraction	Fatty acid (mg/g)					Polyphenols (mg/g)			
	Yield (%)	Palmitic	Linoleic	Oleic	Stearic	Gallic acid	Catechin	Epicatequin	Coumaric acid
1st day	14±2	12±2	100±20	21±3	7±1	2.28±0.01	7.2±0.2	4.9±0.1	0.15±0.01
2nd day	15±2	13±2	110±10	23±2	7±1	2.1±0.2	7.9±0.6	4.5±0.3	0.13±0.02

Regarding the quantification, good linearity was found out over the wide range of the tested concentrations (1 and 200 µg/mL for fatty acids and 20 and 500 ng/mL for polyphenols) being the coefficients of determination higher than 0.998 for FAMEs and polyphenols. The instrumental limits of detection (LODs) were set at the lowest concentration where the signal-to-noise ratio of the analytes were > 3; the LODs were between 0.1 and 0.5 µg/mL for fatty acids and between 1 and 10 ng/mL for polyphenols.

3.5 Application to real samples

The optimized extraction method was applied to two different samples to observe its potential for recovering bioactive compounds from wine wastes. In this sense, red wine and white wine ground wastes were extracted consecutively with the optimized conditions in order to isolate fatty acids and polyphenols.

Table 4 summarizes the concentration of fatty acids and polyphenolds found in the analyzed white and red wines. On the one hand, it can be observed that in both varieties of grapes the proportion between the four fatty acids was the same, being the most concentrated the linoleic acid and then the oleic acid. This is a very promising result for the revalorization of wine industry wastes because the linoleic acid and the oleic acid are highly valued MUFA and PUFA respectively. Moreover, being the proportion between fatty acids the same in different varieties may be helpful for an industrial revalorization of different wastes all together. Furthermore, the obtained yields (6 % oil yield in red wine wastes and 2 % in white wine wastes) were high in comparison with previous works, where yields were from 6 % to 15 % (in this case in a seed mixture the yield was 14 %) by means of SFE and from grape seeds fatty acids [26-28].

On the other hand, it can be observed that white wine wastes have less fatty acids but more polyphenols than red wine wastes (see Table 4). However, literature reviews that red wine grapes have more polyphenols than white wine grapes [29-30]. This may be due to *Ostatu winery* red wine grape wastes are below the average in polyphenol content and on the contrary, *Mokoroaga Txakoli winery* above the average in comparison with other authors.

Table 4
Fatty acid and polyphenol content of samples (n=5)

Extraction		Fatty acid (mg/g)				Polyphenols (mg/g)			
	Yield (%)	Palmitic	Linoleic	Oleic	Stearic	Gallic acid	Catechin	Epicatequin	Coumaric acid
Red wine wastes	5.9±0.4	5.6±0.5	42±2	9±1	2.9±0.5	2.1±0.1	3.6±0.5	3.1±0.1	0.23±0.01
White wine wastes	1.7±0.1	1.5±0.1	12.6±0.7	2.3±0.1	0.4±0.1	3.4±0.2	56±2	100±4	0.046±0.003

4 Conclusions

In order to provide a quantitative method to isolate bioactive compounds simultaneously from grape wastes, a SFE method has been fully optimized. The developed method allows the consecutive extraction of fatty acids and polyphenols from the same wine wastes with very good yields, at very short time (i.e., 10 min taking into account all the procedure) and at low temperatures (i.e., 35 °C) comparing with literature, where extraction time higher than 100 minutes was required [22]. The proposed method is a promising alternative for a full revalorization of wine wastes before giving them any other application. The optimized method was successfully applied to two different wine wastes obtaining very good yields and the results obtained from grape residues were particularly promising.

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5 References

[1] Shahidi F. 2012. Nutraceuticals, functional foods and dietary supplements in health and disease. J Food Drug Anal, 20, 226-230.

[2] Dugas AJ, Castañeda-Acosta J, Bonin GC, Price KL, Fisher NH, Winston GW. 2000. Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: structure-activity relationships. J Nat Prod, 63, 327-331.

[3] Tikhonov I, Roginsky V, Pliss E. 2009. The chain-breaking antioxidant activity of phenolic compounds with different numbers of O-H groups as determined during the oxidation of styrene. Int J Chem Kinet, 41, 92-100.

[4] Leopoldini M, Russo N, Toscano M. 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. Food Chem, 125, 288-306.

[5] Kim J, Lee KW, Lee HJ. 2014. Polyphenols suppress and modulate inflammation: Possible Roles in Health and Disease. In Watson RR, Preedy VR, Zibadi S, (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 393-408.

[6] Hertog MGL. 1994. Flavonols and Flavones in Foods and Their Relation with Cancer and Coronary Heart Disease Risk. Ph.D. Thesis, The Netherlands: Agricultural University Wageningen.

[7] Cordova FM, Watson RR 2014. Food and supplement polyphenol action in cancer recurrence. In Watson, RR, Preedy VR, Zibadi S (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 191-195.

[8] Jean-Gilles D, Li I, Vaidyanathan VG, King R, Cho B, Worthen DR, Chichester CO, Seeram NP. 2013. Inhibitory effects of polyphenol punicalagin on type-II collagen degradation in vitro and inflammation in vivo. Chem-Biol Interact, 205, 90-99.

[9] Menaa F, Menaa A. 2014. Skin photoprotection by polyphenols in animal models and humans. In Watson RR, Preedy VR, Zibadi S (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 831-838.

[10] Gollücke AP, Peres RC, Odair A Jr, Ribeiro DA. 2013. Polyphenols: a nutraceutical approach against diseases. Rec Pat Food, Nutr Agric, 5, 214-219.

[11] Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, Lamari FN. 2009. Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. Behav Brain Res, 198, 352–358.

[12] Fedačko J, Pella D, Mechírová V, Horvath P, Rybár R, Varjassyová P, Vargová V. 2007. N-3 PUFAs From dietary supplements to medicines. Pathophysiology, 14, 127-132.

[13] Tousoulis D, Plastiras A, Siasos G, Oikonomou E, Vergeniotis A, Kokkou, E, Maniatis K, Gouliopoulos N, Miliou A, Paraskevopoulos T, Stefanadis C. 2014. Omega-3 PUFAs improved endothelial function and arterial stiffness with a parallel antiinflammatory effect in adults with metabolic syndrome. Atherosclerosis, 232, 10-16.

[14] Stein O, Dabach Y, Ben-Naim M, Halperin G, Stein Y. 2008. Effects of oleic acid and macrophage recruitment on cholesterol efflux in cell culture and in vivo. Nutrition Metab and Cardiovas, 18, 596-601.

[15] Gnoni GV, Natali F, Geelen MJH, Siculella L. 2010. Oleic acid as an inhibitor of fatty acid and cholesterol synthesis. In Preedy VR, Watson RR (Eds.) Olives and olive oil in health and disease prevention. Academic Press, Massachusetts, 1365-1373.

[16] Mensink RP. 2013.Fatty Acids: health effects of saturated fatty acids. In Allen LH, Prentice A, Caballero B (Eds.) Encyclopedia of Human Nutrition. Academic Press, Massachusetts, 215-219.

[17] Arvanitoyannis IS, Varzakas TH. 2007. Fruit/Fruit Juice Waste Management: Treatment Methods and Potential Uses of Treated Waste. In Arvanitoyannis IS, (Ed.) Waste Management for the Food Industries. Academic Press, Massachusetts, 569-628.

[18] Raventós M, Duarte S, Alarcón R. 2002. Application and Possibilities of Supercritical CO2 Extraction in Food Processing Industry: An Overview. Food Sci Tech Int, 8, 269-284.

[19] Sahena F, Zaidul ISM, Jinap S, Karim AA, Abbas KA, Norulaini NAN, Omar AKM. 2009. Application of supercritical CO2 in lipid extraction – A review. J Food Eng, 95, 240-253.

[20] Mendiola JA, Herrero M, Castro-Puyana M, Ibañez E. 2013. Supercritical Fluid Extraction. In Rostagno MA, Prado JM (Eds.) Natural Products Extraction. Royal Society of Chemistry, London, 196-230.

[21] Chaudot X, Tambuté A, Caude, M. 2000. Selective extraction of hydrocarbons, phosphonates and phosphonic acids from soils by successive supercritical fluid and pressurized liquid extractions. J Chromatogr A, 866, 231–240.

[22] Ashraf-Khorassani M, Taylor LT. 2004. Sequential fractionation of grape seeds into oils, polyphenols, and procyanidins via a single system employing CO2-based fluids. J Agr Food Chem, 52, 2440-2444.

[23] Omar J, Alonso I, Garaikoetxea A, Etxebarria N. 2013. Optimization of focused ultrasound extraction (FUSE) and supercritical fluid extraction (SFE) of citrus peel volatile oils and antioxidants. Food Anal Method, 6, 1244-1252.

[24] Dayhuff L, Wells MJM. 2005. Identification of fatty acids in fishes collected from the Ohio River using gas chromatography–mass spectrometry in chemical ionization and electron impact modes. J Chromatogr A, 1098, 144–149.

[25] Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phospho-molybdic–phosphotungstic acid reagents. Am J Enol Viticult, 16, 144–158.

[26] Gómez AM, López CP, Martínez de la Ossa E. 1996. Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: a comparison with conventional solvent extraction. Chem Eng J, 61, 227-231.

[27] Cao X, Ito Y. 2003. Supercritical fluid extraction of grape seed oil and subsequent separation of free fatty acids by high-speed counter-current chromatography. J Chromatogr A, 1021, 117–124.

[28] Agostini F, Bertussi RA, Agostini G, Atti dos Santos AC, Rossato M, Vanderlinde R. 2012. Supercritical Extraction from Vinification Residues: Fatty Acids, α-Tocopherol, and Phenolic Compounds in the Oil Seeds from Different Varieties of Grape. ScientificWorldJournal, Article ID 790486.

[29] Alonso Borbalán AM, Zorro L, Guillén DA, Barroso DG. 2003. Study of the polyphenol content of red and white grape varieties by liquid chromatography-mass spectrometry and its relationship to antioxidant power. J Chromatogr A, 1012, 31-38.

[30] Makris DP, Boskou G, Andrikopoulos NK. 2007. Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. J Food Compos Anal, 20, 125-132.

Microencapsulation and storage stability of polyphenols from *Vitis vinifera* grape wastes

Food Chemistry, 190, 614-621 (2016)

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CHAPTER 4

Abstract

Wine production wastes are an interesting source of natural polyphenols. In this work, wine wastes extracts were encapsulated through vibration nozzle microencapsulation using sodium alginate as polymer and calcium chloride as hardening reagent. An experimental design approach was used to obtain calcium-alginate microbeads with high polyphenol content and good morphological features. In this way, the effect of pressure, frequency, voltage and the distance to the gelling bath were optimized for two nozzles of 150 and 300 μm. Long-term stability of the microbeads was studied for 6 months taking into account different storage conditions: temperatures (4 °C and room temperature), in darkness and in presence of light, and the addition of chitosan to the gelling bath. Encapsulated polyphenols were found to be much more stable compared to free polyphenols regardless the encapsulation procedure and storage conditions. Moreover, slightly lower degradation rates were obtained when chitosan was added to the gelling bath.

Keywords: wine wastes; vibration nozzle methodology; (+)catechin; (-)epicatechin; long-term stability.

1 Introduction

The growing rate of industrial food consumption in western societies comes along with the deep changes in nutritional habits and food culture [1]. Surely as a consequence of these changes, the interest of functional and enriched food is growing, either to enhance the health effects or to replace the losses of key compounds during the manufacturing processes [2].

Polyphenols are among these supplements [3] since they can neutralize free radicals turning them in less dangerous and thereby, stopping side reactions [4-6]. This feature makes polyphenols an interesting family of compounds with broad applications such as the treatment of cancer [7-8] or inflammations [9], anti-aging aims in cosmetics [10-11] or nutraceutical purposes [12]. Moreover, polyphenols are known to be nootropics, owing to be helpful in several functions of the brain, such as learning, memory, attention or motivation, thereby protecting the brain against neurodegenerative diseases [13-15].

Wine production wastes are an interesting source of many by-products of high added-value, including polyphenols. In fact, the interest of wine wastes comes not only from the high polyphenols concentrations [16-17] but also from the high waste volumes which are yearly produced [18] and only a small percentage of this it is revalorized in Europe. The production of biofuel and bioenergy, or the preparation of manure or feed for animals are the most used alternatives to avoid costs overrun of waste management. Owing to these drawbacks, grape pomace may become a promising and economically profitable source of polyphenols.

Unfortunately, polyphenols have a poor long-term stability, as they are affected by pH variation, presence of metal ions, light, temperature, oxygen, and enzymatic activities [19]. Moreover, due to low water solubility, they often present a poor bioavailability [20] and they are unstable in alkaline conditions encountered in biological fluids [21]. The emergent microencapsulation techniques allow, in this sense, to improve the stability and bioavailability of the products and control the rate of active agent release [20-22]. In this way, the shelf-life of encapsulated compounds can be considerably extended [23-24] thanks to the coating material which protects the bioactive compounds against degradation reactions and controls release of the compounds.

Among the different microencapsulation techniques, vibration nozzle microencapsulation (VNM) technology is a simple, able to scale-up industrially and suitable technique for the encapsulation of hydrophilic drugs [25]. When this microencapsulation technique is used for alimentary or medical purposes, alginate is often used as wall material for bead formation [25-26]. This encapsulation agent is obtained from brown seaweed and it has free carboxylic groups which react with calcium cations forming stable gels [27]. Moreover, the addition of stabilizing agents such as chitosan could improve the stability of the formed microcapsules [28]. Chitosan is obtained from crab exoesquelets and it has many –OH and $–NH_2$ groups that act as electron donors. Among several encapsulation agents alginate and chitosan are very promising due to their absent of toxicity and their excellent biocompatibility, as they can react together by coacervation because of their opposite charges [29-30].

Taking into account the number of variables that might affect the formation of uniform spherical smooth microbeads of calcium-alginate and calcium-alginate-chitosan, the main aim of this work was to optimize the VNM process through an experimental design approach. In this sense, different nozzles and microbead drying methods were assessed to obtain microcapsules of different sizes. Furthermore, in order to assure the mid-term stability of the encapsulated microbeads, the stability of microcapsules formed under optimized conditions was evaluated for 6 months at different conditions of temperature and light. The effect of chitosan addition and microbead size on long-term stability of encapsulated polyphenols was also studied.

2 Materials and methods

2.1 Chemicals and materials

Encapsulating agents used, low molecular weight sodium alginate from brown algae (viscosity of 1 % solution at 25 °C, 4-12 cps) and medium molecular weight chitosan (deacetylation degree 75-85 %, viscosity of 1 % solution with 1 % acetic acid, 200-800 cps), as well as the standards (+)catechin (≥ 96 %) and (-)epicatechin (≥ 90 %) were obtained from Sigma-Aldrich (Steinheim, Germany). Analysis grade calcium chloride 2-hydrate used for creating calcium alginate beads and ethanol (96 %) used to extract polyphenols were purchased from Panreac (Barcelona, Spain).

HPLC grade methanol (MeOH) and analysis grade glacial acetic acid used as mobile phase in chromatographic analysis and tri-sodium citrate used for microbead destabilization were obtained from Merck (Billerica, USA). HPLC grade ethanol (EtOH) used for the extract preparation was purchased from Romil (Cambridge, UK). Ultra-pure water (5 Ω at 25 °C) was generated with a Milli-Q water purification system Element 10 from Millipore (Billerica, USA).

2.2. Preparation of wine wastes extract

Wine wastes were selected from two most important Basque designations of origin: D.O. Rioja and D.O. Txakoli. Regarding the first one, the wastes were collected from Samaniego, Araba (Ostatu winery and Tempranillo red grapes) and the second ones were collected from Getaria, Gipuzkoa (Mokoroaga winery and Hondarribi zuri white grapes), both in the Basque Country (North of Spain).

The wine wastes were air dried at room temperature for two days and then mixed to obtain a representative waste sample of the wine production in the Basque Country. Air dried wastes were ground in a cryogenic mill (SPEX SamplePrep 6770 Freezer/Mill) under liquid nitrogen for 10 minutes at 11 cps. Grounded wastes were mixed with 1:1 ethanol/water (v/v) solution and sonicated in an ultrasonic bath for 1 hour. Afterwards, ethanol was evaporated in a rotary evaporator and the remaining water extract was stored in the refrigerator until analysis and encapsulation.

2.3 Optimization of microbeads formation

Microbeads were obtained by a vibrating nozzle method using a Buchi B-390 encapsulator (Flawil, Switzerland). An equipment scheme is shown in Figure 1. Alginate was used as encapsulation agent (1.6 % w/v for 150 µm nozzle and 1.8 % w/v for 300 µm nozzle) which were prepared in an ultrasonic bath dissolving the low viscosity sodium alginate in the wine wastes extract. 100 mL of hardening solution was used, which consisted of wine wastes extract in the same concentration as in the alginate solution and of calcium chloride at 0.2 M. For complete hardening, microcapsules were allowed to stir in the hardening solution for 10 min.

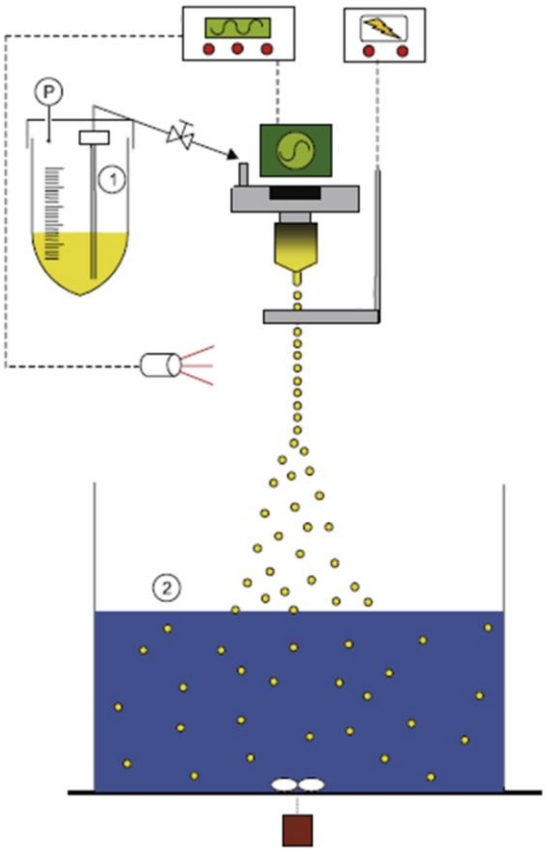

Figure 1: Buchi B-390 encapsulator equipment scheme. (1) Sodium alginate solution along with the extract. (2) Hardening solution: calcium chloride along with the extract at the same concentration.

Among all the instrumental parameters that can affect the appropriate formation of microbeads, in this work the effect of (i) vibration frequency (Hz) used to break up the laminar liquid, (ii) voltage (V) required to create an additional electrostatic field between the nozzle and the hardening solution in order to prevent the coalescence of the microbeads, (iii) air-pressure (bar) to deliver the sodium alginate solution and (iv) the distance between the nozzle and the hardening solution (mm) was assessed for nozzles of 150 µm and 300 µm using an experimental design approach. A central composite design (CCD) was carried out with the The Unscrambler® (9.7 Camo Asa, Oslo, Norway) software. The design matrix consisted of 27 randomized experiments allowing the study of the effects of these variables within

the following ranges: 100-2000 Hz, 250-2000 V, 140-320 bar and 30-150 mm for nozzle of 150 µm and 100-1500 Hz, 500-2000 V, 56-200 bar and 30-150 mm for nozzle of 300 µm. The amplitude was not optimized and it was fixed at 2 in both cases.

These ranges were established considering the equipment restrictions and the characteristics of each nozzle to maintain laminar flow breakup. The results were obtained by counting the well formed and not-well formed microbeads in 3 different pictures obtained by optical microscopy (Olympus BH2, Tokyo, Japan) with an adapted digital camera (Nikon DS-Fi2, Tokyo, Japan) operating at 2.5 magnification. Between 100 and 300 microbeads were counted systematically in each sample.

Three different responses were considered to get the optimum conditions: (i) the percentage of coalescence formation (clusters of microbeads partially fused), (ii) the percentage of tail formation (microcapsules with an elliptic shape), and (iii) the percentage of well-formed spherical smooth microbeads formation. Some of the microbeads formed in each experiment are illustrated in Figure 2. The goal of the optimization process was the minimization of the coalescence and tail formation and the maximization of well-formed microbeads specimens. The precision was estimated from the three replicates of the central point.

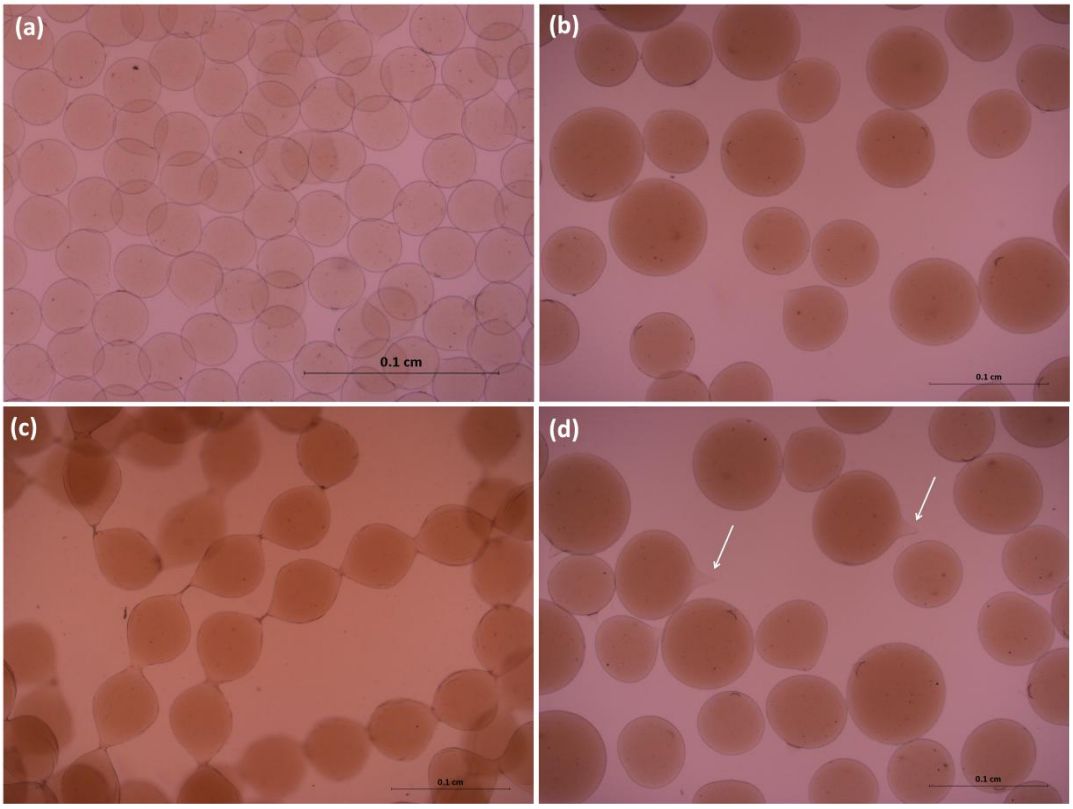

Figure 2: Optical microscopy photographs of empty alginate microbeads. (a) Well formed microcapsules using optimized conditions with nozzle of 150 µm. (b) Well formed microcapsules using optimized conditions with nozzle of 300 µm. (c) Evidence of coalescence in microbeads obtained with nozzle of 300 µm. (d) Evidence of tails in microbeads obtained with nozzle of 300 µm.

2.4. Determination of polyphenols content by means of High Performance Liquid Cromatography with Diode Array Detector (HPLC-DAD)

The content of polyphenols entrapped in the microparticles was estimated by dissolving 350 mg of wet capsules or 20 mg of dry capsules in 1.5 mL of 0.2 M sodium citrate sonicating for an hour in an ultrasonic bath. Using sodium citrate, Ca^{2+} ions of the microbeads were exchanged by Na^{+} ions of the citrate, destabilizing the calcium alginate gel structure [31]. Afterwards, alginate was precipitated by adding 3 mL of MeOH and the solution was filtered through a GHP Acrodisc 0.2 μm filter (Pall, NY, USA) prior to the extract analysis by means of HPLC-DAD. This last process was carried out to avoid alginate precipitation with the mobile phase. In order to check that no analyte loss happens during the precipitation step, Empty alginate microcapsules were spiked with polyphenol standards and submitted to the whole process. Wine waste extract polyphenol content was analyzed directly by means of HPLC-DAD after filtering through a 0.2 μm filter.

The analysis of polyphenols in microcapsules and wine waste extracts was carried out in a HPLC system consisted of an Agilent 1100 series equipped with a quaternary pump, an autosampler and a DAD. The chromatographic separation was achieved using a Phenomenex Synergy Hydro-RP C_{18} column (4 μm, 150 mm x 4.6 mm i.d.) with a guard column (4 μm Depth Filter x 2 mm) (Phenomenex, Torrance (CA), USA) and a binary A/B gradient (Solvent A was Milli Q water with 3 % acetic acid and solvent B was 100% MeOH). The gradient program was as follows: initial conditions were 0 % B; rise to 80 % B over 11 minutes, decrease to 0 % B over the next 0.5 minutes and hold at 0 % B until 18 minutes for re-equilibration of the system prior to the next injection. A flow rate of 1 mL/min was used; the column was set at room temperature and the injection volume was 100 μL. Major polyphenols present in wine wastes, (+)catechin and (-)epicatechin were quantified at a detection wavelength of 280 nm.

2.5 Assessment of microencapsulation approach

The microencapsulation approach was assessed taking into account the efficiency of the process as well as the drying method and the stability of the microbeads at different storage conditions. Previously extracted and determinated (with known concentration of (+)catechin and (-)epicatechin polyphenols) wine wastes extract was used to evaluate the proposed microbeads formation.

2.5.1 Encapsulation efficiency

The wine waste extract containing 278 ± 6 μg/g of (+)catechin and 386 ± 8 μg/g of (-)epichatechin was diluted in 1:1, 3:4, 1:2, 1:4 and 1:8 ratios using Milli-Q water in order to have extracts containing between 35–278 μg/g and 48–386 μg/g of (+)catechin and (-)epichatechin respectively. The encapsulation efficiency was evaluated at these concentration levels. For that purpose, the wine waste extract was diluted in water prior to the addition of low viscosity sodium alginate. The hardening solution was always prepared in the same concentration as in the alginate solution. After incubation in the hardening solution, microcapsules containing different concentrations of polyphenols were rinsed with 100 mL of ultrapure water and analyzed by means of HPLC-DAD as described in

section 2.4. Three microbeads batches were prepared for each wine waste dilution and the analyses were carried out in triplicate.

2.5.2 Microbeads drying

Once hardened, microcapsules were filtered, rinsed with water and dried with three different techniques: freeze drying (FreeZone Plus Labconco, Kansas, USA) during 48 h, air drying at room temperature during 72 h, and drying at constant temperature of 50 °C during 24 h. The best drying method condition was established by the determination of polyphenol content after each drying procedure, which was determined as described in section 2.4.

2.5.3 Long-term stability of microbeads

Long-term stability of the microbeads formed with optimized conditions was studied for 6 months at different conditions: a) -4 °C dark b) room temperature (r.t.) dark, c) r.t. light. To reinforce the microbeads shell, the addition of 0.05 % (w/v) medium molecular weight chitosan to the hardening solution was studied. In order to dissolve the chitosan the hardening solution was prepared at 1 % acetic acid concentration.

The different assays were performed using both types of nozzles according to the optimum conditions described in section 2.3 and freeze-dried afterwards. The (+)catechin and (-)epicatechin content of the different assays was analyzed at different days over time (185 days) as described in section 2.4. In order to investigate the improvement of the encapsulation, free wine wastes extract stability was also studied at the same conditions.

With the purpose of comparing the degradation of loaded microcapsules and free polyphenol extract, a first-order reaction kinetic model was used. Degradation rate constants (λ) and half-lives ($t_{1/2}$) were calculated according to the equations 1 and 2:

$$\mathrm{Ln}\left(\frac{C_t}{C_0}\right) = -\lambda t \qquad \text{eq. (1)}$$

$$t_{1/2} = \frac{ln2}{\lambda} \qquad \text{eq. (2)}$$

where c_0 is the initial polyphenol content and c_t is the polyphenol content at time t.

3. Results and discussion

3.1 Optimization of microbeads formation

In a first attempt, the response surfaces were built by multiple linear regression (MLR) including linear and squared terms and the interactions of any two variables and considering the percentage of well

formed microbeads as response. The relative standard deviation (RSD %) values were below 10 % for all the studied responses with the two nozzle sizes. Model fitness was confirmed by the analysis of variance (ANOVA) of the residuals (lack of fit significantly lower than pure experimental uncertainty) and the uncertainties of each parameter.

MLR data obtained with the experimental design assays of 150 µm nozzle revealed that the significant variables (p-level < 0.05) affecting the formation of spherical smooth microbeads were the voltage, the frequency and the pressure. The distance to the hardening solution had no significant effect at this confidence level, but it was fixed at the maximum value (120 mm). As illustrated in Figure 3, a relative maximum was found at high voltage and frequency values and at low pressure values with respect to the percentage of well formed microbeads. Hence, high voltage (1565 V) and frequency (1525 Hz) values and low pressure values (185 mbar of air-pressure) were established as optimum conditions when nozzle of 150 µm was used.

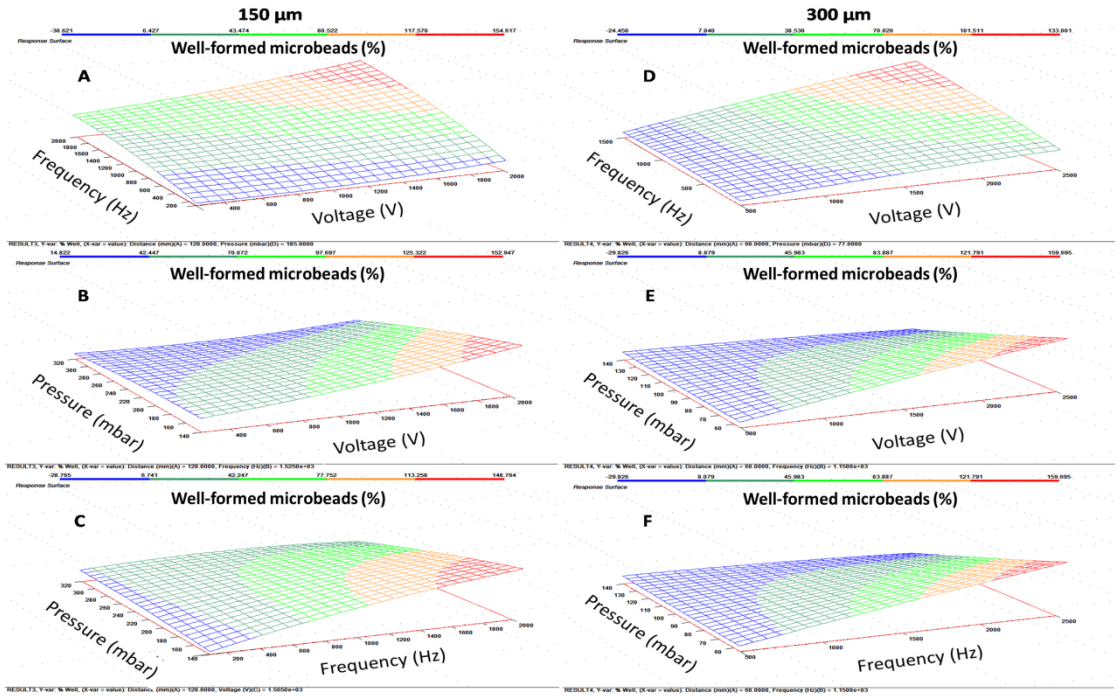

Figure 3: Response surfaces of significant variables for the formation of spherical smooth microbeads being the assessed response the percentage of well formed microbeads. For microbeads formed with the 150 µm nozzle: (a) frequency (Hz) and voltage (V), (b) pressure (mbar) and voltage and (c) pressure and frequency. For microbeads formed with the 300 µm nozzle: (d) frequency and voltage, (e) pressure and voltage and (f) pressure and frequency.

Regarding the 300 µm nozzle microbeads formation, similar results were obtained after the analysis of the response surfaces. In fact, MLR data revealed that the significant variables affecting the well formation of microcapsules were also the voltage, the frequency and the pressure (p-level < 0.05). Again, the distance to the hardening solution was not significant (p-level> 0.05) and it was fixed at 60 mm. Similarly to the results found for 150 µm nozzle microbeads, a local maximum was found at high voltage and frequency values and at low pressure values (see Figure 3). Thus, the optimal conditions for

300 µm nozzle were established as follows: 1150 Hz of frequency, 2000 V of voltage and 77 mbar of air-pressure.

Once the standard working conditions were established just considering the percentage of well formed microbeads, the coalescence and tail formation were evaluated. In general, the percentage of tail formation was found to be the highest at low frequencies and at high voltages using both nozzles; and with high working distances in the case of 300 µm nozzle. Regarding the coalescence, maximum values were found at low voltage and distance values; and in the case of 300 µm nozzle also at low frequencies. These results are consistent with the optimal conditions obtained for both microcapsules formation.

Digital photographs of at least 50 microbeads formed with these optimized conditions were assessed and the average values of diameters were measured. The average size of the obtained wet microcapsules were 278 ± 8 µm and 600 ± 90 µm with the 150 µm and 300 µm nozzles respectively.

3.2 Assessment of microencapsulation process

3.2.1 Encapsulation efficiency

The encapsulation efficiency was calculated by comparing the concentration of polyphenols in the loaded capsules with the total amount of polyphenols present in the sodium alginate solution used for the encapsulation. The later was calculated taking into account the flow of alginate used during the encapsulation time.

Figure 4 shows the encapsulation efficiency obtained for each dilution using the two types of studied nozzles. As it can be observed, there were no significant differences (p-level > 0.05) in encapsulation efficiencies between the different dilutions with any of the nozzles. Therefore, it can be concluded that the polyphenol concentration used in the sodium alginate solution and in the hardening solution does not affect to the encapsulation efficiency, at least, in the studied range.

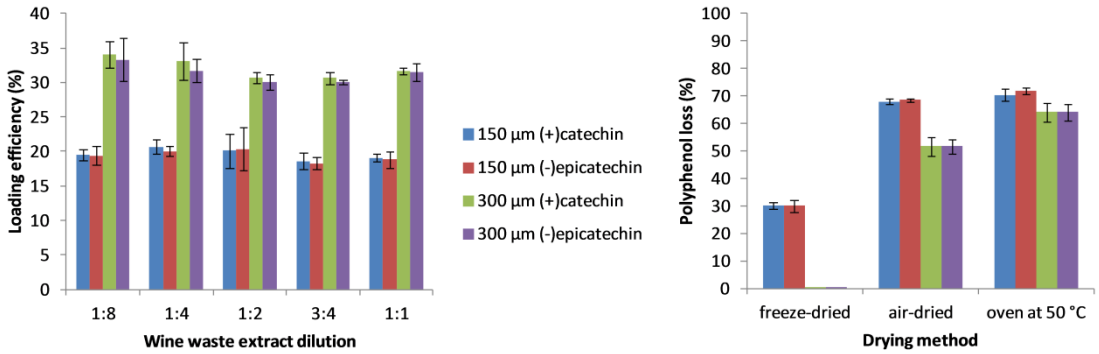

Figure 4: (Left) Loading efficiencies obtained using different initial wine wastes extract dilutions to prepare the sodium alginate and the hardening solutions. (Right) The effect of different drying procedures on polyphenol loss during drying, compared to non-dried microbeads. n=3, 95 % confidence level.

Besides, according to the results shown in Figure 4, similar encapsulation efficiencies were obtained for (+)catechin and (-)epicatechin regardless the size of nozzle. However, higher encapsulation efficiencies were obtained using the nozzle of bigger size. In fact, the encapsulation efficiencies for both compounds were around 30 % for the 300 µm nozzle microbeads and around 20 % for the 150 µm nozzle ones. When bigger nozzle diameters were used bigger microbead sizes were obtained whereas the surface area-to-volume ratio decreased. Consequently, the amount of polyphenols released during the microbeads hardening process was smaller, which favored the encapsulation efficiency. Similar results were previously obtained in other works published in the literature where higher encapsulation efficiencies were obtained when the diameter of the nozzle was increased [32-33]. In addition to the size, another reason to explain the poor encapsulation efficiencies is the used low molecular weight alginate. Microcapsules created with medium or high molecular weight alginate have higher encapsulation efficiencies in comparison to low molecular weight ones [34]. However, the microcapsule size is also bigger and the active ingredient release rate is much slower [35]. Because of this, low molecular weight alginate is more appropriate in applications when the active ingredient degradation must be avoided but fast in vivo release is desired.

3.2.2 Selection of drying method of microbeads

The effect of different drying methods on polyphenol encapsulation is shown in Figure 4. The results are expressed as the percentage of polyphenol loss occurred during the drying techniques. According to the results, the best strategy to dry the microbeads was found to be freeze-drying, in which no decrease in polyphenol content was observed for the microcapsules produced with the 300 µm nozzle. Although a slight decrease of polyphenol content was observed when the nozzle of 150 µm was used, freeze-drying method rendered the lowest losses (30 %) in comparison to the other two assessed strategies (> 70%). In addition, the freeze-dried microbeads were finely granulated whereas microbeads dried in air and at constant temperature were bound together. This is important in food applications where mouth-feel is a crucial factor.

3.2.3 Long-term stability of microbeads

Microbeads storage stability was studied along with the non-encapsulated wine wastes extract at different conditions of temperature (i.e., at 4 °C and at room temperature) and light (i.e., in presence of light and in darkness) for 6 months. They all presented a first order degradation curve for (+)catechin and (-)epicatechin for all storage conditions (Table 1). The changes in (+)catechin and (-)epicatechin content of microbeads formed with 2 nozzles in presence or absence of chitosan are shown in Figure 5.

Table 1
First order degradation kinetic model parameters and half-lives of free wine wastes extract and the different loaded microbeads (150 µm or 300 µm nozzle, and, with or without chitosan) at different environments (at 4 °C in the freezer and at room temperature (r.t.) with light or in darkness).

	Environm.	R^2		$t_{(1/2)}$ (days)	
		(+)Catechin	(-)Epicatechin	(+)Catechin	(-)Epicatechin
Free extract	4 ºC	0.943	0.720	396	408
	r.t. light	0.973	0.984	14	15
	r.t. dark	0.976	0.981	19	20
150 µm, chi.	4 ºC	0.875	0.788	764	857
	r.t. light	0.980	0.987	89	85
	r.t. dark	0.958	0.966	152	146
150 µm, no chi.	4 ºC	0.814	0.624	619	680
	r.t. light	0.973	0.980	74	72
	r.t. dark	0.952	0.947	139	139
300 µm, chit.	4 ºC	0.949	0.929	478	510
	r.t. light	0.971	0.936	462	478
	r.t. dark	0.993	0.992	163	157
300 µm, no chi.	4 ºC	0.879	0.855	472	465
	r.t. light	0.678	0.647	373	361
	r.t. dark	0.965	0.965	157	159

In the case of microbeads formed with the 150 µm nozzle, at 4 °C in darkness half-lives between 619 and 857 days were obtained, whereas at room temperature in light and in darkness were 72-89 days and 139-152 days respectively. When chitosan was added to the gelling bath relatively small differences were found, decreasing the encapsulation efficiency by 3-7 % but also decreasing the degradation rate by 3-7 %.

In contrast, half-lives of the microbeads formed with the 300 µm nozzle at 4 °C were between 465 and 510 days, whereas at room temperature in light and in darkness were 361-478 days and 157-163 days respectively. The addition of chitosan improved the encapsulation efficiency by 31-36 % and decreased the degradation rate by 1-16 %. The different effect of chitosan on the encapsulation efficiency of microbeads for small and large nozzles may lie in the two opposite effects that are happening when chitosan is added to the harderning solution. On the one hand, a membrane is formed through ionic interactions between the carboxylic residues of the alginate and positively charged amino groups of chitosan, reducing the active compounds leaking during encapsulation and decreasing the degradation during storage [36]. On the other hand, the microcapsules loss some water during this process, leading to a size reduction and therefore to a lower loading efficiency [31]. This size reduction effect is much more pronunciated in microbeads created with the 150 µm nozzle, as they have higher surface area-to-volume ratio. As a result, their encapsulation efficiency is lower.

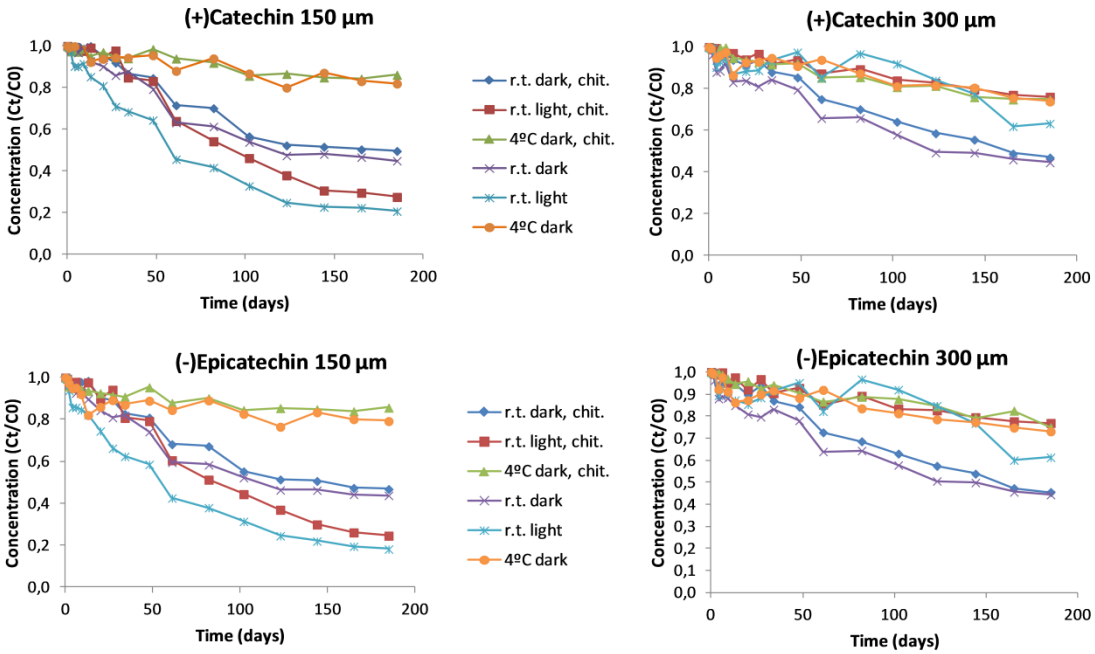

Figure 5: Storage stability of two main polyphenol targets (+)catechin and (-)epicatechin encapsulated with 150 and 300 µm nozzle and in the presence or absence of chitosan (chit.) at 4 °C in the freezer in darkness and at room temperature (r.t.) in darkness and with light (n=3). RSD values were always below 10 %.

As it is illustrated in Figure 5, (+)catechin and (-)epicatechin showed close degradation rates. In all cases, much slower degradation rates were obtained at low temperatures. Precisely, the longest stability in this study was obtained at 4 °C in the smallest microcapsules (150 µm nozzle). The addition of chitosan increased slightly the stability of small microcapsules, especially at low temperatures.

In the same way, 16-22 % less degradation was observed when microbeads formed with the 150 µm nozzle were stored in darkness. These results were expected as polyphenols are prone to degradate under several environmental conditions, such as light and temperature. Surprisingly, 17-31 % lower degradation rates were observed when 300 µm nozzle microbeads were stored in light. It is possible that polyphenols become cross-linked in light, either to the alginate or to each other [20, 37-38], thereby slowering the degradation.

On the other hand, due to lesser surface area-to-volume ratio microbeads formed with the 300 µm nozzle and stored at room temperature were less vulnerable to degradation than the ones formed with the 150 µm nozzle. This pattern was not observed at 4 °C.

Finally, Table 1 shows that the degradation of free wine wastes extract took place much faster than the encapsulated ones, for all environment conditions. This way, due to the protection of polyphenols by the encapsulating agent preventing oxidation, between 5 and 33 times higher half-lives were obtained when microbeads were stored at room temperature. The protection improvement was less when they were stored at 4 °C, between 1.2 and 2.1.

4 Conclusions

The encapsulation technique can be a valuable methodology to improve the stability and bioavailability of different type of products. In this work, microencapsulation of wine wastes extract rich in polyphenols was successfully optimized and evaluated using two different types of nozzles. Encapsulation efficiencies in calcium alginate microbeads were found to be independent to the extract concentration in the studied range but higher efficiencies were rendered by the high size nozzle.

The stability of microcapsules containing polyphenols was studied at different conditions of light and temperature. The assays showed that all encapsulated systems were much more stable than the free extract at all conditions, with half-life between 72 and 857 days. Lower degradation rates were obtained when chitosan was added to the gelling bath. This study will be helpful for the industrial application of wine wastes polyphenols.

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5 References

[1] Jaworowska A, Blackham T, Davies IG, Stevenson L. 2013. Nutritional challenges and health implications of takeaway and fast food. Nutr Rev, 71, 310-318.

[2] Howlett J. 2008. Functional Foods: From Science to Health and claims. Brussels, Belgium: ILSI Europe.

[3] Shahidi F. 2012. Nutraceuticals, functional foods and dietary supplements in health and disease. J Food Drug Anal, 20, 226-230.

[4] Dugas AJ, Castañeda-Acosta J, Bonin GC, Price KL, Fisher NH, Winston GW. 2000. Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: structure-activity relationships. J Nat Prod, 63, 327-331.

[5] Tikhonov I, Roginsky V, Pliss E. 2009. The chain-breaking antioxidant activity of phenolic compounds with different numbers of O-H groups as determined during the oxidation of styrene. Int J Chem Kinet, 41, 92-100.

[6] Leopoldini M, Russo N, Toscano M. 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. Food Chem, 125, 288-306.

[7] Hertog MGL. 1994. Flavonols and Flavones in Foods and Their Relation with Cancer and Coronary Heart Disease Risk. Ph.D. Thesis, The Netherlands: Agricultural University Wageningen.

[8] Cordova FM, Watson RR. 2014. Food and supplement polyphenol action in cancer recurrence. In Watson RR, Preedy VR, Zibadi S (Eds). Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 191-195.

[9] Kim J, Lee KW, Lee HJ. 2014. Polyphenols suppress and modulate inflammation: Possible Roles in Health and Disease. In Watson RR, Preedy VR, Zibadi S (Eds). Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 393-408.

[10] Jean-Gilles D, Li I, Vaidyanathan VG, King R, Cho B, Worthen DR, Chichester CO, Seeram NP. 2013. Inhibitory effects of polyphenol punicalagin on type-II collagen degradation in vitro and inflammation in vivo. Chem-Biol Interact, 205, 90-99.

[11] Menaa F, Menaa A. 2014. Skin photoprotection by polyphenols in animal models and humans. In Watson RR, Preedy VR, Zibadi S (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 831-838.

[12] Gollücke AP, Peres RC, Odair A Jr, Ribeiro DA. 2013. Polyphenols: a nutraceutical approach against diseases. Rec Pat Food, Nutr Agric, 5, 214-219.

[13] Mandel S, Youdim MBH. 2004. Serial Review: Flavonoids and Isoflavones (Phytoestrogens): Absorption, Metabolism, and Bioactivity. Free Radical Bio Med, 37, 304-317.

[14] Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, Lamari FN. 2009. Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. Behav Brain Res, 198, 352–358.

[15] Vauzour D, Kerr J, Czank C. 2014. Plant Polyphenols as Dietary Modulators of Brain Functions. In Watson RR, Preedy VR, Zibadi S (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 357-370.

[16] Makris DP, Boskou G, Andrikopoulos NK. 2012. Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. J Food Compos Anal, 20, 125-132.

[17] Aizpurua-Olaizola O, Ormazabal M, Vallejo A, Olivares M, Navarro P, Etxebarria N, Usobiaga A. 2015. Optimization of supercritical fluid consecutive extractions of fatty acids and polyphenols from *Vitis Vinifera* grape wastes. J Food Sci, 80, 101-107.

[18] FAO. (2013). Faostat webpage. http://faostat.fao.org.

[19] Bakowska AM, Kucharska AZ, Oszmianski J. 2003. The effects of heating, UV irradiation and storage on stability of anthocyanin-polyphenol copigment complex. Food Chem, 81, 349-355.

[20] Munin A, Edwards-Lévy F. 2011. Encapsulation of Natural Polyphenolic Compounds; a Review. Pharmaceutics, 3, 793-829.

[21] Dube A, Ng K, Nicolazzo JA, Larson I. 2010. Effective use of reducing agents and nanoparticle encapsulation in stabilizing catechins in alkaline solution. Food Chem, 122, 662–667.

[22] Zvonar A, Berginc K, Kristl A, Gasperlin M. 2010. Microencapsulation of self-microemulsifying system: Improving solubility and permeability of furosemide. Int J Pharm, 388, 151-158.

[23] Robert P, Gorena T, Romero N, Sepulveda E, Chavez J, Saenz C. 2010. Encapsulation of polyphenols and anthocyanins from pomegranate (Punica granatum) by spray drying. Int J Food Sci Tech, 45, 1386-1394.

[24] Bakowska-Barczak AM, Kolodziejczyk PP. 2011. Black currant polyphenols: their storage stability and microencapsulation. Ind Crop Prod, 34, 1301-1309.

[25] Dorati R, Genta I, Modena T, Conti B. 2013. Microencapsulation of a hydrophilic model molecule through vibration nozzle and emulsion phase inversión technologies. J Microencapsul, 30, 559-570.

[26] De Prisco A, Maresca D, Ongeng D, Mauriello G. 2015. Microencapsulation by vibrating technology of the probiotic strain Lactobacillus reuteri DSM 17938 to enhance its survival in foods and in gastrointestinal environment. LWT-Food Sci Tech, 61, 452-462.

[27] King AH. 1983. Brown Seaweed Extracts (Alginates). In Glicksman M(Ed.) Food Hydrocolloids. CRC Press, Boca Raton, 115.

[28] Krasaekoopt W, Bhandari B, Deeth H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. Int Dairy J, 13, 3-13.

[29] Xu Y., Zhan C, Fan L, Wang L, Zheng H. 2007. Preparation of dual crosslinked alginate-chitosan blend gel beads and in vitro controlled release in oral site-specific drug delivery system. Int J Pharm, 336, 329-337.

[30] George M, Abraham E. 2006. Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan- a review. J Control release, 114, 1-14.

[31] Deladino L, Anbinder PS, Navarro AS, Martino MN. 2008. Encapsulation of natural antioxidants extracted from Ilex paraguariensis. Carbohyd Polym, 71, 126-134.

[32] Khorram M, Samimi M, Samimi A, Moghadam H. 2014. Electrospray preparation of propranolol-loaded alginate beads: Effect of matrix reinforcement on loading and release profile. J Appl Polym Sci, 131, 413-434.

[33] Genç L, Büyüktiryaki S. 2014. Preparation and characterization of methotrexate-loaded microcapsules. Pharm Dev Technol, 19, 42-47.

[34] Bojana I, Verica D, Viktor N, Branko B. 2013. Liposome-in-alginate systems for encapsulation of natural antioxidants. Inside Food Symposium, 9-12 April 2013, Leuven, Belgium.

[35] Alsberg E, Kong HJ, Hirano Y, Smith MK, Albeiruti A, Mooney DJ. 2003. Regulating bone formation via controlled scaffold degradation. J Dent Res, 82, 903-908.

[36] Lertsutthiwong P, Rojsitthisak P, Nimmannit U. 2009. Preparation of turmeric oil-loaded chitosan-alginate biopolymeric nanocapsules. Mater Sci Eng C, 29, 856-860.

[37] McManus JP, Davis KG, Lilley TH, Haslam E. 1981. The association of proteins with polyphenols. J Chem Soc Chem Comm, 7, 309-311.

[38] Nishad Fathima N, Ansari T, Raghava rao J. 2007. Role of green tea polyphenol crosslinking in alleviating ultraviolet-radiation effects on collagen. J Appl Polym Sci, 106, 3382-3386.

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5. ATALA

Laburpena

Lan honetan ardo hondakinetatik konposatu bioaktiboak erauzteko bi urratseko jariakin gainkritiko bidezko erauzketa (SFE Supercritical Fluid Extraction) optimizatu da, eta ondorioz, lehen urratsean gantz azidoak soilik eta bigarren urratsean polifenolak soilik erauztea lortu da. Polifenolek egonkortasun baxua dute ordea. Hori dela eta, zurrusta-bibrazio bidezko mikrokapsularatzea (VNM Vibration Nozzle Microencapsulation) erabili da 150 eta 300 µm-ko zurrustekin kaltzio-alginatozko eta kaltzio-alginato-kitosanezko tamaina ezberdinetako polifenolen mikrokapsulak eratzeko. Prozesu honetan eragina duten aldagaiak optimizatu, kapsulak sortu eta haien egonkortasuna aztertu da 6 hilabetetan zehar hainbat kontserbazio baldintzatan (4 °C-tara ilunpean eta giro tenperaturan ilunpean eta argipean). Kapsulatutako polifenolen egonkortasuna baldintza guztietan kapsulatu gabeena baino askoz ere handiagoa dela ikusi da.

Hitz-gakoak: Ardo hondakinak, Jariakin gainkritiko bidezko erauzketa, mikrokapsularatzea, gantz azidoak, polifenolak.

1 Sarrera

Bukatu zen bi gauza batera emakumeek soilik egin zitzaketen garaia. Gaur egun tximistaren abiaduran bizi gara gehientsuonok, ia geure itzalak aurreratuko gaituen beldurrez, eta noski, gorputzari aireaz gain zerbait eman behar diogula zeharo ahaztuta. Ondorioz, janari lasterrak eta industrialki prozesatutako elikagaiak gure eguneroko ogi bilakatu dira, baita horiek duten balio nutrizionalaren galera konpentsatzeko gehigarrien beharra ere. Gehigarri horien artean gantz azido asegabeak eta polifenolak aipatu nahi ditugu [1].

Polifenolek gaitasun antioxidatzaile handia dute, horri esker erradikal askeak neutralizatzeko gai dira arrisku txikiagoko konposatuak emanez [2-4]. Gaitasun hori dela eta hainbat aplikazio ezberdinetarako egokiak dira konposatu hauek; hala nola hanturak tratatzeko [5], minbiziaren gisako endekapenezko gaixotasunetarako [6-7] edo kosmetikan zahartzearen aurkako tratamenduetarako [8-9]. Bestalde, nootropiko bezala zerrendatuak daude, garunean oroimena, arreta eta motibazioa gisako funtzioak bultzatzen laguntzen baitute [10].

Gantz azidoak 3 taldetan sailkatzen dira: poliasegabeak (PUFA), monoasegabeak (MUFA) eta asetuak (SAFA). Azido linoleikoaren gisako PUFAk oso garrantzitsuak dira gaixotasun kardiobaskularren prebentzioan [11-12]; azido oleikoaren gisako MUFAk aldiz oinarrizkoak dira odoleko triazilglizerol eta kolesterol mailak jaisteko eta ondorioz minbizien prebentziorako [13-14]. Azido estearikoa eta palmitikoaren gisako SAFAk alderantzizko efektuaren erantzule dira ordea, odoleko kolesterol maila igotzen baitute eskuarki [15].

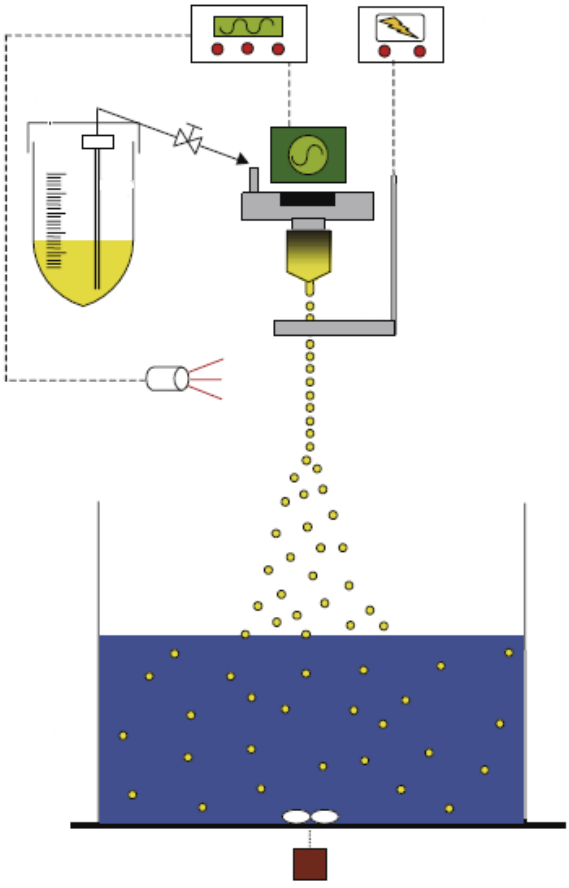
Hondakin agroindustrialen inguruko interesa haziz joan da azken urteetan, balio erantsi handiko albo-produktuen iturri garrantzitsu izan baitaitezke. Ildo horretatik, ardoaren produkzioko hondakinen ustiapena aukera bikaina izan daiteke, bertan aurkitzen diren gantz azido asegabeen eta polifenolen kontzentrazioa altua izateaz gain sortzen den hondakin kopurua izugarria baita eta ustiapena, berriz, benetan mugatua [16].

Konposatu bioaktiboak erauzterako eta jakiak prozesatzeko orduan aukerarik egokienetako bat da jariakin gainkritikoen bidezko erauzketa. SFE jariakin gainkritikoen ezaugarrietan oinarritzen da. Gas bat presio eta tenperatura kritikotik haratago jartzen denean egoera gainkritikoa batera pasa daiteke, hala likidoen eta gasen arteko ezaugarriak hartzen ditu. Likidoen antzeko dentsitatea dute eta hargatik disolbatzaile onak dira; bestalde, gasen likatasuna eta mugikortasuna dutenez, material solidoaren poroetara hobeto heltzen dira eta erauzketak azkarrago lortzen dira. Erauzketak egiteko hainbat disolbatzaile erabili daitezke; dena den, ohikoena karbono dioxidoa erabiltzea da, hainbat abantaila esanguratsu aurkezten baititu [17-19]. Batetik inertea eta ingurumenarentzat garbia izateaz gain erauzketa tenperatura eta presio baxuetan gauzatzea ahalbidetzen duenez baldintza gogorrekiko sentikorrak diren konposatuen degradatzea sahiesten du. Bestetik, lortzen diren erauzkinak purutasun altukoak dira eta ondorioz elikagaien industrian arazo gabe erabili daitezke. Erauzketa eskala industrialean egiteko arazorik ez dago gainera. Hortaz gain, nahiz eta CO_2 disolbatzaile apolarra izan, etanola edo metanolaren gisako albo-disolbatzaile polar bat gehitu dakioke, eta modu horretan polartasun ezberdineko konposatuak

elkarrengandik bereizita erauztea lortu, lehenik apolarrak CO_2 soilik erabiliz eta ostean polarrak albo-disolbatzailea gehituz [20-21].

Zoritxarrez, polifenolak erraz degradatu daitezke argiaren, tenperaturaren, oxigenoaren eta aktibitate entzimatikoen ondorioz [22]. Bestalde, sarritan ez dira oso bioeskuragarriak uretan disolbagarritasun baxua baitute eta jariakin biologikoetan dagoen baldintza alkalinoetan ezegonkorrak baitira gainera [23]. Ildo honetatik dator konposatu hauek kapsulatu beharra, modu horretan konposatuen egonkortasuna eta bioeskuragarritasuna egiazki hobetzea lortzen baita [23-24].

Mikrokapsulatzeko teknika ugari daude. Horien artean aurkitzen da zurrusta-bibrazio bidezko mikrokapsularatzea, teknika erraza, eskala industrialean erabilgarria eta konposatu hidrofilikoentzat egokia baita [25]. Teknika horretan kapsulatu nahi diren konposatuak agente kapsulatzailearekin nahastu eta nahastearen fluxu laminar bat zulo txiki batetik pasarazten da, aplikaturiko bibrazioari esker tanta nimiñoak osatuz. Boltaje bat ezartzean tanta horiek dispertsatu egiten dira eta gogortze disoluziora erortzean mikrokapsulak osatzen dira (1. irudia).



1. irudia: erabilitako kapsulatze sistemaren azalpen eskematikoa (Buchi B-390).

Kapsulatzaile agente ohikoena alginatoa da. Alginatoa alga marroietatik lortzen da eta kaltzio katioiekin erreakzionatu dezaketen talde karboxiliko ugari ditu, eta modu horretan konposatu aktiboak

babesteko gai den gel egonkorrak osatzen ditu [26]. Hori dela eta, agente gisa alginatoa erabiltzen denean gogortze disoluzioa kaltzio klorurozko disoluzioa izan ohi da.

Kapsulen egonkortasuna are gehiago hobetzeko aukera gehigarri bat alginatoarekin batera kitosan bezalako agente egonkortzaile bat erabiltzea da [27]. Kitosana karramarroen exoeskeletoetatik erdiesten da eta elektroi emaile gisa diharduten –OH eta $–NH_2$ talde ugari ditu. Ondorioz, alginatoak eta kitosanak bateragarritasun bikaina dute, aurkako kargak dituztenez elkarrekin erreakzionatu baitezakete [28].

Lan honetan ardo hondakinetatik gantz azidoak eta polifenolak bakoitza bere aldetik erauzi ahal izateko SFE bidezko metodoa optimizatu da. Bestalde, polifenolen egonkortasuna hobetzeko VNM bidezko mikrokapsularatzea ere optimizatu da. Osatutako mikrokapsulen egonkortasuna aztertu da 6 hilabetetan zehar kontserbazio baldintza ezberdinetara.

2 Jariakin gainkritiko bidezko erauzketa

2.1 Gantz azidoen eta polifenolen erauzketaren optimizazioa

Gantz azidoak apolarrak dira, polifenolak ordea polarrak. Ezberdintasun horretan oinarritzen da diseinatutako erauzketa metodoa konposatu bakoitza bere aldetik erauzteko. Lehenik, CO_2 soilik erabiliz ardo hondakinetatik gantz azidoak erauztea lortu nahi da, eta ostean albo-disolbatzaile gisa metanola gehituz polifenolak erauztea. Ahalik eta konposatu gehien erauzteko beharrezkoa da ordea bi urrats hauek bata bestearen atzetik egin aurretik bakoitza bere aldetik optimizatzea. Horretarako, lehendabizi tenperatura, CO_2 fluxua, presioa eta erauzketa denboraren eragina aztertu dira urrats bakoitzean, eta polifenolen kasuan baita gehitutako albo-disolbatzailearen ehunekoa ere.

Optimizazioak diseinu konposatu zentrala (CCD Central Composite Design) erabiliz gauzatu dira, 4 aldagai eta 27 esperimentuko matrize banaren bidez erauzketa maximoa deneko gainazal erantzunak erdietsiz hain zuzen ere. Polifenolen kasuan erauzketa denbora diseinutik kanpo utzi da eta optimizazioaren ostean aztertu da erauzketa jarraien bidez. Gainazal erantzunak lortzeko emaitza gisa masa-espektrometriari akoplaturiko gas-kromatografia (GC-MS) bidez lortutako gantz azidoen kontzentrazioak erabili dira gantz azidoen kasuan eta polifenolen kasuan aldiz Folin-Ciocalteu [29] metodoaren bidez lortutako polifenolen kontzentrazio totala. Aztertutako tarteak eta erdietsitako balio optimoak 1. taulan daude erakusgai.

1. taula
SFE erauzketako 2 urratsetan aztertutako tarteak eta erdietsitako balio optimoak.

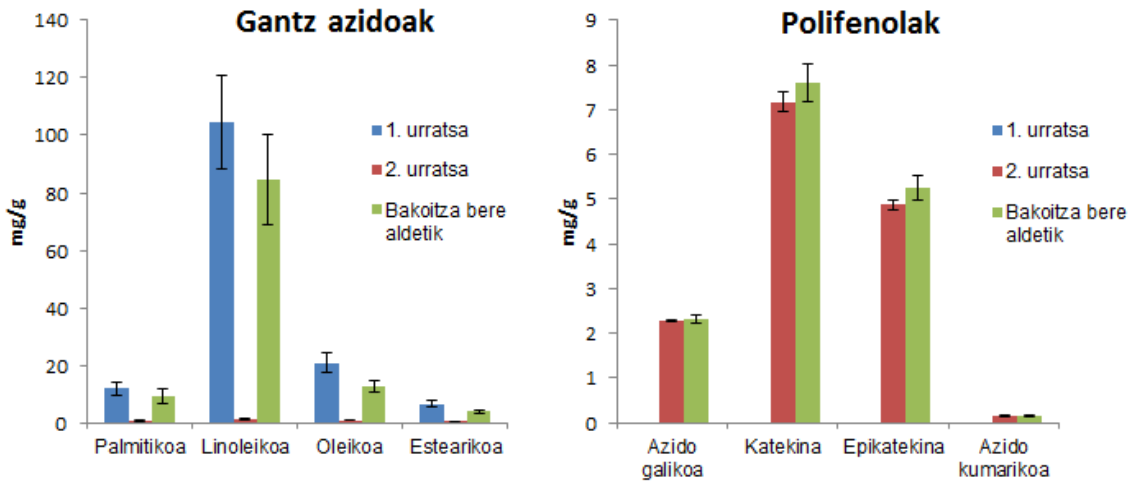
Aldagaiak	Aztertutako tartea Gantz azidoak	Balio optimoak	Aztertutako tartea Polifenolak	Balio optimoak
Tenperatura (°C)	35-55	35	35-55	35
Fluxua (mL/min)	1-2	2	1-2	2
Presioa (bar)	103-253	253	103-253	103
Denbora (min)	5-79	5	Erauzketa jarraiak	5
Metanola (%)	-	-	0-40	40

Gantz azidoen erauzketan estatistikoki eragina duten aldagaiak CO_2 fluxua eta presioa direla ikusi da. Hala, horiek balio altuetan finkatu dira eta denbora eta tenperatura aldiz balio baxuetan, ez baitute eraginik.

Polifenolen kasuan eragina dutenak CO_2 fluxua eta erabilitako albo-disolbatzailearen portzentaia izan dira. Ondorioz, horiek balio altuetan finkatu dira eta eraginik ez duten aldagaiak, kasu honetan tenperatura eta presioa balio baxuetan. Erauzketa jarraietan 5 minutuko erauzketak aski direla ikusi da.

2.2. Bi urratseko erauzketaren balidazioa

Behin urrats bakoitza bere aldetik optimizatu ostean, bata bestearen atzetik aplikatu dira ardo hondakinetatik lehenik gantz azidoak eta ostean polifenolak erauzi ahal izateko. Ardo hondakinetan gehien agertzen diren 4 konposatu nagusiak kuantifikatu dira talde bakoitzean, gantz azidoen kasuan GC-MS bidez eta polifenolen kasuan fragmentazio bikoitzeko masa-espektometriari akoplaturiko eraginkortasun handiko likido kromatografia bidez (LC-MS/MS). Lorturiko emaitzak 2. irudian laburbiltzen dira.



2. irudia: Gantz azidoen eta polifenolen kontzentrazioak lehen urratsean (CO_2 soilik), lehenengoaren jarraian bigarren urratsa gauzatzean (CO_2 + metanola) eta urrats bakoitza bere aldetik egitean (alde batetik CO_2 soilik eta beste aldetik CO_2 + metanola soilik), dagokien desbiderapen estandarrekin (n=3).

2. irudian ikus daiteken bezala, bi urratsak jarraian egitearen edo bakoitza bere aldetik egitearen artean ez dago ezberdintasunik. Hala, lehen urratsean CO_2 soilik erabiliz inongo polifenolik erauzi gabe gantz azido ia denak erauztea lortu da, eta ostean, metanola gehituta polifenol guztiak erauztea bigarren urratsean.

3 Polifenolen kapsularatzea

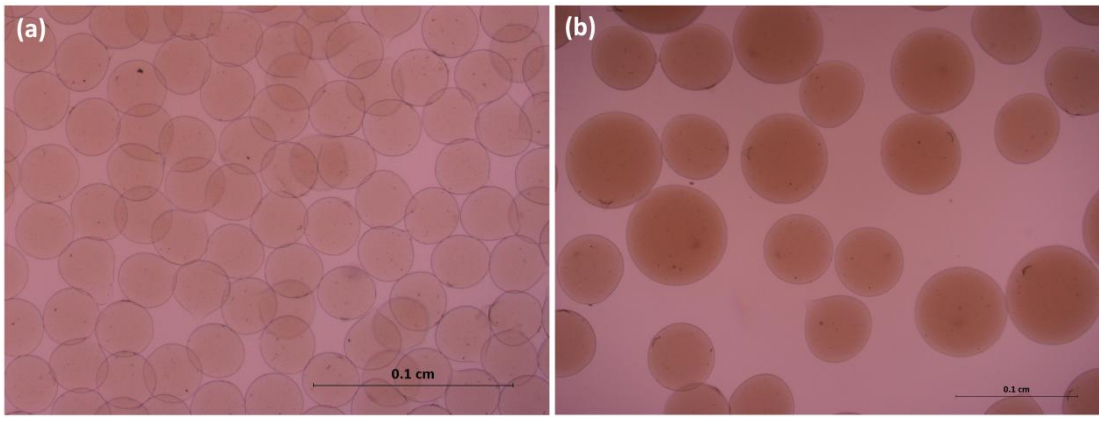
3.1 Polifenolen mikrokapsularatzearen optimizazioa

1. irudian ikusgai dagoen zurrusta-bibrazio bidezko mikrokapsularatzearen bidez kapsulak eratzerako orduan 4 aldagai nagusi daude eragina izan dezaketenak: agente kapsulatzailearen eta kapsulatu nahi diren konposatuen nahastearen fluxu laminarra osatzeko ezarririko presioa, fluxua banakako tanta nimiñoetan bihurtzen duen bibrazioaren maiztasuna, tantatxo hauek banatzeko aplikaturiko boltajea, eta gogortze disoluziora dagoen distantzia. Bestalde, sisteman jarritako zurrustaren arabera hainbat tamainako mikrokapsulak osatu daitezke, zurrusta bakoitzarentzat aldagaien balio optimoak ezberdinak izango dira ordea. Lan honetan 150 eta 300 µm-ko zurrustak erabili dira mikrokapsulak osatzeko, ostean emango zaien aplikazioaren arabera tamaina bateko edo besteko mikrokapsulak izatea komeni baita. Zurrusta bakoitzarekin lan egiteko baldintza egokienak aurkitzeko 27 esperimentuko matrize bana erabili da aurreko kasuan bezala. Gainazal erantzunak lortzeko 3 erantzun hartu dira kontuan: ondo eratutako kapsula biribilen ehunekoa, gaizki eratutako kapsula ez biribilen ehunekoa eta elkarri itsatsiriko kapsulen ehunekoa.

Hala, ondo eratutako kapsulen kopurua maximoa izatea eta gaizki eratutakoena minimoa izatea bilatu da. Diseinuan aztertutako tarteak eta erdietsitako balio optimoak 2. taulan daude erakusgai. Baldintza optimoekin erdietsitako mikrokapsula hezeen batazbesteko tamaina 278 ± 8 µm-koa izan da 150 µm-ko zurrusta erabiliz, 300 µm-ko zurrustaren kasuan aldiz 600 ± 90 µm-koa (3. irudia)

2. taula
150 eta 300 µm-ko zurrustekin kapsula optimoak osatzeko aztertutako aldagaien tarteak eta balio optimoak

Aldagaiak	Aztertutako tartea 150 µm	Balio optimoak	Aztertutako tartea 300 µm	Balio optimoak
Presioa (mbar)	140-320	185	56-200	77
Bibrazioa (Hz)	100-2000	1525	100-1500	1150
Boltajea (V)	250-2000	1565	500-2000	2000
Distantzia (mm)	30-150	120	30-150	60

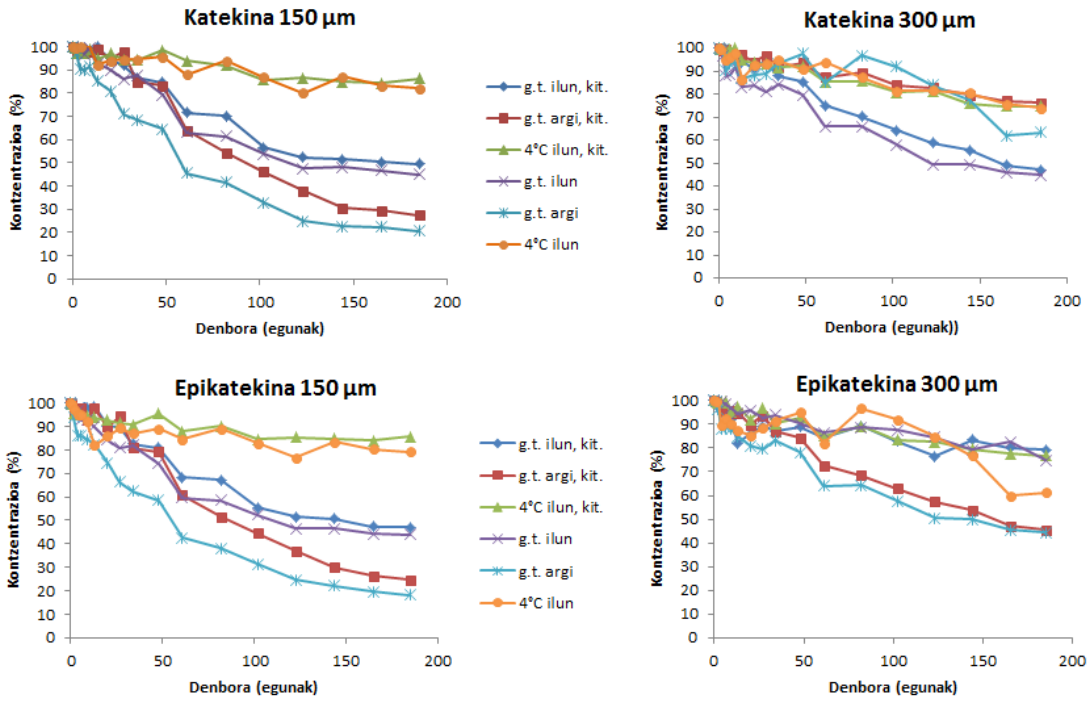


3. irudia: Mikroskopio optiko bidez ateratako mikrokapsulen argazkiak. (a) 150 µm-ko zurrustarekin baldintza optimoetan lortutako kapsulak. (b) 300 µm-ko zurrustarekin baldintza optimoetan lortutako kapsulak.

3.2 Mikrokapsulen egonkortasun azterketa

Baldintza optimoekin erdietsitako mikrokapsulak liofilizazio bidez lehortu eta haien egonkortasuna aztertu da zenbait baldintzetan (4 °C-tara ilunpean eta giro tenperaturan ilunpean eta argipean) 6 hilabetetan zehar. Horretarako ardo hondakinetako polifenol nagusiak diren katekinaren eta epikatekinaren kontzentrazioa jarraitu da kapsuletan eta emaitzei zentzua eman ahal izateko kapsulatu gabeko erauzkin berdinaren degradazioa ere jarraitu da. Baldintza ezberdinetan kapsulek jasandako kontzentrazioen bilakaera 4. irudian dago erakusgai. 3. taulan aldiz mikrokapsulen eta kapsulatu gabeko erauzkinaren bizitza erdiak azaltzen dira; hots, kontzentrazioak hasierakoaren erdira jaisteko kasu bakoitzean behar diren denborak.

4. irudian ikus daitekeenez, katekinak eta epikatekinak antzerako degradazio abiadura mantentzen dute kasu guztietan, txikiena tenperatura baxuetan delarik. Kitosana gehigarri gisa erabiltzeak degradazioa apur bat mantsotzen duela ikusi da, 150 µm-ko zurrustarekin % 3-7 artean eta 300 µm-koarekin % 1-16 artean hain justu. Bestalde, nahiz eta 4 ºC-tara joera hau ez ikusi, orokorrean mikrokapsula txikienak azkarrago degradatzen direla ikusi da, ziurrenik inguruarekin kontaktuan duten gainazalaren azalera handiagoa dutelako.



4. irudia: Katekina eta epikatekinaren egonkortasuna haserako kontzentrazioarekiko 150 eta 300 µm-ko zurrustekin osatutako mikrokapsuletan kitosanaren (kit.) presentzian edo absentzian hurrengo baldintzetan: 4 °C-tara hozkailuan ilunpean eta giro tenperaturan (g.t.) ilunpean eta argipean (n=3). Desbiderapenak % 10-etik beherakoak izan dira kasu guztietan.

3. taulan argi ikus daitekeen bezala, orokorrean eta laburbilduz esan daiteke kapsulatu gabeko erauzkina kapsulatutakoa baino askoz ere azkarrago degradatzen dela baldintza guztietan. Giro tenperaturan gordetzen direnean 5-33 aldiz azkarrago degradatzen da kapsulatu gabeko erauzkina, hozkailuan gordetzen direnean aldiz 1.2-2.1 aldiz azkarrago.

3. taula
Kapsulatu gabeko erauzkineko eta erauzkin berdinarekin sortutako mikrokapsula ezberdinetako katekinaren eta epikatekinaren bizitza erdiak aplikaturiko baldintza ezberdinetan. zit.: zitosan; g.t.: giro tenperatura.

	Baldintzak	$t_{(1/2)}$ (egunak)	
		Katekina	Epikatekina
Erauzkin askea	4 ºC	396	408
	g.t. argi	14	15
	g.t. ilun	19	20
150 µm, zit.	4 ºC	764	857
	g.t. argi	89	85
	g.t. ilun	152	146
150 µm, zit. ez	4 ºC	619	680
	g.t. argi	74	72
	g.t. ilun	139	139
300 µm, zit.	4 ºC	478	510
	g.t. argi	462	478
	g.t. ilun	163	157
300 µm, zit. ez	4 ºC	472	465
	g.t. argi	373	361
	g.t. ilun	157	159

4 Ondorioak

Ardo hondakinetatik gantz azido asegabeak eta polifenolak bereizita erauzteko SFE metodoa garatu da. Garatutako metodoak, azkarra (10 min) izateaz gain, tenperatura baxuetan (35 °C) gauzatzen denez konposatu aktiboak degradatzea ekiditen du.

Bestalde, polifenolak erraz degradatu daitekeenez, hauek kapsulatzeko metodo bat garatu da VNM bidez. Janari munduko aplikazio ezberdinetarako egokiak izan daitezken tamaina ezberdineko alginato mikrokapsulak garatu dira eta sortutako mikrokapsulek izugarri hobetu dute polifenolen egonkortasuna. Kitosana gehigarri gisa erabiltzea aztertu da eta egonkortasuna are gehiago hobetzen duela ikusi.

Laburbilduz, lan honen bidez ardo hondakinetatik bi produktu interesgarri erdietsi direla esan daiteke, batetik gantz azido asegabeetan oso aberatsa den erauzkina eta bestetik egonkortasun handiko polifenol mikrokapsulak. Modu honetan ardo hondakinen balioa handitzea lortu daiteke, eta Euskal Herrian sortzen den bolumena zenbatekoa den kontuan hartuz, litekeena da etorkizun hurbileko aukera

izatea. Arrazoia izango du azkenean hondakinak txerrikeria bat direla dionak, kasu honetan ere, txerrian bezala, dena aprobetxatu baitaiteke.

Esker onak

Egileek esker ona adierazi nahi diete IDOKI SCF S.L. enpresari jariakin gainkritiko bidezko erauzketa sistema erabiltzen uzteagatik, Ostatu eta Mokoroaga ardotegiei ikerketarako beharrezko materiala eskaintzeagatik eta Eusko Jaurlaritzari SAIOTEK (SAI12/136) proiektuagatik. O. Aizpuruak esker onak adierazi nahi dizkio Eusko Jaurlaritzari baita bere aurre-doktoradutzako bekarengatik.

5 Bibliografia

[1] Shahidi F. 2012. Nutraceuticals, functional foods and dietary supplements in health and disease. J Food Drug Anal, 20, 226-230.

[2] Dugas AJ, Castañeda-Acosta J, Bonin GC, Price KL, Fisher NH, Winston GW. 2000. Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: structure-activity relationships. J Nat Prod, 63, 327-331.

[3] Tikhonov I, Roginsky V, Pliss E. 2009. The chain-breaking antioxidant activity of phenolic compounds with different numbers of O-H groups as determined during the oxidation of styrene. Int J Chem Kinet, 41, 92-100.

[4] Leopoldini M, Russo N, Toscano M. 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. Food Chem, 125, 288-306.

[5] Kim J, Lee KW, Lee HJ. 2014. Polyphenols suppress and modulate inflammation: Possible Roles in Health and Disease. In Watson RR, Preedy VR, Zibadi S, (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 393-408.

[6] Hertog MGL. 1994. Flavonols and Flavones in Foods and Their Relation with Cancer and Coronary Heart Disease Risk. Ph.D. Thesis, The Netherlands: Agricultural University Wageningen.

[7] Cordova FM, Watson RR 2014. Food and supplement polyphenol action in cancer recurrence. In Watson, RR, Preedy VR, Zibadi S (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 191-195.

[8] Jean-Gilles D, Li I, Vaidyanathan VG, King R, Cho B, Worthen DR, Chichester CO, Seeram NP. 2013. Inhibitory effects of polyphenol punicalagin on type-II collagen degradation in vitro and inflammation in vivo. Chem-Biol Interact, 205, 90-99.

[9] Menaa F, Menaa A. 2014. Skin photoprotection by polyphenols in animal models and humans. In Watson RR, Preedy VR, Zibadi S (Eds.) Polyphenols in Human Health and Disease. Academic Press, Massachusetts, 831-838.

[10] Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, Lamari FN. 2009. Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. Behav Brain Res, 198, 352–358.

[11] Fedačko J, Pella D, Mechírová V, Horvath P, Rybár R, Varjassyová P, Vargová V. 2007. N-3 PUFAs From dietary supplements to medicines. Pathophysiology, 14, 127-132.

[12] Tousoulis D, Plastiras A, Siasos G, Oikonomou E, Vergeniotis A, Kokkou, E, Maniatis K, Gouliopoulos N, Miliou A, Paraskevopoulos T, Stefanadis C. 2014. Omega-3 PUFAs improved endothelial function and arterial stiffness with a parallel antiinflammatory effect in adults with metabolic syndrome. Atherosclerosis, 232, 10-16.

[13] Stein O, Dabach Y, Ben-Naim M, Halperin G, Stein Y. 2008. Effects of oleic acid and macrophage recruitment on cholesterol efflux in cell culture and in vivo. Nutrition Metab and Cardiovas, 18, 596-601.

[14] Gnoni GV, Natali F, Geelen MJH, Siculella L. 2010. Oleic acid as an inhibitor of fatty acid and cholesterol synthesis. In Preedy VR, Watson RR (Eds.) Olives and olive oil in health and disease prevention. Academic Press, Massachusetts, 1365-1373.

[15] Mensink RP. 2013.Fatty Acids: health effects of saturated fatty acids. In Allen LH, Prentice A, Caballero B (Eds.) Encyclopedia of Human Nutrition. Academic Press, Massachusetts, 215-219.

[16] Aizpurua-Olaizola O, Ormazabal M, Vallejo A, Olivares M, Navarro P, Etxebarria N, Usobiaga A. 2015. Optimization of supercritical fluid consecutive extractions of fatty acids and polyphenols from *Vitis Vinifera* grape wastes. J Food Sci, 80, 101-107.

[17] Raventós M, Duarte S, Alarcón R. 2002. Application and Possibilities of Supercritical CO2 Extraction in Food Processing Industry: An Overview. Food Sci Tech Int, 8, 269-284.

[18] Sahena F, Zaidul ISM, Jinap S, Karim AA, Abbas KA, Norulaini NAN, Omar AKM. 2009. Application of supercritical CO2 in lipid extraction – A review. J Food Eng, 95, 240-253.

[19] Mendiola JA, Herrero M, Castro-Puyana M, Ibañez E. 2013. Supercritical Fluid Extraction. In Rostagno MA, Prado JM (Eds.) Natural Products Extraction. Royal Society of Chemistry, London, 196-230.

[20] Chaudot X, Tambuté A, Caude, M. 2000. Selective extraction of hydrocarbons, phosphonates and phosphonic acids from soils by successive supercritical fluid and pressurized liquid extractions. J Chromatogr A, 866, 231–240.

[21] Omar J, Alonso I, Garaikoetxea A, Etxebarria N. 2013. Optimization of focused ultrasound extraction (FUSE) and supercritical fluid extraction (SFE) of citrus peel volatile oils and antioxidants. Food Anal Method, 6, 1244-1252.

[22] Bakowska AM, Kucharska AZ, Oszmianski J. 2003. The effects of heating, UV irradiation and storage on stability of anthocyanin-polyphenol copigment complex. Food Chem, 81, 349-355.

[23] Munin A, Edwards-Lévy F. 2011. Encapsulation of Natural Polyphenolic Compounds; a Review. Pharmaceutics, 3, 793-829.

[24] Aizpurua-Olaizola , O., Navarro, P., Vallejo, A., Olivares, M., Etxebarria, N., Usobiaga, A. 2016. Microencapsulation and storage stability of polyphenols from Vitis vinifera grape wastes. Food Chem, 190, 614–621.

[25] Dorati R, Genta I, Modena T, Conti B. 2013. Microencapsulation of a hydrophilic model molecule through vibration nozzle and emulsion phase inversión technologies. J Microencapsul, 30, 559-570.

[26] King AH. 1983. Brown Seaweed Extracts (Alginates). In Glicksman M(Ed.) Food Hydrocolloids. CRC Press, Boca Raton, 115.

[27] Krasaekoopt W, Bhandari B, Deeth H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. Int Dairy J, 13, 3-13.

[28] Xu Y., Zhan C, Fan L, Wang L, Zheng H. 2007. Preparation of dual crosslinked alginate-chitosan blend gel beads and in vitro controlled release in oral site-specific drug delivery system. Int J Pharm, 336, 329-337.

[29] Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phospho-molybdic–phosphotungstic acid reagents. Am J Enol Viticult, 16, 144–158.

Identification and quantification of cannabinoids in *Cannabis sativa L.* plants by high performance liquid chromatography-mass spectrometry

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CHAPTER 6

Abstract

High Performance Liquid Chromatography tandem mass spectrometry (HPLC-MS/MS) has been successfully applied to cannabis plant extracts in order to identify cannabinoid compounds after their quantitative isolation by means of supercritical fluid extraction (SFE). MS conditions were optimized by means of a Central Composite Design (CCD) approach and the analysis method was fully validated. 6 major cannabinoids (Tetrahydrocannabinolic acid (THCA), Tetrahydrocannabinol (THC), Cannabidiol (CBD), Tetrahydrocannabivarin (THCV), Cannabigerol (CBG) and Cannabinol (CBN)) were quantified (RSD < 10 %) and 7 more cannabinoids were identified and verified by means of a liquid chromatographer coupled to a quadrupole-time-of-flight (Q-ToF) detector. Finally, based on the distribution of the analysed cannabinoids in 30 *Cannabis sativa L.* plant varieties and the principal component analysis of the resulting data it was observed a clear difference between outdoor and indoor grown plants which was attributed to a higher concentration of THC, CBN and CBD in outdoor grown plants.

Keywords: Cannabinoid analysis; Liquid chromatography; Mass spectrometry; Fingerprinting.

1 Introduction

The widespread consumption of *Cannabis sativa L.* as a recreational drug, competes with a more interesting and promising use of it as a medicinal plant. In fact, Cannabis has been applied as a therapeutical drug in many diseases such as multiple sclerosis, chronic pain, glaucoma, asthma, etc. [1].

The fact that the plant has more than 500 compounds makes it a complex matrix, even though the main focus of interest of this plant is in the content and distribution of the phytocannabinoids, which increases the complexity of any herbal extract [2-5]. Moreover, the varieties of species (sativa, indica and rudelaris), all the hybridized strains that have been spread all over the world; the uptake ways and the posologies of it complicated the use of standard protocols. In addition to this, cannabinoids are biosynthesized in an acidic form among which the most abundant are cannabidiolic acid (CBDA) and Δ9-tetrahydrocannabinolic acid A (THCA-A). However, this acidic cannabinoids are not stable since they may decompose in the presence of light or heat. Overall, acidic cannabinoids are decarboxylated to their neutral homologues, as in the case of tetrahydrocannabinolic acid (THCA) which is decarboxylated to tetrahydrocannabinol (THC) [6].

THC is accepted to be the main psychoactive agent and it possesses analgesic, anti-inflammatory, appetite stimulant and antiemetic properties and it can also protect the brain from cognitive deficits at very low doses [7-8]. However, regular use of the plant may cause cognitive deficits at least in adolescents, since until the early 30s they are having significant neurodevelopmental changes [9-10]. The neurocognitive effects of extended use in adults are still somewhat inconsistent [9]. Cannabidiol (CBD) can modulate euphoric effects of THC and has antipsychotic, neuroprotective, anticancer, antidiabetic and other effects such as reducing the anxiety induced by fear or reducing cigarette consumption in tobacco smokers [11-16]. Other minor cannabinoids present in cannabis are known to have diverse biological activities. Cannabigerol (CBG), for example, has antibacterial, antiproliferative and bone-stimulant properties. Cannabinol (CBN) has sedative or stupefying effect. Tetrahydrocannabivarin (THCV) has anorexic and antiepileptic effects and, also, it may be clinically effective in migraine treatment [12, 14]. Thus, cannabis preparations may provide advantages over other single-compound synthetic drugs. The therapeutic effects of major constituents may be enhanced by other cannabinoids or non-cannabinoids whereas some unwanted side effects may be mitigated [12, 17-21].

As mentioned before, one of the major drawbacks of using cannabis plants medically is the lack of standardization. As an example, in a study developed by Hazekamp et al. reported that the nominal concentrations of target cannabinoids obtained for the same plant variety but from different coffee shops varied more than 25 % [22]. To overcome this problem there are at least two approaches: one is leaned towards the tight control of the varieties and strains and the way they are grown in order to assure the highest homogeneity in the final plants, and the other one is focused on the extracts blending to offer the desired products [22, 23].

To address the extraction of bioactive compounds, Supercritical Fluid Extraction (SFE) is one of most suitable technique [24] due to the safe use of CO_2 as the main solvent and ethanol as a cosolvent compared to other commonly used solvents in the extraction of cannabinoids [24-25]. Moreover, it

assures the stability of thermo labile and light sensitive compounds and it is scalable up to industrial size [24]. In addition, SFE can be used with cannabis samples with very good yields [26].

In addition to the development of more efficient methods to extract major and trace elements from complex plant matrices, a great effort has been made in order to develop robust and sensitive chromatographic methods able to resolve complex mixtures. Two separation methods (gas-chromatography (GC) and high performance liquid chromatography (HPLC)) have been mainly used in most researches dealing with the analysis of cannabinoids [23, 26-28]. Some works have pointed that one dimension GC does not offer enough resolution to analyze such complex mixtures. In this sense, hyphenated techniques such as Comprehensive Two Dimensional Gas Chromatography (GCxGC) have already been successfully employed to establish chemical profiles of different cannabis and heroin samples [29, 30]. Nonetheless, GC analysis requires a derivatization step to measure the thermo labile acidic cannabinoids (due to the decarboxylation process in the injection port). On the contrary, these compounds can be directly analyzed by means of HPLC without any derivatization step [28], which is a major advantage in order to obtain a more complete chemical profile of the cannabis samples. In addition to this, other techniques, such as Supercritical Fluid Chromatography (SFC) have been used to analyze acidic cannabinoids. SFC with Photodiode Array Detection (PDA) is less sensitive than GC or HPLC coupled to mass spectrometry (MS) but the analysis time is much shorter [31].

The performance of HPLC-MS/MS depends on the type of mass analyzer used in the analysis [32]. Nowadays, the most widely used analyzers are the triple quadrupole instruments (QqQ), which have excellent sensitivity and selectivity to quantify analytes. However, they do not allow structural identification of non-target compounds as they provide only nominal mass measurements. In this respect, time-of flight analyzers (ToF) are often used in order to obtain the structural information of the target compounds [33-34]. Coupled to a quadrupole mass filter, Q-ToF mass spectrometers provide accurate mass identification (< 5 ppm, accepted accuracy threshold for confirmation of elemental composition) for both, the precursor and the product ions [32]. This allows differentiating between two different compounds with the same nominal mass but with different elemental composition.

In the framework of a full characterization of ecologically grown Cannabis plants of 30 different varieties under tight control of the strains and growing conditions, the main aim of this work was to develop and validate an HPLC-MS/MS method to quantify the major cannabinoids, and to identify and extract the fingerprints of the less abundant cannabinoids by a HPLC-qToF approach.

2 Materials and methods

2.1 Materials and reagents

Cannabinoid standard compounds (THCA, CBG and THCV) were purchased from Echo Pharmaceuticals BV (Weesp, The Netherlands) and reference standards (THC, CBD, CBN and THC-d_3) were purchased from Cerilliant (Round Rock, Texas, USA). HPLC grade EtOH and MeOH were obtained from Panreac (Barcelona, Spain), LC/MS grade formic acid from Thermo Fisher Scientific

(Erembodegem, Belgium), Leucine Enkephalin acetate hydrate from Sigma-Aldrich Chemie (Steinheim, Germany), 99.9995 % purity CO_2 from Air Liquide (Madrid, Spain) and washed thin sea sand from Scharlau (Sentmenat, Spain). De-ionized water was generated with a Milli-Q water purification system Element 10 from Millipore (< 0.057 S/cm, Milli-Q model, Millipore, Billerica, USA).

2.2 Samples

Cannabis plant material was provided by Ganjazz Art Club (Donostia, Basque Country, Spain). Ten types of plants were chosen to develop and validate the analytical method (MW: Medicine Woman, AM: Amnesia, GW: Great White Sarck, TI: Tijuana and SO: Somango from indoor and BU: Bubba Kush, BL: Blueberry SS: Super Skunk, GR: Grapefruit and TR: Trainwreck X HP from outdoor) and 20 more for fingerprinting (Parmir, Power plant, AK 47, N.Y.C Diesel, Jaggen, Cheese, Chocolope, Deep Chunk, OG Kush, Soul Diesel, Skunk Green, Super Lemon Haze, Super Silver Haze and Neviles Haze from indoor and Amnesia, Critical, Chocolope, Cream Caramel, Super Lemon Skunk, Trainwreck and Grapefruit from outdoor).

The plants were grown from clones of a motherplant and each one had its optimum flowering growth time. For indoor plants, vegetative growth was carried out from the 1st of October of 2012 to 25th of October, and the flowering growth until 7th of January of 2013. This last phase was developed under 600 watt lamps with 70000 lx. They were dried in closets during 15 days and then carried in wood boxes to an air-tight container and stored in a cool dry place during at least another 15 days. Though no pesticide was used, the occurrence of plants diseases like mildew was less than 5 %. In the case of outdoor plants, their vegetative growth was carried out indoor from 15th of July of 2013 to 15th of October of 2013 under a complete lamp supply with micro and macro-organisms but without pesticides. The flowering phase was developed outdoor in greenhouses and plants were pulverized with neem oil and other no-chemical preventives like bacilus turigensis against larvas. The mildew level was between 10-15 % depending on cannabis variety.

The plant samples were cryo-milled under liquid nitrogen at 660 rpm during 4 min (SPEX SamplePrep, 6770 Freezer/Mill, Madrid, Spain) and stored frozen (-20 °C) in amber glass vials until their analysis (maximum one month).

2.3 SFE of cannabis plants

SFE was performed on a Thar SFC, Waters company (Saint-Quentin, France) Method Station SFC system, consisting of a Fluid Delivery Module (CO_2 pump and solvent pump), a high speed Alias autosampler, an Analytical-2-Prep column, a Photodiode Array Detector (PAD, Waters 2998), an Automated Back Pressure Regulator (ABPR) and a high pressure extraction vessel of 1 mL (EV-1 Jasco). Extraction conditions employed were previously optimized by our research group in a previous work [26]. Briefly, Cannabis plant was accurately weighted (0.05 g) in the extraction vessel and 1 g of sea sand was added to fill it. CO_2 was used as extraction solvent and EtOH (20 %) as co-solvent in order to modify polarity. Samples were extracted for 10 minutes at 35 °C with a total flow of 1 mL/min at a fixed pressure

of 100 bar. Extracts were collected in amber glass vials with 4 mL of EtOH and kept at -20 °C until their analysis (maximum one week).

2.4 Liquid chromatography tandem mass spectrometry with triple quadrupole detection (HPLC-MS/MS)

The HPLC-MS/MS analyses were performed using an Agilent Technologies (Santa Clara (CA), USA) Infinity liquid 1260 chromatographic system, consisting of an autosampler, a column thermostat and a binary solvent management system coupled to a triple quadrupole (Agilent Technologies 6430) equipped with an Atmospheric Pressure Chemical Ionization ion source (APCI).

The chromatographic separation was achieved using a Kinetex C18 column (2.6 µm, 150 mm x 3 mm i.d.) with a guard column (0.5 µm Depth Filter x 0.1 mm) (Phenomenex, Torrance (CA), USA) and a binary A/B gradient (Solvent A: Milli Q water with 0.1 % formic acid, and, solvent B: MeOH with 0.1 % formic acid). The gradient program was established as follows: initial conditions were 50 % B; raised to 80 % B over the first minute, held at 80 % B until 11 minutes, increased to 95 % B over the next 2 minutes, held at 95 % B until 16 minutes, decreased to 50 % B over the next 2 minutes and held at 50 % B until 28 minutes for re-equilibration of the system prior to the next injection. A flow rate of 0.25 mL/min was used; the column temperature was 30 ºC and the injection volume was 10 µL.

MS acquisition was carried out in the APCI positive ionization mode. The conditions were set as follows: corona discharge current of 5 µA, capillary voltage of 3500 V, heated vaporizer at 280 °C, a nitrogen flow rate of 7 L/min, a source temperature of 210 °C and nebulizer pressure of 32 psi. For the quantification of the target cannabinoids, multiple reaction monitoring (MRM) mode was used and two transitions were monitored (one used as quantifier and the other one used as qualifier) (see Table 1).

The quantification of the extracts by means of LC-MS/MS was performed with external calibration; i.e., a set of standards containing target compounds at concentrations ranging from 0.5 to 1000 ng/mL in MeOH were analyzed in the same conditions of the samples. System fluctuations were corrected with an isotopically labeled standard (THC-d_3) used as internal standard.

Due to no reference material was available to validate the HPLC-MS/MS analysis method it was decided to compare it with a SFC method. SFC analyses were carried out in the same Thar SFC system used for extraction. The compounds were separated in a Kromasil normal phase analytical DIOL column (5 µm, 250 mm x 4.6 mm i.d.) (Teknokroma, Spain). 5 µL were injected in the loop injection system. MeOH was chosen as co-solvent to increase the polarity of the supercritical CO_2. Different MeOH percentages (2-18 %) and flows (1.0 – 1.5 mL/min) were tried in order to fit the best separation of the target compounds. Under optimum conditions, the samples were analyzed at 40 °C using a 15 % MeOH at a flow of 1.5 mL/min. System pressure was fixed at 150 bar and compounds were monitored at 220 nm in the PDA. The analysis time was 7 min.

Calibration curves were built in MeOH in the range of 0.5-5 µg/mL for THC and 2-50 µg/mL for THCA. Under these conditions, dilution of the samples (1:50 in MeOH) was required in order to avoid chromatographic column saturation.

2.5 Ultra-performance liquid chromatography - quadrupole time of flight mass spectrometry (UPLC-qTOF)

The identification of unknown cannabinoids was performed using an ACQUITY UPLCTM system from Waters (Milford, MA, USA), equipped with a binary solvent delivery pump an autosampler and a column compartment. The same phase column and pre-column used in low resolution analysis were used at 30 ºC for separation of cannabinoids. Flow rate was 0.25 mL/min and injection volume was 10 µL. Mobile phases consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in methanol (B). Separation was carried out in 22 minutes. Initial conditions were 50 % B, raised to 100 % B over 15 minutes, held at 100 % B until 17 minutes, decreased to 50 % B over the next 2 minutesand held at 50 % B until 22 minutes for re-equilibration of the system prior to the next injection. All samples were kept at 4 ºC during the analysis.

All MS data acquisitions were performed on a SYNAPT^{TM}G2 HDMS with a Q-ToF configuration (Waters, Milford, MA, USA) equipped with an APCI source operating at 450 ºC in positive mode. The capillary voltage was set to 0.7 kV and corona discharge to 5 µA. Nitrogen was used as the desolvation and cone gas at flow rates of 800 L/h and 20 L/h, respectively. The source temperature was 120 ºC and the desolvation temperature was 300 ºC. Leucine-enkephalin solution was used for the lock mass correction, monitoring the ions at mass-to-charge ratio (m/z) 556.2771 and 278.1141. All of the acquired spectra were automatically corrected during acquisition based on the lock mass. Data was acquired in the mass range 50-1200 u in resolution mode (FWHM ≈ 20,000). Before analysis, the mass spectrometer was calibrated with a sodium formiate solution.

Low collision energy MS experiments and high collision energy MS/MS assays were performed over a single experimental run using an acquisition mode called MSE. In this way, molecular ions data and fragment ions data were obtained in the same run, essential for structure elucidation. In positive mode, only the protonated molecules were able to form adducts with mobile phase species, so their presence in the low collision energy spectra provide an unequivocal identification of the $[M+H]^+$ ions. The sodium adducts $[M+Na]^+$ at 22 u above the proposed protonated molecule were also used for this purpose.

2.6 Data treatment

Based on the analytical results obtained from the 30 plant samples a multivariate data treatment was carried out to assess the differences between varieties of *Cannabis sativa L*. Unsupervised pattern recognition was accomplished with the statistical software The Unscrambler® (9.7 Camo Asa, Oslo, Norway) in order to identify the main variation sources and the distribution of the collected samples. LC-MS/MS data were normalized sample-wise in order to avoid any systematic trend due to higher or lower concentrations and then treated by principal component analysis (PCA). PCA uses an orthogonal transformation to convert a number of possibly correlated variables into linearly uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the matrix data as possible and the next principal components accounts for as much of the remaining

variability as possible. This way, the dimensionality of the data set can be reduced and the underlying variables identified.

Firstly, a PCA was performed with only quantified cannabinoid concentration data (30 sample x 6 analytes), and secondly, a PCA with all cannabinoids corrected area data (30 sample x 13 analytes) was performed to see whether exist any difference between them. Both models were built by leverage correction.

3 Results and discussion

3.1 Optimization of HPLC-MS/MS analysis

According to previous works, it is well-known that different solvent combinations can be employed for the separation and analysis of cannabinoids in HPLC-MS/MS [35-36]. In this work, different solvent modifiers were tested: (i) water and MeOH both with 2-10 mM NH_4Ac; (ii) water and MeOH both with 0.1-0.2 % (v/v) formic acid;(iii) water and MeOH both with 0.1-0.2 % (v/v) formic acid and 2-10 mM NH_4Ac. The influence of flow rate (0.1-0.25 mL/min) and injection volume (2-20 µL) were also tested. The most appropriate conditions for target analytes separation taking into account the best peak shape and the highest sensitivity were obtained using a mobile phase consisted of a mixture of water with 0.1 % formic acid (A) and MeOH with 0.1 % formic acid (B), a flow rate of 0.25 mL/min and 10 µL of injection volume. The column temperature was maintained constant at 30 ºC during the run.

The effect of MS acquisition parameters was optimized by means of a Central Composite Design (CCD) approach covering the following factor spaces: capillary voltage 1120-5880 V, heated vaporizer temperature 155-485 °C, nitrogen flow 1.05-12.95 L/min, nitrogen temperature 65-350 °C and nebulizer pressure 3-60 psi. Peak areas obtained for a standard mixture containing CBD, THC and CBN at 1 µg/mL were used as the design responses. Though a CCD with 5 variables is a rather demanding approach, all the experiments (i.e., 45 experiments including 3 replicates of the central point) were carried out without the analytical column and therefore they were accomplished during the same day. As a result, we were able to build and analyze the response surface to find the maximum sensitivity and the highest resolution. The precision, in terms of relative standard deviation (RSD) was estimated from the 3 replicates of the central point (RSD < 2 % for all the target compounds) and the data was treated with The Unscrambler® software in order to build the response surfaces by multiple linear regression (MLR).

MLR data revealed that the significant factors affecting the peak areas were the heated vaporizer temperature and the nebulizer pressure (p-level < 0.05) for all the studied compounds. As shown in Figure 1 for CBD, the local maximum was found at the low-medium heated vaporizer values and medium nebulizer values within the factor space. This pattern was also observed for the rest of the analytes. The conditions of the non-significant parameters were established also according the obtained response surfaces for each compound. In this way, MS parameters optimal conditions were fixed as follows:

3500 V of capillary voltage, 280 °C of vaporizer temperature, 7 L/min of nitrogen at 210 °C and 32 psi of nebulizer. Capillary voltage and nitrogen flow and temperature were set at the medium value as they were not significant and for practicality. Once these parameters were optimized, the influence of corona discharge intensity was assessed in the 2-10 µA range, and the maximum sensitivity was attained at 5 µA of corona for the majority of the target compounds.

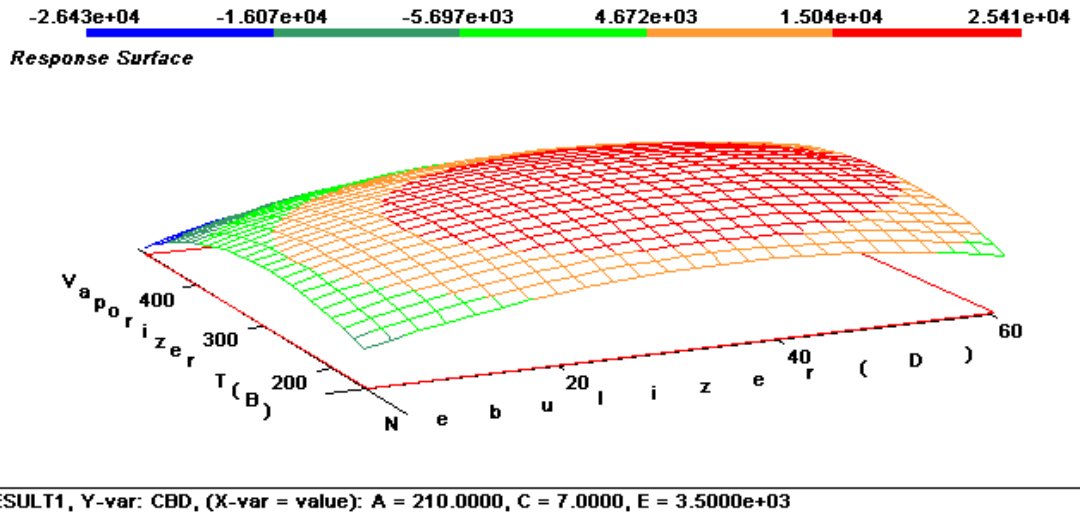

Figure 1: Response surface of vaporizer temperature (ºC) and nebulizer pressure (psi) for the MS parameter optimization for CBD analyte.

Regarding the MRM detection mode, it was fully optimized using a standard solution of each target compound at 500 ppb in MeOH in order to find out the optimum precursor ions, which corresponded to the most intense ions with the highest m/z ratio, the product ions and the collision energies. 8 different collision energies were assessed in the 5-40 eV range in order to opt for the most intense product ions. Two different transitions were selected accordingly; one use for quantification purposes and the second one for qualification purposes (see Table 1).

Table 1
MRM transitions, optimized potentials, qualifier/quantifier area ratios and retention times of the analytes and the internal standard for HPLC-MS/MS analysis. Mass/charge ratio of the precursor ion (Q1), mass/charge ratio of the fragment ion (Q2), fragmentor potential (FP), collision energy (CE), qualifier/quantifier ratio (Q/Q), retention time (t_R).

Analyte	Q1 (u)	Q2 (u)	FP (V)	CE (eV)	Q/Q (%)	t_R (min)
Known compounds		192.8	40	20		
CBD	315.1	259.0	40	15	67	8.7
THCV	287.1	165.0	80	20		
		231.0	80	15	28	8.9
CBG	317.2	193.0	120	10		
		123.0	120	25	24	8.9
CBN	311.0	222.9	50	15		
		293.0	50	10	48	10.8
THC	315.0	193.0	70	20		
		259.0	70	20	38	12.1
THCA	315.1	193.0	100	20		
		259.1	100	15	49	17.0
Unknown compounds		316.9	60	10		
C1	372.9	180.7	60	10	23	6.9
C2	359.0	341.0	100	10		
		218.8	100	30	22	7.7
C3	375.0	209.0	100	20		
		251.0	100	5	68	8.7
C4	315.0	193.0	100	20		
		258.9	100	15	45	9.3
C5	317.1	193.1	100	10		
		123.0	100	30	28	10.8
C6	375.0	251.0	80	5		
		209.0	80	20	81	11.3
C7	287.2	165.1	120	20		
		123.1	120	30	44	12.7
Internal standard		195.9	120	25		
THC-d3	317.8	262.0	120	20	54	12.0

3.2 Figure of merits

HPLC-MS/MS analysis method was fully validated (see Table 2) over a range of 0.5-2000 ng/mL. The calibration curves were linear ($R^2 > 0.999$) in the concentration ranges studied for each analyte.

The instrumental limits of detection (LODs) were set at the lowest concentration where the signal-to-noise ratios of the analytes were higher than 3. All the obtained LODs were between 0.02 and 0.2 ng/mL.

To check the trueness of the developed method, different aliquots of MeOH were spiked at two different concentration levels within the calibration curve range: 3 replicates in the low concentration range (10 ng/mL) and another 3spiked samples in the high concentration range (1500 ng/mL). Trueness, expressed in terms of relative bias (%), was acceptable for all cannabinoids at both low and high concentration levels (see Table 2).

Table 2
Figures of merit of the developed HPLC-MS/MS method: Coefficients of determination (R^2), low (10 ng/mL) and high (1500 ng/mL) concentration range trueness expressed in terms of bias (%), instrumental limits of detection (LOD) and repeatability and reproducibility expressed as relative standard deviations (RSD).

Analyte	R^2	Trueness (%)		LOD	Precision (%)	
		Low	High	(ng/mL)	Repeatability.	Reproducibility.
CBD	0.9998	4.9	0.1	0.2	10	7
THCV	0.9999	2.9	0.7	0.05	6	2
CBG	0.9998	4.2	3.2	0.02	7	2
CBN	0.9998	1.0	1.6	0.05	7	4
THC	0.9998	1.2	1.7	0.05	5	5
THCA	0.9992	1.9	1.5	0.2	5	4

The precision of the whole method was assessed calculating the relative standard deviations (RSDs) for repeatability and reproducibility. Repeatability was quantified by intra-day variation carrying out SFE extractions from the same sample (Somango) and analyzing them (n = 5), and reproducibility by inter-day variation, extracting the same sample in triplicate at three different days. The RSD values ranged from 5 to10 % in the case of the samples analyzed in the same day whereas the RSD values obtained from the analyses performed in different days varied from 2 to 7 %.

As no reference material was available, the accuracy of the developed method was assessed comparing the concentration of cannabinoid compounds in real cannabis plants with SFC-PDA. To this end, ten varieties of cannabis were analyzed in triplicate by SFC-PDA and the results were compared with the HPLC-MS/MS method. Unfortunately, due to the lower sensitivity of the SFC-PDA method (i.e., instrumental limits of detection higher than 500 ng/mL), the concentration of the major cannabinoids, i.e. THC and THCA, were only determined. The concentration of these analytes obtained by both methods were statistically comparable (p-level < 0.05) as can be seen in Figure 2.

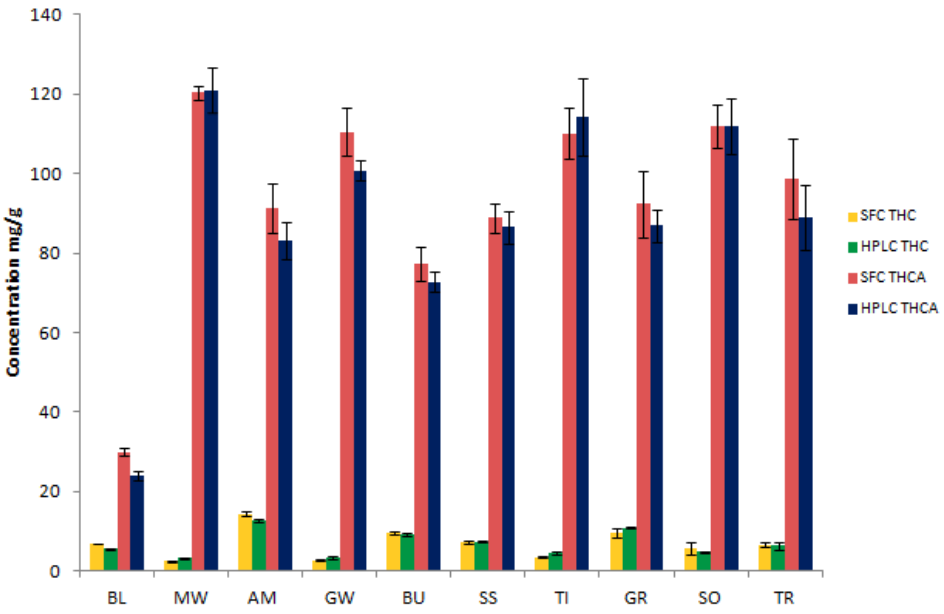

Figure 2: Representation of THC and THCA concentrations (mg/g) of different cannabis plants extracted by SFC and analyzed by HPLC-MS/MS (n=3). BL: *Blueberry*, MW: *Medicine Woman*, AM: *Amnesia*, GW: *Great White Sarck*, BU: *Bubba Kush*, SS: *Super Skunk*, TI: *Tijuana*, GR: *Grapefruit*, SO: *Somango* and TR: *Trainwreck X HP*.

3.3 Identification of the cannabis extracts by HPLC-MS/MS and UPLC-qToF

Since more than 70 cannabinoids can be found in the extracts of cannabis [3, 5], and the distribution of these cannabinoids can be extended in a very wide range of concentrations, sample extracts were initially analyzed by HPLC-MS/MS in scan mode in order to obtain their mass spectra with complete information and so, ease the compound identification. In the first approach two different distributions were observed in the Somango variety of a real cannabis plant. On the one hand, some analytes were found at concentrations close to the limit of detection such as CBD or THCV. On the other hand, major cannabinoids such as, THC and THCA were found at high concentrations and they could saturate the detector. Therefore, three different dilutions (i.e., 1:10, 1:500 and 1:5000) of the sample extracts were injected in the HPLC-MS/MS system in order to detect properly all the analytes of interest. When less diluted samples were injected, the most concentrated analytes (i.e. THCA and THC) were sent to waste after the chromatographic separation and, thereby, they did not enter to the detector. Although they passed though the column it was not observed any saturation or carry-over problem after a sample cleaning injection with MeOH.

This way, as can be seen in Table 1, 6 different cannabinoids were identified and quantified using pure standards in the Somango cannabis plant. Besides, the known compounds, other 7 unknown compounds were detected based on the mass spectra collected in the scan mode (see Figure 3) and based on the information found in the literature [5, 37]. In the later case, after the identification of the precursor ions ($[M+H]^+$) of the 7 unknown cannabinoids, the fragmentation pattern at different collision energies was assesed in order to identify the unknown cannabinoids.

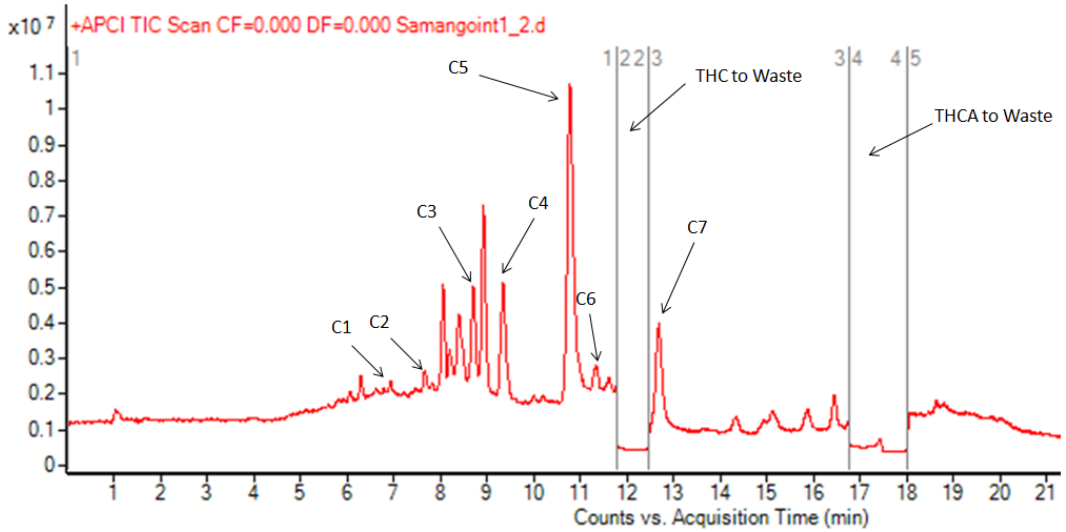

Figure 3: Scan chromatogram of *Somango* (SO) sample extract (dilution 1:10) where THCA and THC were not analyzed.

The identification of unknown compounds (C1-C7) was verified by means of UPLC–qToF, which provides high resolution and accurate mass measurements of the precursor and fragments ions [32]. Moreover, the formation of positively charged adducts $[M+Na]^+$ instead of the precursor ion $[M+H]^+$ is often observed in the positive ionization mode. This sodium adducts are valuable to confirm the $[M+H]^+$

identification owing to the 23 u higher molecular mass. This strategy was successfully applied to identify the unknown seven cannabinoids. Their experimental, theoretical masses and the suggested molecular formula as well as the compound name are shown in Table 3. The errors obtained in the identification for all the compounds were ≤ 1.2 mDa.

Table 3
UPLC-qToF verification in the identification of unknown (C1-C7) cannabinoid compounds.

	$[M+H]^+$	Calc. Mass	% error mDa	% error ppm	Molecular formula	Compound name
C1	373.2011	373.2015	0.4	1.1	$C_{22}H_{29}O_5$	Cannabicoumaric acid
C2	359.2113	359.2222	0.9	2.5	$C_{22}H_{31}O_4$	CBCA
C3	375.2537	375.2535	0.2	0.5	$C_{23}H_{35}O_4$	10-ethoxy-9-hydroxy-Δ^6a-THC
C4	373.2375	373.2380	0.5	1.3	$C_{23}H_{33}O_4$	4-Acetoxycannabichrome
C5	361.2367	361.2379	1.2	3.3	$C_{22}H_{32}O_4$	CBGA
C6	365.2538	375.2535	0.3	0.8	$C_{23}H_{35}O_4$	CBGAM
C7	345.2064	345.2066	0.2	0.6	$C_{21}H_{29}O_4$	THCA-C4

C1 was identified as Cannabicoumaric acid as $[M+Na]^+$ reassured the molecular weight and among all known cannabinoids the $C_{22}H_{29}O_5$ formula is unique for this compound. C2 was identified as Cannabichromenic acid (CBCA) as it showed a loss of 44 u (359→315) and $[M+Na]^+$ aduct confirmed the molecular weight. The loss of 44 u is due to the loss of carboxyl groups of the acid cannabinoids, which are not very stable since their carboxyl group is cleaved off as CO_2 under the influence of heat or light, resulting in their corresponding neutral cannabinoids. The mass of the molecular ion could have been attributed to Cannabidiolic acid (CBDA) but, the fragmentation spectrum is quite different from its neutral compound, CBD, and moreover, in female flowers the presence of CBD-type cannabinoids is expected to be low. C5 and C6 were identified also as acid cannabinoids becauseall of them showed a loss of 44 u. Their fragmentation pathways were similar to CBG and their quasimolecular ion $[M+H]^+$, reassured by the sodium adducts, proved that the unknown compounds C5 and C6 were Cannabigerolic acid (CBGA) and Cannabigerolic acid monomethylether (CBGAM) respectively. The loss of the methyl group (331→317 m/z) supported also this identification (Figure 4). C3 showed the same chemical formula of C6 but, based on the different fragmentation patterns, this compound was identified as10-ethoxy-9-hydroxy-Δ6a-THC. The chemical formula assigned to C4 was also unique among all known cannabinoids, that enabled the identification of this compound as 4-Acetoxycannabichrome. Finally, C7 was identified as THCA-C4 because its fragmentation patterns were very similar to THCA, which was also supported by the $[M+H]^+$ adduct.

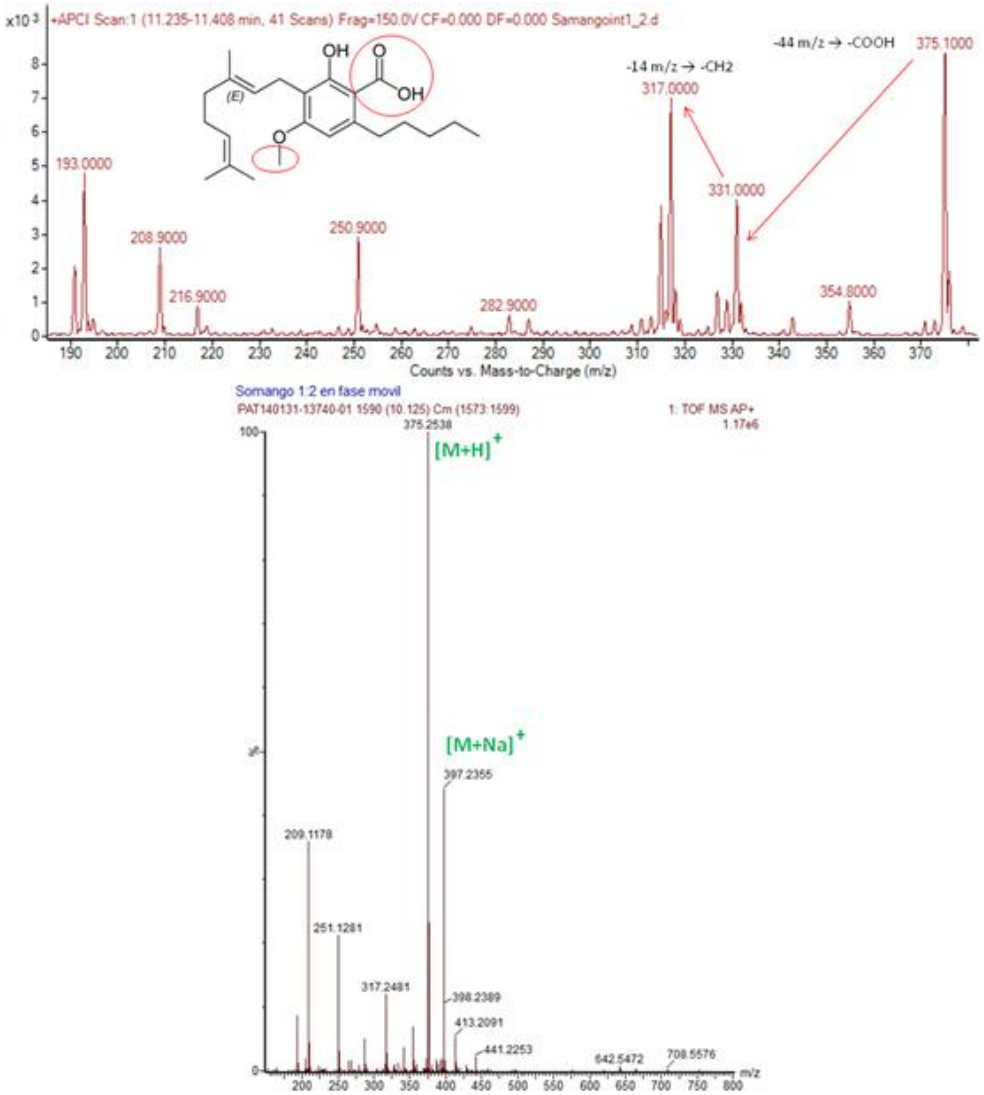

Figure 4: C6 compound, identified as cannabigerolic acid monomethylether (CBGAM). Up spectra is from HPLC-MS/MS and down spectra from UPLC-qToF.

3.4 Quantification of the cannabis extracts by HPLC-MS/MS and statistical data treatment

Once the analysis method was validated and the unknown cannabinoids were identified in Somango, 30 different *Cannabis sativa L.* varieties were subsequently analyzed. 6 major cannabinoids were quantified (Table 4) and the other previously identified 7 compounds were qualified correcting the areas with the areas of the deuterated internal standard THC-d3. Relative standard deviations were calculated carrying out 5 extractions from the Somango cannabis extract sample (RSD < 10 %) (Table 2).

As it can be observed in Table 4, overall, indoor grown plants have less concentration of CBN. This may be attributed to the controlled ambient conditions in which the indoor plants were grown because it is known that CBN is produced when THC is exposed to air and consequently degraded to CBN [2]. In the same way, outdoor plants have also higher degradation of acidic cannabinoids to their corresponding neutral compounds. Owing to this, they have more THC and CBD than indoor plants. On the contrary, the adverse meteorological conditions do not affect the growth of indoor plants, so that they retain better the resin where cannabinoids are in higher concentration, resulting in a lower degradation

[2]. Nonetheless, concentrations found for THCA, CBG and THCV varied a lot from one variety to another.

Table 4
Quantification results of cannabinoids in HPLC-MS/MS of different *Cannabis sativa L.* varieties. I = Indoor, O = Outdoor.

Sample	I/O	THCA (mg/g)	THC (mg/g)	CBG (mg/g)	THCV (µg/g)	CBN (µg/g)	CBD (µg/g)
Parmir	**I1**	81±4	2.6±0.2	0.37±0.03	35±2	11.2±0.8	1.6±0.2
G. White Sarck	**I2**	99±5	3.7±0.2	0.37±0.03	56±3	7.5±0.5	2.9±0.3
Power Plant	**I3**	107±5	2.0±0.1	0.39±0.03	70±4	11.6±0.8	1.8±0.2
AK 47	**I4**	74±4	1.2±0.1	0.30±0.02	33±2	7.4±0.5	0.67±0.07
N.Y.C. Diesel	**I5**	114±6	2.2±0.1	1.14±0.08	35±2	7.0±0.5	2.4±0.2
Jaggen	**I6**	91±5	2.9±0.2	0.67±0.05	62±4	10.2±0.7	2.1±0.2
Medicine Woman	**I7**	119±6	3.6±0.2	1.23±0.08	60±4	11.1±0.8	2.5±0.2
Amnesia	**I8**	117±6	2.7±0.2	1.04±0.07	97±6	18±1	3.9±0.4
Cheese	**I9**	70±4	1.1±0.1	0.54±0.04	13.7±0.8	4.6±0.3	1.5±0.2
Chocolope	**I10**	94±5	2.9±0.2	0.55±0.04	12.4±0.8	10.9±0.8	3.4±0.3
Deep Chunk	**I11**	71±4	1.3±0.1	0.16±0.01	31±2	6.1±0.4	1.9±0.2
OG Kush	**I12**	67±3	1.8±0.1	0.34±0.02	27±2	2.4±0.2	1.9±0.2
Soul Diesel	**I13**	70±4	1.4±0.1	0.19±0.01	26±2	4.5±0.3	2.5±0.2
Skunk Green	**I14**	80±4	2.0±0.1	0.076±0.005	38±2	15±1	2.8±0.3
S. Lemon Haze	**I15**	69±3	3.5±0.2	0.30±0.02	310±20	13.0±0.9	3.6±0.4
S. Silver Haze	**I16**	105±5	3.2±0.2	0.53±0.04	134±8	9.1±0.1	3.5±0.4
Tijuana	**I17**	92±5	3.6±0.2	0.73±0.05	135±8	13.0±0.9	4.5±0.4
Neviles Haze	**I18**	63±3	1.9±0.1	0.067±0.005	63±4	5.9±0.4	2.2±0.2
Somango	**I19**	86±4	4.6±0.3	0.68±0.05	240±10	10.0±0.1	3.7±0.4
Amnesia	**O1**	91±5	16±1	0.74±0.05	94±6	91±6	9.1±0.9
Critical	**O2**	112±6	7.6±0.5	0.38±0.03	153±9	61±4	5.0±0.5
Blueberry	**O3**	30±2	6.5±0.4	0.100±0.007	28±2	60±4	3.3±0.3
Chocolope	**O4**	80±4	25±2	0.75±0.05	5.8±0.3	84±6	14±1
Cream Caramel	**O5**	113±6	10.8±0.7	1.17±0.08	103±6	63±4	6.9±0.7
Bubba Kush	**O6**	69±3	9.1±0.5	0.018±0.001	52±3	61±4	6.0±0.6
S. Lemon Skunk	**O7**	51±3	17±1	0.54±0.04	4.5±0.3	91±6	10±1
Super Skunk	**O8**	76±4	5.0±0.3	0.39±0.03	69±4	59±4	6.0±0.6
Trainwreck	**O9**	65±3	22±1	0.48±0.03	3.6±0.2	73±5	12±1
Trainwreck X HP	**O10**	71±4	6.0±0.4	0.33±0.02	98±6	58±4	3.4±0.3
Grapefruit	**O11**	73±4	9.6±0.6	0.39±0.03	107±6	470±30	10±1

Principal component analysis was carried out to reduce the dimensionality of the data set and identify better the variation of the cannabinoid concentrations between plant varieties, Thus, the PCA analysis of the 30 samples required two PCs to explain up to 76 % of the total variance when the quantified cannabinoids were considered and 62 % when all the cannabinoids (quantified and qualitatively identified compounds) were included.

Figure 5 shows the score plot of the PCA model with the quantified cannabinoid data-set and, as can be seen, the indoor and outdoor plants are clearly distinguished mostly by CBN. As mentioned before, it can be observed that outdoor grown plants have more CBN, THC and CBD probably due to the effect of the weather alterations. However, the distribution of THCA, THCV and CBG did not offer any meaningful pattern, probably because the differences between varieties are much higher than the degradation differences.

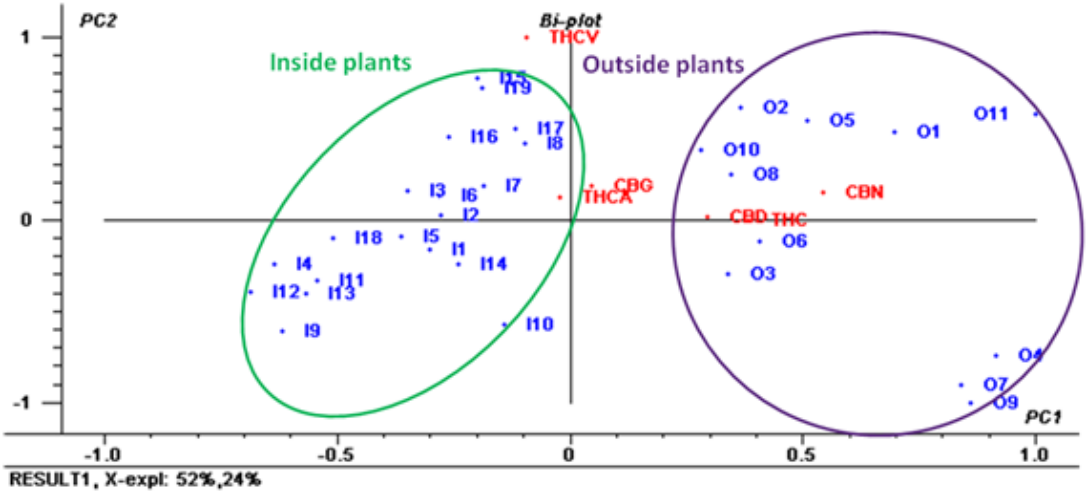

Figure 5: Score and loadings biplot (PC1 *vs* PC2) of *Cannabis sativa L.* samples performed by principal components analysis according to the concentration of the main quantified cannabinoid compounds. (I: refers to indoor grown plants; O: refers to outdoor grown plants)

Moreover, differences between Sativa and Indica varieties were studied but no clear results were obtained from the treated data. This can be due to the majority of the plants are not purely *sativa* or *indica* and moreover there is a lack of cannabis plant varieties standardization [22].

As 6 cannabinoids were quantified and other 7 identified, this methodology provided accurate cannabinoid profiles and can be easily used for differentiate between plant varieties as it has been done in other works [23, 26].

4 Conclusions

An HPLC-MS/MS method was fully optimized and validated to determine the major cannabinoids present in extracts obtained by supercritical fluid extraction of *Cannabis Sativa L.* plants. In addition to this, the identification of 7 minor cannabinoids was achieved by means of UPLC-qToF. This methodology can be useful to establish an accurate cannabinoid profile of cannabis varieties in order to correlate to therapeutic effectiveness.

Based on the application of both analytical techniques the analysis of 30 different cannabis strains grown under controlled conditions was carried out. The first results showed that the distribution patterns of indoor and outdoor grown plants were different enough to their differentiation. The found

difference was attributed to a higher concentration found for THC, CBN and CBD in outdoor grown plants.

Acknowledgements

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5 References

[1] Clarke RC, Watson DP. 2007. Cannabis and Natural Cannabis Medicines, in ElSohly MA (ed) Marijuana and the Cannabinoids, Humana Press, Totowa, 1-15.

[2] Hazekamp A. 2007. Cannabis; extracting the medicine, PhD thesis, Universiteit Leiden, The Netherlands.

[3] ElSohly MA, Slade D. 2005. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. Life.Sci, 78, 539-548.

[4] Ahmed SA, Ross SA, Slade D, Radwan MM, Zulfiqar F, ElSohly MA. 2008. Cannabinoid ester constituents from high-potency Cannabis sativa. J Nat Prod, 71, 536-542.

[5] Radwan MM, ElSohly MA, Slade D, Ahmed SA, Khan IA, Ross SA. 2009. Biologically active cannabinoids from high-potency Cannabis sativa. J Nat Prod, 72, 906-911.

[6] Russo EB. 2007. History of Cannabis and Its Preparations in Saga, Science, and Sobriquet. Chem Biodiv, 4, 1614-1648.

[7] Grotenhermen F. 2002. Effects of Cannabis and the cannabinoids, in Cannabis and Cannabinoids-Pharmacology, Toxicology, and Therapeutic Potential (F. Grotenhermen, E. Russo, eds), Haworth Press, New York, 55-65.

[8] Fishbein M, Gov S, Assaf F, Gafni M, Keren O, Sarne Y. 2012. Long-term behavioral and biochemical effects of an ultra-low dose of Δ9-tetrahydrocannabinol (THC): neuroprotection and ERK signaling. Exp Brain Res, 221, 437–448.

[9] Schweinsburg AD, Brown SA, Tapert SF. 2008. The influence of marijuana use on neurocognitive functioning in adolescents. Curr Drug Abuse Rev, 1, 99-111.

[10] Lisdahl KM, Wright NE, Medina-Kirchner C, Maple KE, Shollenbarger S. 2014. Considering the cannabis: the effects of regular cannabis use on neurocognition in adolescents and young adults. Curr Addict Rep, 1, 144-156.

[11] Consroe P. 1998. Brain cannabinoid systems as targets for the therapy of neurological disorders. Neurobiol Dis, 5, 534-551.

[12] McPartland JM, Russo EB. 2001. Cannabis and cannabis extracts: greater than the sum of their parts? J Cann Therap, 1, 103-132.

[13] Mechoulam R, Parker LA, Gallity R. 2002. Cannabidiol: an overview of some pharmacological aspects. J Clin Pharmacol, 42, S11-S19.

[14] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci, 30, 515-527.

[15] Bergamaschi MM, Queiroz RH, Chagas MH, De Oliveira DC, De Martinis BS, Kapczinski F, Quevedo J, Roesler R, Schröder N, Nardi AE, Martín-Santos R, Hallak JE, Zuardi AW, Crippa JA. 2011. Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. Neuropsychopharmacol, 36, 1219-1226.

[16] Morgan CJ, Das RK, Joye A, Vurran HV, Kamboj SK. 2011. Cannabidiol reduces cigarette consumption in tobacco smokers: preliminary findings. Addict Behav, 38, 2433-2436.

[17] Ryan D, Drysdale AJ, Pertwee RG, Platt B. 2006. Differential effects of cannabis extracts and pure plant cannabinoids on hippocampal neurons and glia. Neurosci Lett, 408, 236-341.

[18] Farrimond JA, Whalley BJ, Williams CM. 2012. Non-Δ 9tetrahydrocannabinol phytocannabinoids stimulate feeding in rats. Behav Pharmacol, 23, 113-117.

[19] Roser P, Gallinat J, Weinberg G, Juckel G, Gorynia I, Stadelmann AM. 2009. Psychomotor performance in relation to acute oral administration of Δ9-tetrahydrocannabinol and standardized cannabis extract in healthy human subjects. Eur Arch of Psy Clin N, 259, 284-292.

[20] Whalley BJ, Wilkinson JD, Williamson EM, Constanti A. 2004. A novel component of cannabis extracts potentiates excitatory synaptic transmission in rat olfactory cortex in vitro. Neurosci Lett, 365, 58-63.

[21] Wilkinson JD, Whalley BJ, Baker D, Pryce G, Constanti A, Gibbons S, Williamson EM. 2003. Medicinal Cannabis: is Δ9-tetrahydrocannabinol necessary for all its effects? J Pharm Pharmacol, 55, 1687-1694.

[22] Hazekamp A, Fischedick JT. 2012. Cannabis – from cultivar to chemovar. Drug Test Anal, 4, 660-667.

[23] Fischedick JT, Hazekamp A, Erkelens T, Choi YH, Verpoorte R. 2010. Metabolic fingerprinting of *Cannabis sativa L.*, cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes. Phytochemistry, 71, 2058-2073.

[24] Herrero M, Castro-Puyana M, Mendiola JA, Ibañez E. 2013. Compressed fluid for the extraction of bioactive compounds. Trends Anal Chem, 43, 67-83.

[25] Guiochon G, Tarafder A. 2011. Fundamental challenges and opportunities for preparative supercritical fluid chromatography. J Chromatogr A, 1218, 1037-114.

[26] Omar J, Olivares M, Alzaga M, Etxebarria N. 2013. Optimisation and characterisation of marihuana extracts obtained by supercritical fluid extraction and focused ultrasound extraction and retention time locking GC-MS. J Sep Sci, 36, 1397-1404.

[27] Grauwiler SB, Scholer A, Drewe J. 2007. Development of a LC/MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of cannabis sativa extracts. J Chromatogr B, 850, 515-522.

[28] De Backer B, Debrus B, Lebrun P, Theunis L, Dubois N, Decock L, Verstraete A, Hubert P, Charlier C. 2009. Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material. J Chromatogr B, 877, 4115–4124.

[29] Gröger Th, Schäffer M, Pütz M, Ahrens B, Drew K, Eschner M, Zimmermman R. 2008. Application of two-dimensional gas chromatography combined with pixel-based chemometric processing for the chemical profiling of illicit drug samples. J Chromatogr A, 1200, 8–16.

[30] Omar J, Olivares M, Amigo JM, Etxebarria N. 2014. Resolution of co-eluting compounds of Cannabis Sativa in comprehensive two-dimensional gas chromatography/mass spectrometry detection with Multivariate Curve Resolution-Alternating Least Squares. Talanta, 121, 273–280.

[31] Bäckström B, Cole MD, Carrott MJ, Jones DC, Davidson G, Coleman K. 1997. A preliminary study of the analysis of Cannabis by supercritical fluid chromatography with atmospheric pressure chemical ionisation mass spectroscopic detection. Science and Justice: J Forensic Sci Soc, 37, 91-97.

[32] Lacorte S, Fernandez-Alba AR. 2006. Time of flight mass spectrometry applied to the liquid chromatographic analysis of pesticides in water and food. Mass Spectrom Rev, 25, 866-880.

[33] Zhou JL, Qi LW, Li P. 2009. Herbal medicine analysis by liquid chromatography/time-of-flight mass spectrometry. J Chromatogr A, 1216, 7582-7594.

[34] Bobeldijk I, Viessers JPC, Kearney G, Major H, Van Leerdam JA. 2001. Screening and identification of unknown contaminants in water with liquid chromatography and quadrupole-orthogonal acceleration-timeof-flight tandem mass spectrometry. J Chromatogr A, 929, 63–74.

[35] Stolker AAM, Van Schoonhoven J, de Vries AJ, Bobeldijk-Pastorova I, Vaes WHJ, Van den Berg R. 2004. Determination of cannabinoids in cannabis products using liquid chromatography-ion trap mass spectrometry. J Chromatogr A, 1058, 143-151.

[36] Kneisel S, Auwärter V. 2012. Analysis of 30 synthetic cannabinoids in serum by liquid chromatography-electrospray ionization tandem mass spectrometry after liquid-liquid extraction. J Mass Spectrom, 47, 825-835.

[37] Brenneisen R. 2007. Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents. In Mahmoud A, ElSohly MA, Ed. Marijuana and the Cannabinoids. Humana Press, Totowa.

Simultaneous quantification of major cannabinoids and metabolites in human urine and plasma by HPLC-MS/MS and enzyme-alkaline hydrolysis

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CHAPTER 7

Abstract

A High Performance Liquid Chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) method for simultaneous quantification of Δ9-tetrahydrocannabinol (THC), its two metabolites 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), and four more cannabinoids (cannabidiol (CBD), cannabigerol (CBG), tetrahydrocannabivarin (THCV) and cannabinol (CBN)) in human urine and plasma was developed and validated. The hydrolysis process was studied to ensure complete hydrolysis of glucuronide conjugates and the extraction of total amount of analytes. Initially, urine and plasma blank samples were spiked with THC-COOH-glucuronide and THC-glucuronide, and four different pretreatment methods were compared: hydrolysis-free method, enzymatic hydrolysis with *Escherichia Coli* β-glucuronidase, alkaline hydrolysis with 10 M NaOH and enzyme-alkaline hydrolysis. The last approach assured the maximum efficiencies (close to 100 %) for both urine and plasma matrices. Regarding the figures of merit, the limits of detection were below 1 ng/mL for all analytes, the accuracy ranged from 84 % to 115 % was obtained, and both within-day and between-day precision were lower than 12 %. Finally, the method was successfully applied to real urine and plasma samples from cannabis users.

Keywords: Cannabinoids and metabolites, plasma analysis, urine analysis, enzymatic-alkaline glucuronide hydrolysis, High Performance liquid chromatography-mass spectrometry; Solid-phase extraction.

1 Introduction

Cannabis sativa L. is the most commonly used illicit drug throughout the world, but it is also being used as a medicinal plant. In fact, it is being effectively used for many illnesses as multiple sclerosis, chronic pain, glaucoma, asthma, etc [1]. At least 545 compounds have been identified in *cannabis sativa L.* plants, and among them 104 phytocannabinoids, i.e. the terpenophenolic compounds produced by the trichomes of the plant that are unique to cannabis plants [2]. Cannabinoids are mostly biosynthesized in an acidic form but they may decompose in the presence of light or heat to their neutral homologues, as in the case of Δ9-tetrahydrocannabinolic acid (THCA) which is decarboxylated to tetrahydrocannabinol (THC) [3].

THC is accepted to be the main psychoactive agent and it has anti-inflammatory, analgesic, appetite stimulant and antiemetic properties and at very low doses it can also protect the brain from cognitive deficits [4-5]. However, regular use of the plant can cause cognitive deficits especially in adolescents (or teenagers), since they are having significant neural development [6-7]. On the contrary, the neurocognitive effects of chronic use in adults still are somewhat inconsistent [6].

In addition to the psychoactive Δ9-tetrahydrocannabinol (THC), marijuana plants and cannabis-based medicines contain also different amounts of cannabidiol (CBD), cannabigerol (CBG), tetrahydrocannabivarin (THCV) and cannabinol (CBN) among others. CBD can modulate euphoric effects of THC and has antipsychotic, neuroprotective, anticancer, antidiabetic and other effects such as reducing the anxiety induced by stage fear or reducing tobacco consumption [8-13]; CBG has antibacterial, antiproliferative and bone-stimulant properties; cannabinol (CBN) has sedative or stupefying effects and tetrahydrocannabivarin (THCV) has anorectic and antiepileptic effects [9, 11].

The prevalence of illicit use of the cannabis plant and the need of more data about dose/effect relationships for growing medical applications have brought with it a special interest in analyzing major cannabinoids and their metabolites in human samples. In most studies, analyses are limited to THC and its metabolites 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) [14-17]. However, CBD, CBG, THCV and CBN cannabinoids should also be considered as they are major cannabinoids with considerable health effects.

In analogy to THC, the mentioned cannabinoids have also their metabolites. For example, more than 30 metabolites of CBD were identified in urine, biosynthesized by hydroxylation, mostly on C-7, leading to 7-hydroxy-CBD, followed by further oxidations, leading to CBD-7-oic acid, and numerous hydroxylated derivatives of this acid [18-19]. However, there are no reference standards available for these compounds.

THC and its hydroxilated metabolites are exposed to glucuronide conjugation through ether bonds between the hydroxyl group of the phenolic ring and glucuronic acid. For 11-OH-THC another ether bond can be created between the C-11 and glucuronic acid [15, 20]. THCOOH metabolite is also exposed to ether bond conjugation, but principally forms an ester bond between the carboxyl group at C-11 and the glucuronide [15, 20]. Examples of chemical structures of these glucuronides are shown in

Figure 1. In the same way of THC, other cannabinoids undergo also these biotransformations [19]. The rate of glucuronization differs from analyte to analyte and in each biological matrix [21-23]. This conjugate/free ratio have also been proposed for the identification of recent cannabis use [24-25].

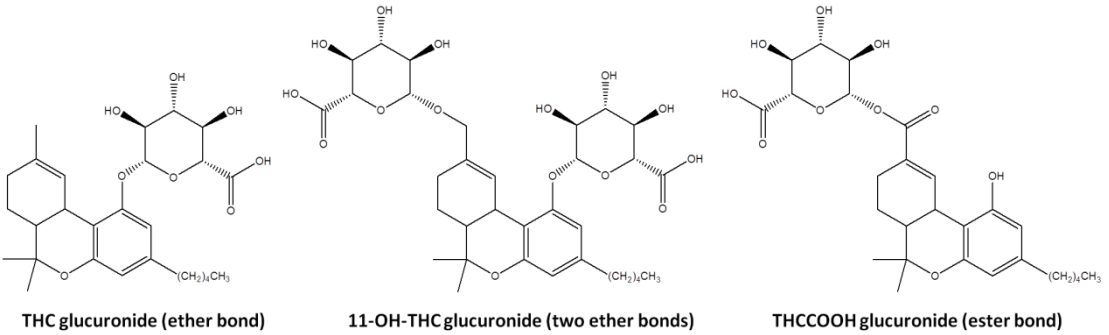

Figure 1: Chemical structures of THC, 11-OH-THC and THC-COOH common glucuronides.

Cannabinoid glucuronides can be analyzed by HPLC-MS/MS [25-27]. However, there are only two available reference standards, THC-COOH-glucuronide and THC-glucuronide. Hence, glucuronide conjugates should be hydrolyzed prior to extraction and analysis to determine the total amount for all analytes.

There are two different ways for glucuronide cleavage: alkaline hydrolysis and enzymatic hydrolysis [28]. Metabolites having an ether glucuronide bond, such as 11-OH-THC and THC glucuronides, are effectively hydrolyzed through enzymatic hydrolysis with *E. coli* β-glucuronidase. However, ester glucuronide metabolites, such as THC-COOH glucuronide, are more effectively hydrolyzed via alkaline hydrolysis [28]. Thus, to ensure the highest recovery of free analytes enzyme-alkaline hydrolysis should be carried out [15, 21, 23].

It is not possible to determine the hydrolysis efficiency in biological specimens because the total amount of cannabinoids is unknown. The only way to determine the efficiency is hydrolyzing spiked samples with known amounts of glucuronidated standards. However, as far as we know there is only one study that ensures the complete hydrolysis of glucuronides in urine and another one in meconium [15, 23].

In this work, the main aim was to fully develop and validate an analysis method by means of HPLC-MS/MS to quantify THC, its two metabolites 11-OH-THC and THC-COOH, and CBD, CBG, THCV and CBN in human urine and plasma. For that, different hydrolysis processes were studied in both matrixes (no hydrolysis, alkaline, enzymatic and enzyme-alkaline) to ensure complete hydrolysis of glucuronide conjugates.

2 Materials and methods

2.1 Materials and reagents

Cannabinoid standard compounds: CBG and THCV were purchased from Echo Pharmaceuticals BV (Weesp, The Netherlands), THC, CBD, CBN, THC-d_3 and THC-COOH-glucuronide were purchased from Cerilliant (Round Rock, Texas, USA), THC-COOH, 11-OH-THC, THC-COOH-d_3 and 11-OH-THC-d_3 were obtained from Lipomed (Arlesheim, Switzerland) and THC-glucuronide was purchased from ElSohly laboratories (Oxford, Mississippi, USA). SPE cartridges (Strata C18-E (55 um, 70 A) 200 mg/3 mL) were obtained from Phenomenex (Aschaffenburg, Germany), LC/MS grade methanol (MeOH) and formic acid used as mobile phase were purchased from Thermo Fisher Scientific (Erembodegem, Belgium). HPLC grade hexane and ethyl acetate were obtained from Labscan (Gliwice, Poland), ultrapure acetonitrile from Teknokroma (Barcelona, Spain), HPLC grade acetic acid and analysis grade hydrochloric acid, sodium hydroxide, sodium acetate and potassium phosphate from Merck (Billerica, USA) and type IX-A E.coli β-glucuronidase from Sigma-Aldrich (Steinheim, Germany). De-ionized water (H_2O) was generated with a Milli-Q water purification system Element 10 from Millipore (Billerica, USA).

2.2 Sample collection

Urine specimens were obtained from the foundation Renovatio (Donostia, Gipuzkoa) and they were collected either from cannabis regular users anonymous volunteers and from healthy not user ones. Plasma samples of cannabis consumers were obtained from the Institute of Biomedicine of Sevilla (Spain) and blank plasma samples were obtained from the Basque Biobank for research (O^+ehun) (Sondika, Bizkaia).

2.3 Specimen hydrolysis

Under optimized conditions, plasma specimens were pretreated to avoid particulate matter during the hydrolysis process. After the addition of internal standards (and glucuronide or cannabinoid standards, if appropriate), 2 mL of MeOH were added to each plasma milliliter followed by 10 min centrifugation at 4500 rpm and 4 ºC. Methanolic supernatants were recovered and evaporated (Caliper Life Sciences TurboVap® LV). From this point, pretreated plasma samples were treated in the same manner as urine samples.

500.000 Fishman units (U) β-glucuronidase were dissolved in 25 mL of 0.1 M potassium phosphate buffer (pH 6.8) to prepare the enzyme-solution. 1 mL buffer and 0.25 mL of the enzyme-solution were added to evaporated plasma samples and properly doped 1 mL urine samples (5000 U/mL specimen) and they were incubated for 16 h at 37 ºC. After that, 80 µL 10 M sodium hydroxide were added and samples were maintained at 60 ºC for 20 min. Once cooled to room temperature, samples were neutralized with 50 µL of acetic acid. Then, 2 mL of cold acetonitrile were added while samples were vortex mixing, followed by 10 min centrifugation at 4500 rpm and 4 ºC. Supernatants were diluted with 1 mL 2 M sodium acetate buffer (pH 4.0) before solid-phase extraction (SPE).

2.4 Solid-phase extraction

SPE method was carried out according UCT applications manual with minor changes [29]. Cartridges were conditioned adding subsequently 3 mL of MeOH; 3 mL of H_2O; and 1 mL of 0.1M sodium acetate buffer (pH 3.0). After sample application at 1 to 2 mL/min, they were washed with 2 mL of H_2O; 2 mL of 0.1 M hydrochloric acid in 95:5 H_2O/acetonitrile mixture; and 200 µL of MeOH. Then, cannabinoid compounds were eluted at 1 to 2 mL/min with 2 mL of hexane and 3 mL of 50:50 hexane/ethyl acetate mixture. Elutions were evaporated, reconstructed in 60 µL of MeOH and centrifugated at 10000 rpm for 5 min prior to inject in HPLC-MS/MS.

2.5 HPLC-MS/MS analysis

HPLC-MS/MS analysis was carried out according to previously optimized method with minor modifications [30]. Analyses were performed using an Agilent Technologies (Santa Clara (CA), USA) Infinity liquid 1260 chromatographic system, consisting of an autosampler, a column thermostat and a binary solvent management system coupled to a triple quadrupole (Agilent Technologies 6430) equipped with an Atmospheric Pressure Chemical Ionization ion source (APCI).

The chromatographic separation was accomplished using a Kinetex C18 column (2.6 µm, 150 mm x 3 mm i.d.) with a guard column (0.5 µm Depth Filter x 0.1 mm) (Phenomenex, Torrance (CA), USA) and a binary A/B gradient (Solvent A was Milli Q water with 0.1 % formic acid, and, solvent B was MeOH with 0.1 % formic acid). The gradient program was as follows: initial conditions were 60 % B; raised to 80 % B over the first minute, held at 80 % B for 7 minutes, increased to 95 % B over the next 2 minutes, held at 95 % B for 10 minutes, decreased to 60 % B over the next 1 minute and held at 60 % B for 7 minutes for re-equilibration of the system prior to the next injection. A flow rate of 0.3 mL/min was used; the column temperature was 30 ºC and the injection volume was 10 µL.

MS acquisition was carried out in the APCI positive ionization mode. The conditions were established as follows: corona discharge current of 5 µA, capillary voltage of 3500 V, heated vaporizer at 280 °C, a nitrogen flow rate of 7 L/min, a source temperature of 210 °C and nebulizer pressure of 32 psi. For the quantification, multiple reactions monitoring (MRM) was used, employing two transitions, one for the quantification and the other for the qualification (Table 1).

The quantification of the specimens was performed with external instrumental calibration; i.e., a set of standards containing target compounds at concentrations ranging from 1 to 4000 ng/mL in MeOH. System fluctuations were corrected with isotopically labeled standards (THC-COOH-d_3 and 11-OH-THC-d_3 for the metabolites and THC-d_3 for the other cannabinoids) used as internal standard.

Table 1
MRM transitions, optimized potentials, qualifier/quantifier area ratios and retention times of the target compounds and the internal standards for HPLC-MS/MS analysis. Mass/charge ratio of the precursor ion (Q1), mass/charge ratio of the fragment ion (Q2), fragmentor potential (FP), collision energy (CE), qualifier/quantifier ratio (Q/Q), retention time (t_R).

Analyte	Q1 (u)	Q2 (u)	FP (V)	CE (eV)	Q/Q (%)	t_R (min)
Target compounds						
11-OH-THC	331.0	313.1 200.9	140 140	10 20	14	11.4
THC-COOH	345.1	299.1 327.1	110 110	15 15	83	12.6
CBD	315.1	192.8 259.0	70 70	20 20	27	12.8
CBG	317.2	193.0 123.0	120 120	10 25	24	13.1
THCV	287.1	165.0 231.0	80 80	20 15	28	13.5
CBN	311.0	222.9 293.0	50 50	15 10	48	14.9
THC	315.0	193.0 259.0	70 70	20 20	38	15.5
Internal standards						
11-OH-THC-d3	334.1	316.2 196.1	140 160	10 25	15	11.4
THC-COOH-d3	348.2	330.1 302.1	80 80	10 15	69	12.5
THC-d3	317.8	195.9 262.0	120 120	25 20	54	15.5

2.6 Hydrolysis optimization and efficiency

Plasma and urine samples were studied under four pretreatment procedures: hydrolysis-free, alkaline, enzymatic, and enzyme-alkaline tandem hydrolysis. In order to calculate the hydrolysis recoveries, 1 mL samples were spiked with a known amount of THC-COOH and THC glucuronides (50 ng/mL) and internal standards. Plasma specimens were pretreated as described in section 2.3 to avoid particulate matter during the hydrolysis process.

Non hydrolyzed samples were prepared adding directly 2 mL of acetonitrile followed by centrifugation and diluting the supernatants with 1 mL of 2 M sodium acetate buffer (pH 4.0) before solid-phase extraction (SPE). Alkaline hydrolysis was carried out as described in section 2.3 but without the enzymatic hydrolysis step. In the same way, enzymatic hydrolysis was developed as described in section 2.3 but without the alkaline hydrolysis step. Enzyme-alkaline hydrolysis was carried out just as described in section 2.3. Each procedure was done in triplicate.

3 Results and discussion

3.1 Hydrolysis optimization and efficiency

Figure 2 shows the efficiencies of studied hydrolysis conditions for urine and plasma samples. As it can be observed, glucuronide compounds subjected to the non-hydrolysis procedure confirmed no cleavage during the process. Applying alkaline hydrolysis, THC-COOH-glucuronide cleavage was total

but no free THC was observed. On contrary, following enzymatic hydrolysis, cleavage of THC-glucuronide was total but only 76 % of free THC-COOH was observed in urine and 68 % in plasma. Finally, tandem hydrolysis maximized cleavage of both glucuronides obtaining efficiencies around 100 % for both compounds in both matrixes.

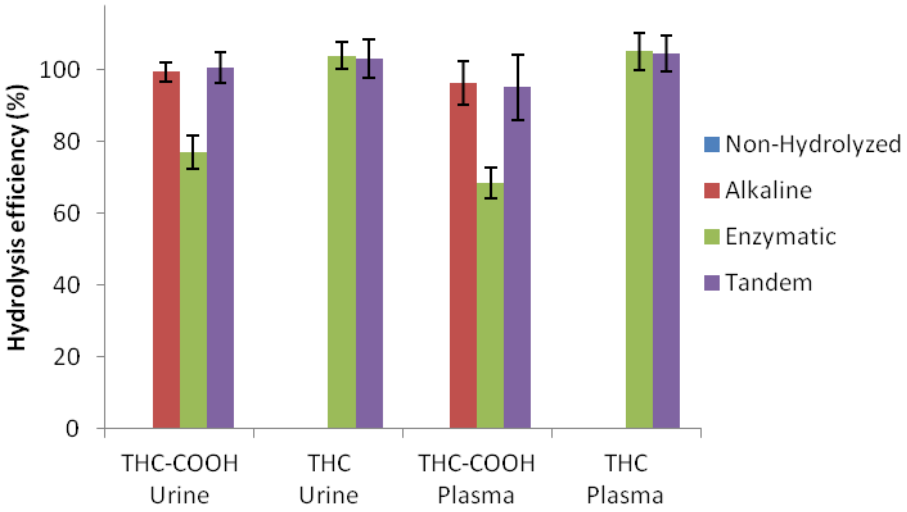

Figure 2: Hydrolysis efficiencies of non-hydrolyzed, alkaline, enzymatic and alkaline-enzymatic hydrolyzed urine and plasma blank samples fortified with THC-COOH and THC glucuronides (n=3).

In conclusion, it can be said that tandem hydrolysis works for the cleavage of both glucuronide bonds, ether bonds, such in THC-glucuronide, and ester bonds, such in THC-COOH-glucuronide. This tandem hydrolysis assures the complete hydrolysis of all glucuronide compounds present in urine and plasma samples.

3.2 Method validation

3.2.1 LODs, LOQs and linearity

Limits of detection (LODs) and quantification (LOQs) were established on the basis of three repeated analyses of urine and plasma blank samples. They were defined as the concentration where the signal to noise ratio (S/N) for the lower MRM transition was 3 in the case of LODs and 10 in the case of LOQs. The obtained values of methodological LODs and LOQs in urine and plasma are shown in Table 2. All LODs are below 1 ng/mL.

Table 2
Correlation coefficients (R^2), limits of detection (LOD) and limits of quantification (LOQ) in urine and plasma.

Analyte	R^2	LOD (ng/mL)		LOQ (ng/mL)	
		Urine	Plasma	Urine	Plasma
11-OH-THC	0.9995	0.2	0.3	0.5	1
THC-COOH	0.9990	0.2	0.2	0.6	0.5
CBD	0.9987	0.3	0.3	1	1
CBG	0.9984	0.2	0.3	0.7	0.9
CBN	0.9996	0.5	0.7	1	2
THCV	0.9991	0.2	0.2	0.9	0.5
THC	0.9993	0.4	0.5	1	1

Moreover, calibration standards were prepared in MeOH and linearity was studied in the instrumental concentration range from 1 to 4000 ng/mL for the two metabolites 11-OH-THC and THC-COOH and from 1 to 1000 ng/mL for the rest of the cannabinoids. As the specimens are reconstituted in a lower volume after SPE, this means that the real ranges go from LOD-s to 240 ng/mL and from LOD-s to 60 ng/mL respectively. The instrumental calibration curve was found to be linear for all analytes in the studied ranges (Table 2).

3.2.2 Intra and inter-batch precision, accuracy and extraction efficiency

On the one hand, accuracy and precision of the method were evaluated at three concentration levels (5, 50, 200 ng/mL) in spiked urine and plasma samples with all the analytes and subjected to the whole analysis procedure. Accuracy was calculated in terms of percent deviation of the calculated concentration average from the corresponding spiked concentration at each concentration level. The repeatability (intra-day precision) and the reproducibility (inter-day precision) were checked in terms of relative standard deviations (RSD %). Samples were analyzed three times over two days.

As it can be observed in Table 3, the RSD values of repeatability (within day precision) and reproducibility (between days precision) were in the 1-10 % and 1-12 % ranges respectively, in both matrixes and for all analytes.

Table 3
Intraday and interday precision (RSD%), accuracy (%) and recovery (%) of the studied analytes in both urine and plasma matrixes (n=3).

Analyte	Concentration (ng/mL)	Intraday(%) Urine	Intraday(%) Plasma	Interday(%) Urine	Interday(%) Plasma	Accuracy (%) Urine	Accuracy (%) Plasma	Recovery (%) Urine	Recovery (%) Plasma
11-OH-THC	5	1	2	1	3	100	97	71	44
	50	4	4	2	1	99	95		
	200	3	1	2	4	95	98		
THC-COOH	5	4	7	1	6	102	107	72	50
	50	1	2	1	1	94	95		
	200	1	1	1	6	98	99		
CBD	5	4	4	5	1	89	88	64	43
	50	2	6	3	5	88	87		
	200	4	10	3	10	84	86		
CBG	5	3	4	3	1	91	92	74	53
	50	6	10	2	7	112	107		
	200	7	5	3	11	92	96		
THCV	5	3	5	7	10	98	96	72	50
	50	1	4	2	4	102	107		
	200	1	2	2	3	105	111		
CBN	5	2	6	6	12	94	93		
	50	1	1	1	4	101	97	71	46
	200	2	1	3	3	105	109		
THC	5	1	8	1	3	106	108		
	50	4	4	1	1	115	109	78	54
	200	1	7	10	6	114	110		

The accuracy values were between 84 % and 115 %. The less accurate values were obtained for CBD and THC, from 84 % to 89 % and from 106 % to 115 % respectively. This can be because of conversion of some CBD into THC in the process. It is well known that CBD can be converted to THC under acid conditions [31], and slightly acid conditions were used in the SPE procedure (pH=3). However, slightly acidic conditions are needed to have good recoveries of carboxilated metabolites as THC-COOH [32].

On the other hand, the extraction recoveries were calculated at the medium concentration level. For each analyte, recoveries were determined comparing the total peak area from the samples spiked before hydrolysis with the total area of samples processed as blanks and spiked after SPE and evaporation.

The extraction efficiencies for urine and plasma samples are shown in Table 3. Higher extraction efficiencies were obtained in urine samples (between 64 % and 78 %) than in plasma samples (between 43 % and 54 %). This is due to the fact that urine is a relatively clean matrix and also because plasma specimens were subjected to a pretreatment to avoid particulate matter during the hydrolysis process.

3.2.3. Stability, selectivity and carry over

The influence of four freezing/thawing cycles was evaluated. For that, samples were spiked at three concentration levels and they were analyzed three times: before any freezing/thawing cycles, after two cycles and after four cycles. Samples were frozen and stored in glass vials at -20 ºC. This way, no statistical differences were found between the sample concentration after four freezing/thawing cycles and the initial sample concentration (P-level > 0.05).

Additionally, interferences from urine and plasma components were evaluated analyzing 2 different drug-free samples. The method was considered selective if analytes were below LOD. Carry-

over effects were checked by analyzing blank samples after injection of the highest calibration point. No interferences were observed in the drug-free samples and no carryover phenomena in blank samples injected after the highest calibration point.

3.3. Application to real samples, plasma and urine

The analysis method was applied to 4 urine regular cannabis user samples and 4 plasma samples. Urine samples happened to be too concentrated as they were obtained from regular cannabis users and not from medical cannabis users. As a consequence, a diluted sample was also injected for the quantification of the major metabolite THC-COOH. The obtained concentrations are shown in Table 4. As it can be observed, the repeatability in real samples was quite similar to the repeatability obtained with spiked blank samples. Moreover, as it can be deduced from Figure 3, even the urine and plasma samples belong to different users and likely the use of different types of marijuana, this method allows not only the determination of several cannabinoids but also the successful comparison of the distribution of the cannabinoids in both fluids.

Table 4
Concentration of the target compounds (ng/mL) in real samples obtained from cannabis users (n=3).

Sample	11-OH-THC	THC-COOH	CBD	CBG	THCV	CBN	THC
Urine							
1	211±8	2300±100	8.1±0.3	37±2	0.86±0.02	5.3±0.3	30±1
2	98±4	650±30	2.2±0.1	38±3	2.89±0.08	3.1±0.3	22.1±0.8
3	30±1	650±40	0.32±0.03	13.2±0.3	1.10±0.03	2.3±0.1	11.5±0.4
4	32.7±0.8	450±30	2.6±0.1	13.3±0.8	0.22±0.01	1.09±0.08	2.84±0.07
Plasma							
5	7.4±0.5	233±9	9.1±0.4	1.5±0.1	0.20±0.02	0.92±0.05	5.4±0.3
6	3.8±0.2	130±4	1.7±0.2	0.42±0.04	<LOD	0.72±0.06	4.1±0.3
7	12.3±0.6	174±8	4.8±0.3	1.08±0.07	<LOD	2.2±0.2	5.9±0.5
8	7.0±0.3	92±4	7.2±0.4	1.6±0.1	<LOD	3.8±0.3	6.5±0.5

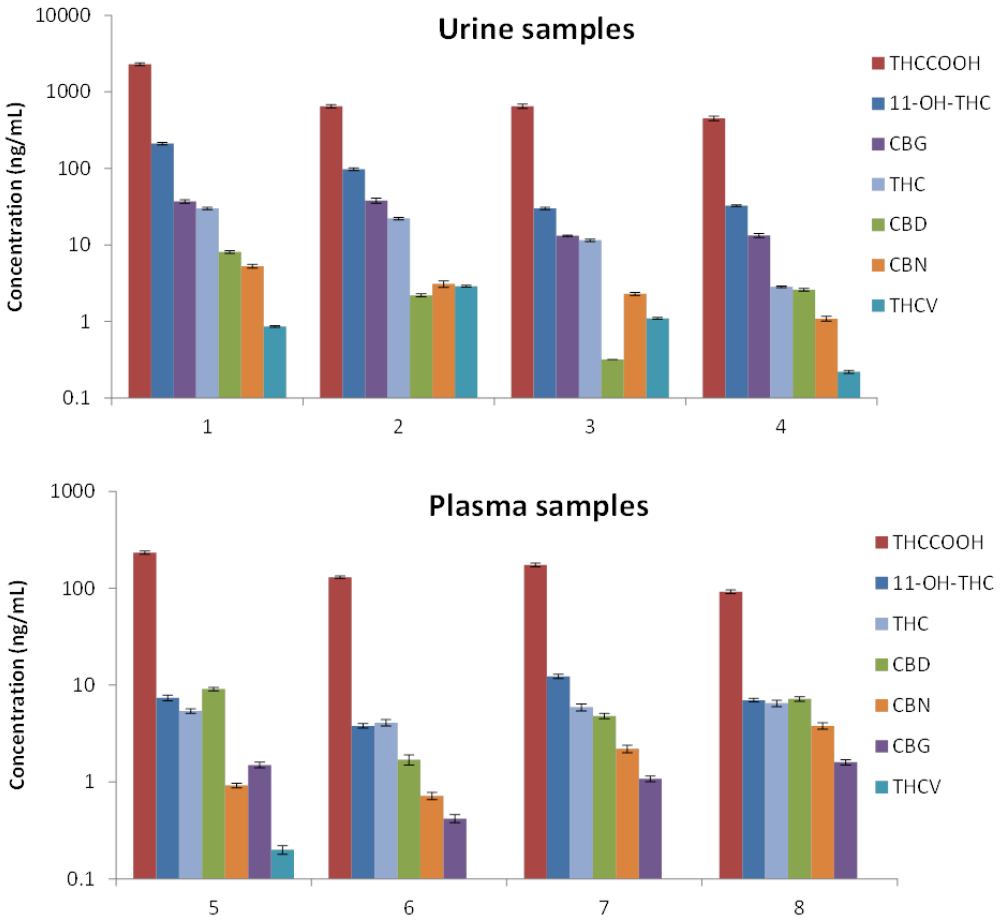

Figure 3: Concentration of THC metabolites and cannabinoids in real urine and plasma samples from cannabis users (n=3).

4 Conclusions

An HPLC-MS/MS method was developed and validated for the quantitative determination of total 11-OH-THC, THC-COOH, THC, THCA, CBD, CBN, CBG and THCV in human urine and plasma. A simple hydrolysis procedure combining the enzymatic and the alkaline hydrolysis has been also developed and validated. Acceptable assay characteristics and limit of detections were obtained and the method was successfully applied to real samples. This way, we have shown the usefulness of the method when the total concentration of the different cannabinoids is analysed in either urine and plasma samples under clinical requirements.

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References

[1] Clarke RC, Watson DP. 2007. Cannabis and Natural Cannabis Medicines, in ElSohly MA (ed) Marijuana and the Cannabinoids, Humana Press, Totowa, 1-15.

[2] ElSohly M, Gul W. 2014. Constituents of cannabis sativa, In Pertwee R (ed). Handbook of Cannabis, Oxford University Press, Oxford (UK), 3-22.

[3] Russo EB. 2007. History of Cannabis and Its Preparations in Saga, Science, and Sobriquet. Chem Biodiv, 4, 1614-1648.

[4] Grotenhermen F. 2002. Effects of Cannabis and the cannabinoids, in Cannabis and Cannabinoids-Pharmacology, Toxicology, and Therapeutic Potential (F. Grotenhermen, E. Russo, eds), Haworth Press, New York, 55-65.

[5] Fishbein M, Gov S, Assaf F, Gafni M, Keren O, Sarne Y. 2012. Long-term behavioral and biochemical effects of an ultra-low dose of Δ^9-tetrahydrocannabinol (THC): neuroprotection and ERK signaling. Exp Brain Res, 221, 437-448.

[6] Schweinsburg AD, Brown SA,. Tapert SF. 2008. The influence of marijuana use on neurocognitive functioning in adolescents. Curr Drug Abuse Rev, 1, 99-111.

[7] Lisdahl KM, Wright NE, Medina-Kirchner C, Maple KE, Shollenbarger S. 2014. Considering the cannabis: the effects of regular cannabis use on neurocognition in adolescents and young adults. Curr Addict Rep, 1, 144-156.

[8] Consroe P. 1998. Brain cannabinoid systems as targets for the therapy of neurological disorders. Neurobiol Dis, 5, 534-551.

[9] McPartland JM, Russo EB. 2001. Cannabis and cannabis extracts: greater than the sum of their parts? J Cann Therap, 1, 103-132.

[10] Mechoulam R, Parker LA, Gallity R. 2002. Cannabidiol: an overview of some pharmacological aspects. J Clin Pharmacol, 42, 11S-19S.

[11] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci, 30, 515-527.

[12] Bergamaschi MM, Queiroz RH, Chagas MH, De Oliveira DC, De Martinis BS, Kapczinski F, Quevedo J, Roesler R, Schröder N, Nardi AE, Martín-Santos R, Hallak JE, Zuardi AW, Crippa JA. 2011. Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. Neuropsychopharmacol, 36, 1219-26.

[13] Morgan CJ, Das RK, Joye A, Vurran HV, Kamboj SK. 2011. Cannabidiol reduces cigarette consumption in tobacco smokers: preliminary findings. Addict Behav, 38, 2433-2436.

[14] Teixeira H, Verstraete A, Proença P, Corte-Real F, Monsanto P, Vieira DN. 2007. Validated method for the simultaneous determination of Δ^9-THC and Δ^9-THC-COOH in oral fluid, urine and whole blood using solid-phase extraction and liquid chromatography–mass spectrometry with electrospray ionization. Forensic Sci Int, 170, 148-155.

[15] Abraham TT, Lowe RH, Pirnay SO, Darwin WD, Huestis MA. 2007. Simultaneous GC–EI-MS Determination of Δ^9-Tetrahydrocannabinol, 11-Hydroxy-Δ^9-Tetrahydrocannabinol, and 11-nor-9-Carboxy-Δ^9-Tetrahydrocannabinol in Human Urine Following Tandem Enzyme-Alkaline Hydrolysis. J Anal Toxicol, 31, 477-485.

[16] Mercolini L, Musenga A, Comin I, Baccini C, Conti M, Raggi MA. 2008. Determination of plasma and urine levels of Δ9-tetrahydrocannabinol and its main metabolite by liquid chromatography after solid-phase extraction. J Pharmaceut Biomed, 47, 156-163.

[17] Röhrich J, Schimmel I, Zörntlein S, Becker J, Drobnik S, Kaufmann T, Kuntz V, Urban R. 2010. Concentrations of delta9-tetrahydrocannabinol and 11-nor-9-carboxytetrahydrocannabinol in blood and urine after passive exposure to Cannabis smoke in a coffee shop. J Anal Toxicol, 34, 196-203.

[18] Harvey DJ, Mechoulam R. 1990. Metabolites of cannabidiol identified in human urine. Xenobiotica, 20, 303-320.

[19] Mechoulam R, Hanus L. 2002. Cannabidiol: an overview of some chemical and pharmacological aspects. Part I: chemical aspects. Chem Phys Lipids, 121, 35-43.

[20] Kemp PM, Abukhalaf IK, Manno JE, Manno BR, Alford DD, Abusada GA. 1995. Cannabinoids in humans. I. Analysis of delta 9-tetrahydrocannabinol and six metabolites in plasma and urine using GC-MS. J Anal Toxicol, 19, 285-291.

[21] Lowe RH, Abraham TT, Darwin WD, Herning R, Cadet JL, Huestis MA. 2009. Extended Urinary Δ9-Tetrahydrocannabinol Excretion in Chronic Cannabis Users Precludes Use as a Biomarker of New Drug Exposure. Drug Alcohol Depend 105, 24-32.

[22] Schwilke EW, Schwope DM, Karschner EL, Lowe RH, Darwin WD, Kelly DL, Goodwin RS, Gorelick DA, Huestis MA. 2009. Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC, and 11-nor-9-carboxy-THC plasma pharmacokinetics during and after continuous high-dose oral THC. Clin Chem, 55, 2180-2189.

[23] Gray TR, Barnes AJ, Huestis MA. 2010. Effect of hydrolisis on identifying prenatal cannabis exposure. Anal Bioanal Chem, 397, 2335-2347.

[24] Mareck U, Haenelt N, Geyer H, Guddat S, Kamber M, Brenneisen R, Thevis M, Schänzer W. 2009. Temporal indication of cannabis use by means of THC glucuronide determination. Drug Test Anal, 11-12, 505-510.

[25] Felli M, Martello S, Chiarotti M. 2011. LC–MS–MS method for simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine: Application to workplace confirmation tests. Forensic Sci Int, 204, 67-73.

[26] Skopp G, Pötsch L. 2004. An investigation of the stability of free and glucuronidated 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid in authentic urine samples. J Anal Toxicol, 28, 35-40.

[27] Scheidweiler KB, Desrosiers NA, Huestis MA. 2012. Simultaneous quantification of free and glucuronidated cannabinoids in human urine by liquid chromatography tandem mass spectrometry. Clin Chim Acta, 413, 1839-1847.

[28] Kemp PM, Abukhalaf IK, Manno JE, Manno BR, Alford DD, McWilliams ME, Nixon FE, Fitzgerald MJ, Reeves RR, Wood MJ. 1995. Cannabinoids in humans. II. The influence of three methods of hydrolysis on the concentration of THC and two metabolites in urine. J Anal Toxicol, 19, 292-298.

[29] United Chemical Technologies (UCT). 2009. Solid Phase Extraction Application Manual. 36-37.

[30] Aizpurua-Olaizola O, Omar J, Navarro P, Olivares M, Etxebarria N, Usobiaga A. 2014. Identification and quantification of cannabinoids in *Cannabis Sativa L.* plants by high performance liquid chromatography-mass spectrometry. Anal Bioanal Chem, 406, 7549-7560.

[31] Adams R, Pease DD, Cain CK, Clark JH. 1940. Structure of Cannabidiol. VI. Isomerization of cannabidiol to tetrahydrocannabinol, a physiologically active product. Conversion of cannabidiol to cannabinol. J Am Chem Soc, 62, 2402-2405.

[32] Sobolevsky T, Prasolov I, Rodchenkov G. 2010. Detection of JWH-018 Metabolites in smoking mixture post-administration urine. Forensic Sci Int, 200, 141-147.

Evolution of the content of cannabinoids and terpenes during the growth of cannabis plants from different chemotypes

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CHAPTER 8

Abstract

The evolution of major cannabinoids and terpenes during the growth of *Cannabis sativa L*. plants was studied. In this work, 7 different plants were selected, 3 from the chemotypes I and III and one from the chemotype II. 50 clones of each mother plant were grown indoor under controlled conditions and every week three plants from each variety were cut and dried, and leaves and flowers were analyzed separately. 8 major cannabinoids content was monitored by means of High-Performance Liquid Chromatography with photodiode array detection (HPLC-DAD) and 28 terpenes were quantified by Gas Chromatography with Flame Ionization Detection (GC-FID) and verified by Gas chromatography–mass spectrometry (GC-MS). The chemotype of the plants, defined by the THCA/CBDA ratio, was clear from the beginning and stable during the growth. The concentration of major cannabinoids and terpenes were determined and different patterns found among chemotypes, as the plants from the chemotype II and III needed more time to reach the peak in THCA, CBDA and monoterpene content. Apparent differences were found also in CBGA development between chemotypes, and between monoterpene and sesquiterpene evolution patterns. Different chemotype plants were clearly differentiated by the terpene content, and characteristic terpenes of each chemotype were identified.

Keywords: *cannabis sativa L.*; plant growing; different chemotypes; cannabinoids; terpenes, principal component analysis.

1 Introduction

Cannabis sativa L. is the most frequently used illicit plant all over the world, but it is also a very powerful medicinal plant as it is effectiveness has been well demonstrated for many different purposes and illnesses as appetite stimulation in anorexia, advanced stages of cancer or HIV/AIDS [1-3], antiemetic effect in cancer chemotherapy [4-6], chronic neuropathic pain in cancer and HIV/AIDS and other types of chronic pain such as fibromyalgia and rheumatoid arthritis [7-9] or multiple sclerosis [10-12]. Moreover, there are much more emerging clinical applications for cannabis, such as treatment for several cancers [13-16], epilepsy [17-18], Alzheimer [19-20], Huntington′s Disease [21-22], diabetes [23-24] and Tourette′s syndrome [25-26].

There are at least 554 identified compounds in *cannabis sativa L.* plants, among them 113 phytocannabinoids [27-28] and 120 terpenes [29]. Cannabinoids are biosynthesized in an acidic form and almost no neutral cannabinoid can be found in fresh plants [30]. However, they may decompose in the presence of light or heat to their neutral homologues, and suffer an oxidation as in the case of tetradydrocannabinol (THC), which can be transformed to cannabinol (CBN) [31]. The first cannabinoid biosynthetized is known to be cannabigerolic acid (CBGA), and from it tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA) are synthetized, each by a particular synthase [32]. In addition to this, monoterpenes and sesquiterpenes are derived from different condensation reactions of two and three assembled isoprene units ($C_{10}H_{16}$ and $C_{15}H_{24}$), respectively [33].

The two major cannabinoids and the best well-knowns for their therapeutic potential are THC and cannabidiol (CBD), thus the neutral homologues of THCA and CBDA respectively. THC is the main psychoactive agent of cannabis and it has anti-inflammatory, analgesic, appetite stimulant and antiemetic properties [34]. On the contrary, CBD can modulate euphoric effects of THC and has antipsychotic, neuroprotective, anticancer, antidiabetic and other effects such as reducing tobacco consumption [35-39]. Moreover, cannabigerol (CBG) and cannabichromene (CBC) seem to be promising for different medical applications. Although they have not been extensively studied, CBG has a promising potential for the treatment of glaucoma [40], inflammatory bowel disease [41] and prostate carcinoma [42]; and CBC might have analgesic effects [43], potential to stimulate the growth of brain cells [44] and the ability to normalize gastrointestinal hypermotility [45].

Terpenes are responsible of the plant aroma but, in addition, they possess specific medical effects and they can work synergistically with cannabinoids. In fact, there are several promising applications based on the combined use of cannabinoids and terpenes such as new acne therapies utilizing CBD with the monoterpenes limonene, linalool and pinene; new antiseptic agents with CBG and pinene; to treat patients with social anxiety disorder using CBD with limonene and linalool or to treat sleeping problems by adding caryophyllene, linalool and myrcene to 1:1 CBD:THC extracts [46-47].

According to major cannabinoid concentration, five different chemotypes of cannabis are recognized. Drug-type plants that have a high THCA/CBDA ratio (>> 1.0) are classified as chemotype I; plants that exhibit an intermediate ratio (usually 0.5-2.0) as chemotype II; typical fiber-type plants that show a low THCA/CBDA ratio (<< 1.0) are classified as chemotype III; chemotype IV plants are fiber-

type plants that contain CBGA as main cannabinoid and chemotype V plants are also fiber-type plants but they exhibit almost no cannabinoid [48].

Genetic analyses demonstrated that the chemotype is determined upon the presence, at B locus, of two codominant alleles, B_D and B_T, responsible of the CBDA and THCA occurrence in plant. This way, plants with B_T/B_T alleles are chemotype I plants, plants with B_D/B_D alleles are from chemotype III, whereas chemotype II plants have two different alleles, B_D/B_T [49-50]. Also at this locus, non-functional alleles, called B_0, can exist. These alleles are unable to convert the CBGA, and this way the plants are CBGA predominant (chemotype IV) [51].

The increasing use of cannabis as medicine and the growing interest on the medicinal effects of non-psychotropic cannabinoids and terpenes has brought the need to produce pure compounds and plants with different content of cannabinoids and terpenes in a large scale. To this end, CBD enhanced plants with more than 15 % CBD and less than 1 % THC have been produced recently [52]. However, in order to optimize the production for each compound a deeper understanding of their development during the growth is necessary. Some works have been published about it, but they were considered from a forensic perspective to differentiate drug type plants from non-drug types in the early stages of the growth [48, 53]. Besides, terpene content was not analyzed and only fiber type and high THC plants were studied.

Therefore, the main aim of this work was to study the time profile evolution of cannabinoid and terpene content during the whole growth of the plant, i.e. from the rooting phase up to the end of the flowering stage. In this case, content of 7 cannabis varieties of 3 different chemotypes (3 from chemotype I and III and , 1 from chemotype II) was studied.

2 Materials and methods

Chemicals

Reference cannabinoids THCA, THC, CBD and CBN were purchased from Lipomed (Arlesheim, Switzerland) and CBDA, CBGA, CBG and CBC were purchased from Echo Pharmaceuticals BV (Weesp, The Netherlands). Reference terpenes α-pinene, β-pinene, myrcene, limonene, eucalyptol, terpinolene, linalool, β-caryophyllene, humulene and guaiol; internal standards phenanthrene and 1-octanol and ≥ 99 % acetic acid were obtained from Sigma Aldrich (Steinheim, Germany). HPLC quality MeOH, EtOH, $CHCl_3$ and H_2O were purchased from Carl Roth (Karlsruhe, Germany). 99.999 % purity nitrogen, hydrogen, helium and synthetic air were obtained from Carbagas (Lausanne, Switzerland).

Plant material

Around 50 clones of each standardized mother plant (3 from chemotype I and III and , 1 from chemotype II) were grown indoor under controlled conditions (20-28 °C and 40-70 % humidity). Cannabis plants were grown in three cycles. In the beginning, they were cultivated in 25 x 25 mm slabs until the roots grow and then were transferred to 2 L pots. During these two steps, plants were grown

under an indoor vegetative lights cycle of 18 h of light, first with a Philips Master TL-D 36 W, and later with Philips Master HPI-T Plus 400 W. When their sizes were appropriate, plants were transferred to 10 L pots and were exposed to a flowering light cycle of 12 h of light (Philips Master Green Power Plus 600 W) until harvest. The soil used was Substract 144 from Ricoter (Aarberg, Switzerland) and the nutrient Plantactiv 18+12+18 Type A- from Hauert (Grossaffoltern, Switzerland). Each week some plants were cut, dried for one week at 20 °C and 45 % humidity, and analyzed for cannabinoid and terpene content.

Sample preparation

0.1 g of cannabis plant materials were weighed in a glass vials and after adding 1 mL of EtOH/$CHCl_3$ 9:1 they were sonicated during 15 min. Afterwards, samples were filtered and diluted in EtOH, 1:100 for the cannabinoid analysis and 1:10 for the terpene analysis. Internal standards were added in the dilution step, phenantrene at the final concentration of 20 µg/mL for cannabinoids and 1-octanol at the final concentration of 100 µg/mL for terpenes. Samples were injected just after the preparation.

HPLC-DAD

The analysis of cannabinoids was carried out in a HPLC system consisted of an Agilent 1100 series equipped with a quaternary pump, an autosampler and a DAD. The chromatographic separation was achieved using a Nucleosil C8 column (3 µm, 125 mm x 4 mm i.d.) with a guard column (3 µm Depth Filter x 4 mm) (Macherey-Nagel, Oensingen, Switzerland) and a binary A/B gradient (solvent A was MeOH and solvent B was H_2O with 0.1 % of acetic acid). The gradient program was as follows: initial conditions were 50 % A; rise to 90 % A over 20 minutes, maintain at 90 % A over the next 1.5 minutes, decrease to 50 % A over the next 0.5 minutes and hold at 50 % A until 27 minutes for re-equilibration of the system prior to the next injection. A flow rate of 0.7 mL/min was used; the column was set at 40 °C and the injection volume was 10 µL. Cannabinoids were quantified at a detection wavelength of 230 nm.

The quantification of cannabinoids was performed with external calibration, using the average values of 3 sets of standards containing target compounds at concentrations ranging from 0.5 to 400 µg/mL in MeOH. Low and high calibration ranges were used in each set, 0.5-25 µg/mL and 10-400 µg/mL respectively. System fluctuations were corrected with the internal standard phenantrene at 20 µg/mL and quality control samples were injected every week along with samples. The limit of detection and the limit of quantification for all compounds were 0.1 µg/mL and 0.5 µg/mL and the correlation coefficients (R^2) were $\geq$ 0.9974.

GC-FID

An Agilent GC 6890 series equipped with a 7683 autosampler, a flame ionization detector (FID) and a DB5 column (0.25 µm, 30 m x 0.25 mm i.d.) from Agilent Technologies (Santa Clara, California, USA) was used for quantitative analysis of terpenes. The analysis was carried out according to (Fischedick et al., 2010) method with minor modifications [54]. The injector temperature was set at 230 °C, the injection volume was 4 µL and a split ratio of 1:20. A carrier gas (N_2) flow rate of 1.2 ml/min was used. The oven temperature program started at 60 °C with a ramp rate of 3 °C/min until reaching 220 °C,

and then was increased to 300 °C with a ramp rate of 40 °C. The temperature was held for 10.67 min at 300 °C making a total run time of 66 min/sample. The FID detector temperature was set to 250 °C and a H_2 flow of 30 mL/min, synthetic air flow of 400 mL/min and N_2 make up flow of 25 mL/min were used.

Thanks to the low variability in response factors of similar molecular mass,[56] the quantification of all terpenes was performed using the average of 3 sets of standards containing ϒ-terpinene at concentrations ranging from 0.5 to 1000 µg/mL in EtOH. Low and high calibration ranges were prepared in each set, 0.5-50 µg/mL and 25-1000 µg/mL respectively. System fluctuations were corrected with the internal standard 1-octanol at 100 µg/mL and quality control samples were injected every week along with samples. The limit of detection and the limit of quantification for all compounds were 0.1 µg/mL and 0.5 µg/mL respectively and the correlation coefficients (R^2) were ≥ 0.9998.

GC-MS

Analysis were performed on an Agilent 6890 series equipped with a 7683 autosampler, a DB5 column (0.25 µm, 30 m x 0.25 mm i.d.) from Agilent Technologies (Santa Clara, California, USA) and a single quadrupole mass spectrometer 5973. The transfer line temperature was set to 280 °C, the MS source to 230 °C and the single quadrupole to 150 °C. Same oven gradient and injection and flow conditions used for GC-FID were applied, but in this case helium was used as a carrier gas instead of nitrogen. The analyzed mass range was 50-400 amu. The GC-MS was controlled by Enhanced Chemstation software MSD D.01.00 Build 75 (Agilent Technologies, Santa Clara, California, USA) and the NIST 11 library (Standard Reference Data Program of the National Institute of Standards and Technology, Distributed by Agilent Technologies) was used to compound identification. The terpenes α-pinene, β-pinene, myrcene, limonene, eucalyptol, terpinolene, linalool, β-caryophyllene, humulene and guaiol were verified with reference standards by comparing the retention time and the obtained mass spectra, while β-ocimene, α-bergamotene, β-farnesene, alloaromandrene, β-selinene, ϒ-selinene, α-bisabolene, β-bisabolene, β-curcumene, β-sesquiphellanderene , α-gurjunene, selina-3,7(11)diene, ϒ-elemene, ϒ-eudesmol, β-eudesmol, α-selinene, bulnesol and α-bisabolol were identified using the NIST library.

Data treatment

Based on the terpene and cannabinoid concentrations, a multivariate data treatment was carried out to find characteristic terpenes for each chemotype plants. Principal component analysis (PCA) and partial least squared regression (PLS) were accomplished with the statistical software The Unscrambler (9.7 Camo Asa, Oslo, Norway) in order to identify specific patterns in the study design and to build regression models against the maturation time. On the one hand, PCA uses an orthogonal transformation to transform a number of possibly correlated variables into linearly uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the matrix data as possible and the next principal component for as much of the remaining variability as possible. Thus, the dimensionality of the data set can be reduced and the underlying variables identified. Moreover, cross-correlation was used to evaluate how the compounds are correlated during the plants growing. On the other hand, PLS makes use of a similar strategy but instead of finding the PCs of minimum variance

between the response and independent variables, the algorithm maximizes the correlation of each PC with the dependent variables (i.e. the time).

Concentration data (X (224 samples x 36 variables) and Y (224 samples x 1 variable (time)) including categorical data such as the type of plant, the phase of plant development, the analysis of leaves or flowers, were uploaded in The Unscrambler. Since in most of the cases the concentration of the cannabinoids and terpenes ranged from values lower than detection limits at the early development stage, the distribution of the concentrations was not normal and there were many < LOD or missing values, so the data was transformed to a log (X+0.005) to eliminate the < LODs and to improve the normality of the distribution.

Initial PCA and PLS models were built with standardized X variables to give equal weigh to all variables and making use of the leverage correction method as the internal validation procedure, and once the putative outliers were removed and a robust model was obtained a final model was built making use of the cross-correlation as the internal validation procedure.

3 Results and discussion

Figure 1 shows the evolution of major cannabinoids, THCA, CBDA and CBGA in leaves and flowers during the growth of plants from chemotypes I, II and III. Several conclusions can be extracted from this figure. On the one hand, the chemotype of the plants, defined by the THCA/CBDA ratio, was clear from the beginning and stable during the growth. This pattern was previously observed in other studies [48, 53], and it is really important for forensic purposes, because it makes senseless to wait for plant flowering to identify if a plant is drug-type or not. On the other hand, THCA and CBDA content evolution in leaves has the same pattern for every chemotype plants. Firstly, the concentrations were clearly decreased during the first weeks of the vegetative phase while the plant was growing. Mother plants are well developed plants that are kept in the vegetative phase. However, as it is the case of this study, usually they are kept smaller than plants that are passing to the flowering phase, so they are more concentrated. Therefore, the decrease of cannabinoid amount during the plant growing is the expected pattern. On the contrary, during the last two weeks of the vegetative phase (days 84 and 94) the concentrations of THCA and CBDA increase slightly though the plants do not grow so we observe the accumulation of the cannabinoids. Later, a decrease is observed during the first weeks of flowering, when the plants were translated from 2 L pots to 10 L pots and the plants grew even more. Finally, when trichomes start to develop an increase is observed not just in the flowers but also in the leaves.

A really important observation from Figure 1 is that the maximum concentration of THCA and CBDA in flowers were attained at different stages or maturation, depending on the chemotype. For the plants of chemotype I the peaks were found in the 9^{th} week of the flowering phase (day 165), followed by a decrease during the onset of senescence, while on chemotype II and III plants the concentrations continued increasing until finishing the study.

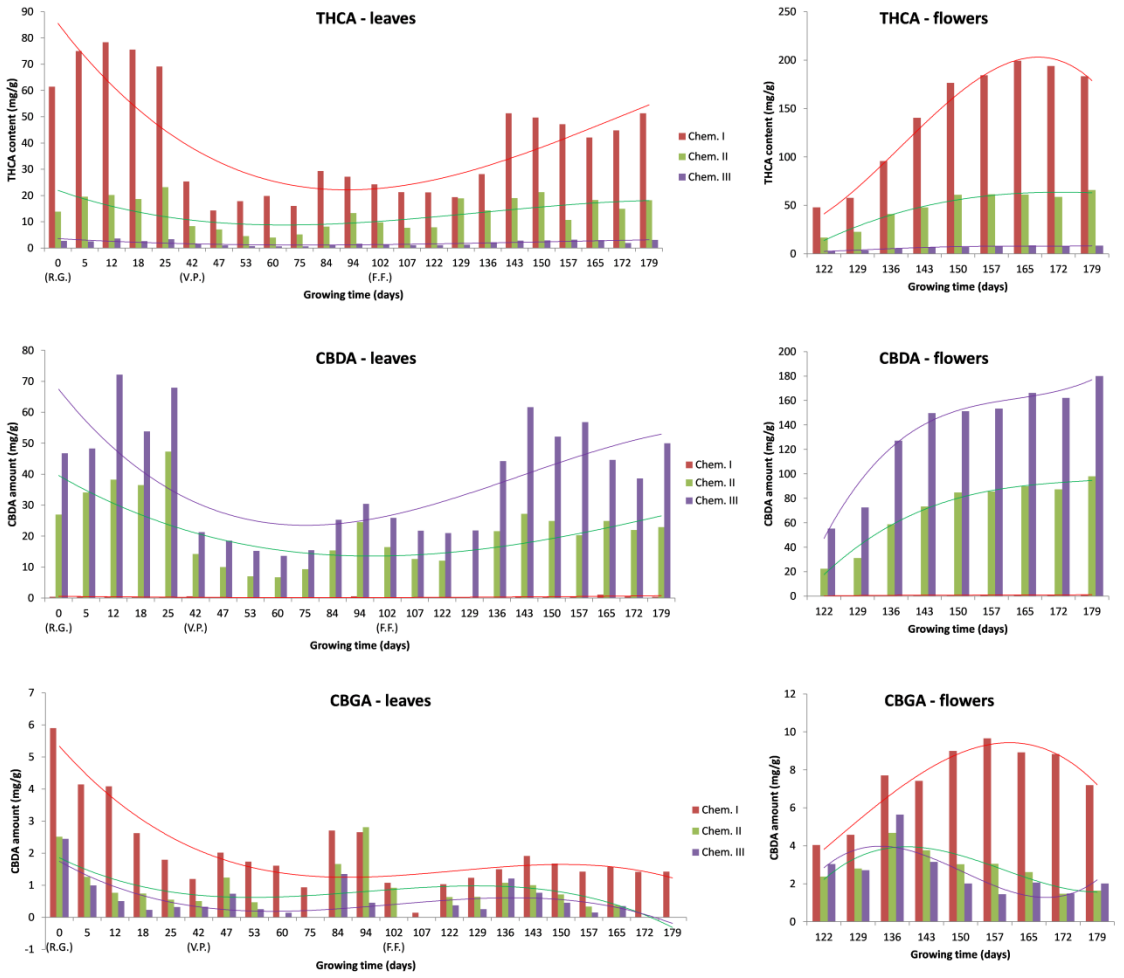

Figure 1: Evolution of THCA, CBDA and CBGA content in leaves and flowers during the growth of plants from chemotype I, II and III. These values are the averages of all studied plants in each chemotype, each one measured in triplicate. (R.G. refers to root growing phase, V.P. to vegetative phase and F.P to flowering phase).

The last discussion is about CBGA. This compound is known to be the first cannabinoid biosynthetized in the plant, and from it, THCA, CBDA and CBCA are synthetized, each by a particular synthase [32]. CBGA evolution in leaves was similar to THCA and CBDA until the flowering phase, but it was not observed the sharp increased shown by THCA and CBDA. It is worth mentioning the differences observed among chemotypes during the flowering phase.As can be seen in Figure 1, the concentration of CBGA remained constant in chemotype I plants while it decreased for the other two chemotypes. This difference was more pronounced in flowers, since an increase in CBGA content was observed in chemotype I plants until the onset of senescence and a slight decrease was observed in chemotype II and III plants. This observation is reassured by statistic data obtained from cross-correlations, where the correlation coefficient of CBGA with THCA and CBDA during the growth of the plants was found to be 0.789 and -0.114, respectively. In conclusion, it can be said that the relation between the CBGA biosynthesis rate and the THCA synthesis rate, controlled by B_T alleles, is somehow stable during the plant growing. On the contrary, this relation is unstable for CBDA synthesis, which is controlled by B_D alleles.

Regarding to other minor cannabinoids, i.e. CBN (a degradation product of THC) was not detected in the plants, showing that plants did not suffer any measurable degradation. THC, CBD and CBG, neutral compounds of above discussed cannabinoids are not included in the Figure 1, as they were found at low concentrations. However, their development was different from the acidic cannabinois. During the root growing and the vegetative phase, their content was really small (below the limit of quantification), followed by a sharp increase, in leaves and in flowers, during the last 6 weeks of the flowering phase due to the decarboxilation of acidic cannabinoids. Small amount of CBC was found in the plants, being clearly in a higher content in chemotype I plants (correlation coefficients of CBC with THCA and CBDA were 0.777 and -0.133 respectively). Complete data about the evolution of all studied cannabinoids and terpenes in each plant is presented as supplementary material.

Figure 2 shows the development of the total content of monoterpenes and sesquiterpenes in the three different studied chemotypes, in leaves and flowers. The total content of monoterpenes and sesquiterpenes was calculated adding up all analyzed terpenes of each kind, 8 for monoterpenes and 20 for sesquiterpenes. The same evolution patterns found for THCA and CBDA in leaves were observed for monoterpenes: a clear decrease during the first weeks of the vegetative phase, a small increase in the last two weeks of the vegetative phase and a slight decrease during the first weeks of flowering phase followed by a clear increase. The maximum concentration in flowers was also chemotype dependant, and as for THCA and CBDA, this maximum for total monoterpenes was found at the 9th week of the flowering phase, while on chemotype II and III plants the concentrations continued increasing until the end of this study.

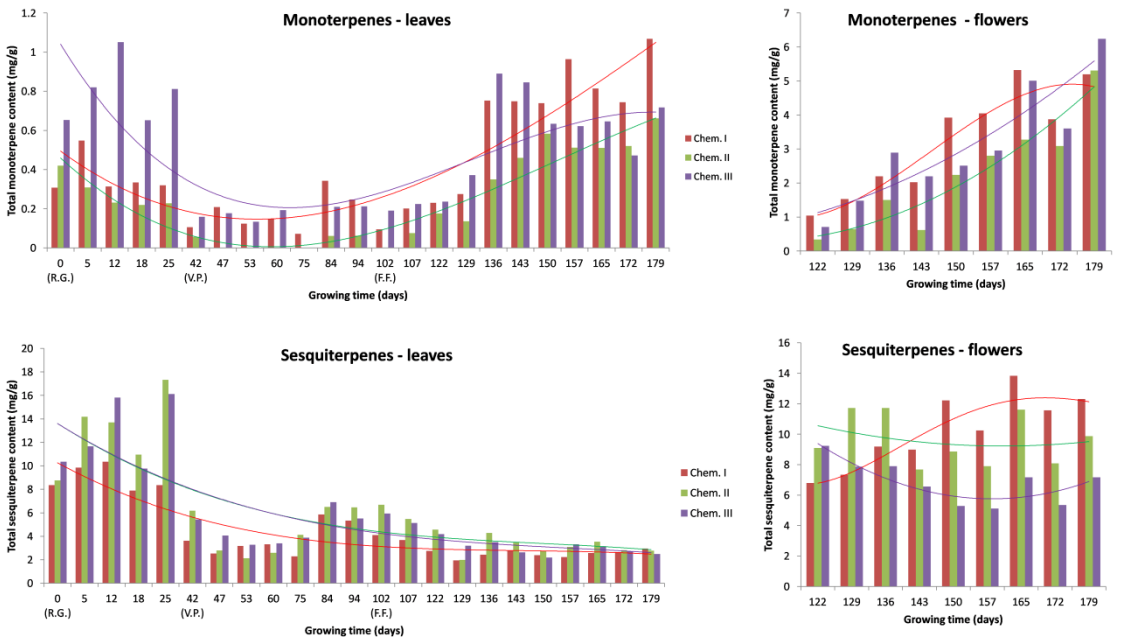

Figure 2: Evolution of total monoterpene and sesquiterpene content in leaves and flowers during the growth of plants from chemotype I, II and III. These values are the averages of all studied plants in each chemotype, each one measured in triplicate. (R.G. refers to root growing phase, V.P. to vegetative phase and F.P to flowering phase).

On the contrary, sesquiterpenes had a different evolution in both plant matrices. In leaves, the pattern was parallel until the first weeks of the flowering phase but after that, the content was maintained stable. In a similar way, in flowers, the amount of sesquiterpenes was not increased during the flowering and was maintained somehow stable. All terpenes are derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The condensation of one DMAPP and two IPP molecules lead to the formation of farnesyl diphosphate (FPP), precursor of sesquiterpenes; whereas the condensation of one DMAPP and one IPP molecules lead to the formation of geranyl diphosphate (GPP), precursor of monoterpenes. Following the formation of FPP and GPP, sesquiterpenes and monoterpenes are generated by the action of many specialized terpene synthases (TPSs) [55]. However, the expression of these TPSs can be different among plant tissues and the different stages of the plant development, resulting in a different terpene content [55]. In this case, it can be deduced that monoterpene synthases expression was more abundant during this phase, leading to an increase in monoterpene content during the flowering phase.

In order to get a broader view of the development of cannabinoids and terpenes, a principal component analysis (PCA) and partial least squared regression (PLS) were carried out taking all the experimental data (X (224 samples x 36 variables), Y(224 samples x 1 time). Though the PCA model required more than 4 PCs to explain the original data structure up to 60% is explained by the first three PCs. As can be seen in Figure 3 in the PC1-PC2 projection we can clearly distinguish the chemotype I plants from the rest (two clusters) and the leaves and flowers in each cluster (leaves in blue and flowers in red). The chemotype II plant is closer to chemotype III plants than chemotype I ones, probably due to a higher CBDA content. From the loading projection we can also identify the featuring cannabinoids and terpenes of each class of samples as those that are close to CBDA and THCA. This way, we can reassured that the higher content of CBGA and CBC is attributed to chemotype I plants, but also that terpenes like β-eudesmol, γ-eudesmol, guaiol, α-bisabolene, α-bisabolol or eucalyptol were much more pronounced in chemotype III plants, while γ-selinene, β-selinene, α-gurjunene, ϒ-elemene, selina-3,7(11)diene or β-curcumene were characteristic of chemotype I plants. This chemotype-dependant terpene distribution isalso observed in the correlation analysis of the data. As shown in Table 1, terpenes that were more pronounced in chemotype III plants have higher correlation coefficients with CBDA than with THCA. On the contrary, characteristic terpenes of chemotype I have high correlation coefficients with THCA and negative coefficients with CBDA.

As shown in the 3D score projection (Figure 4), a closer view of chemotype I plants shows a fine distinction to the three varieties of plants (A, B, C) . Though leaves and flowers are clearly distinguish by colors, it is worth saying that the class features of each plant are shared in both leaves and flowers. Similar results were found with chemotype III plants (data not shown).

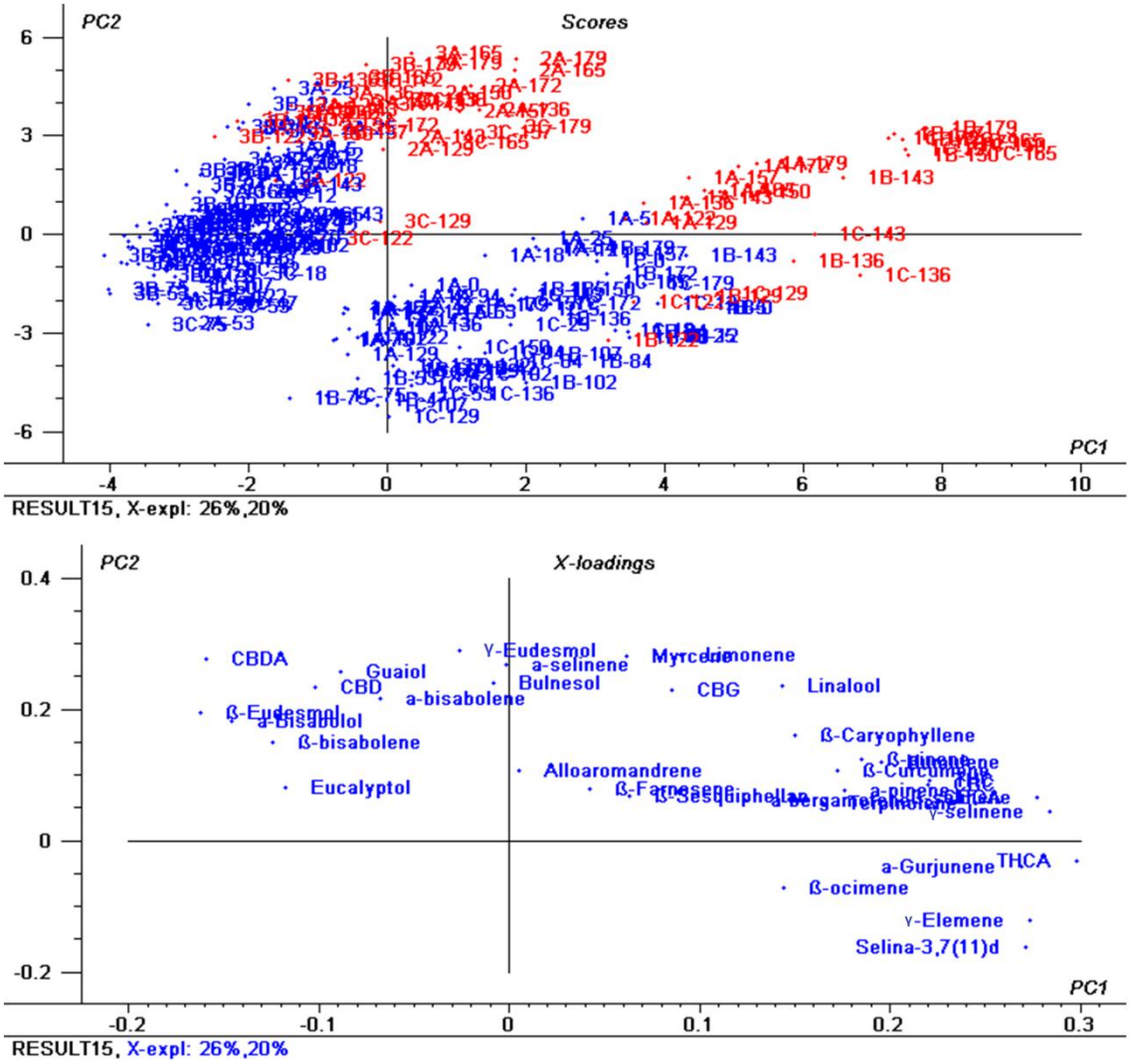

Figure 3: Score and loadings (PC1 *vs.* PC2) performed by principal components analysis according to the concentration of all analyzed compounds during the growth of the plants. (1: refers to chemotype I plants; 2: to chemotype II plants and 3: to chemotype III plants; while A, B and C are the different plants of each chemotype. The number at the end refers to the growing time in days. Leaves are colored in blue and flowers in red).

Table 1

Correlation coefficients between characteristic terpenes of chemotype I and III plants, and THCA and CBDA, obtained by cross-correlation statistical analysis.

Chemotype I	THCA	CBDA	Chemotype III	THCA	CBDA
γ-Selinene	0.921	-0.188	β-Eudesmol	-0.160	0.564
β-Selinene	0.920	-0.128	γ-Eudesmol	0.129	0.517
α-Gurgujene	0.858	-0.346	Guaiol	-0.109	0.487
γ-Elemene	0.790	-0.323	α-Bisabolene	-0.151	0.452
Selina-3,7(11)diene	0.704	-0.404	α-Bisabolol	-0.293	0.369
β-Curcumene	0.702	-0.091	Eucalyptol	-0.387	0.365

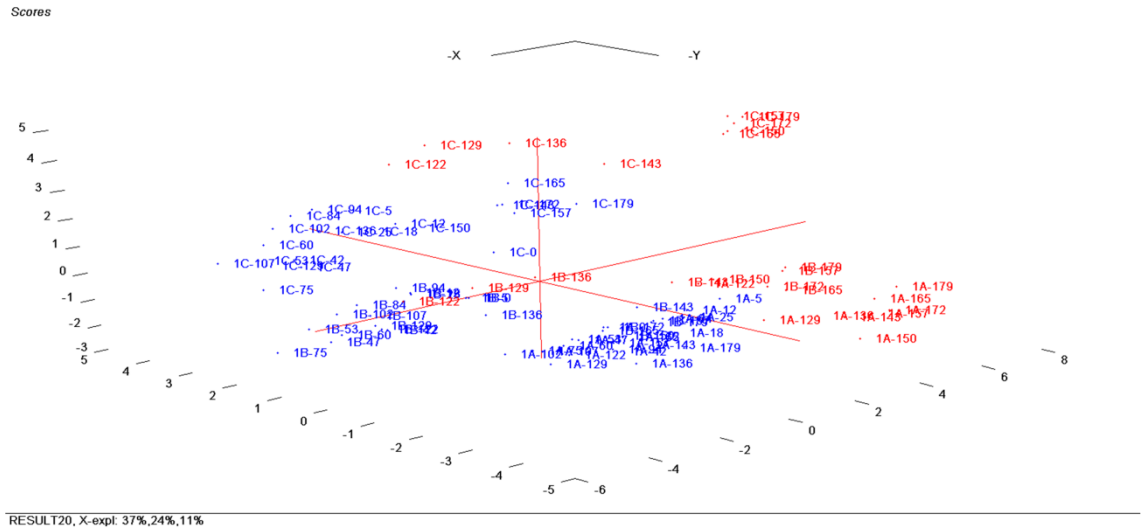

Figure 4: Score loadings (PC1 *vs.* PC2) performed by principal components analysis according to the concentration of all analyzed compounds during the growth of chemotype I plants. (1: refers to chemotype I plants; A, B and C are the different plants of each chemotype; and the number at the end refers to the growing time in days. Leaves are colored in blue and flowers in red).

By means of the PLS analysis we want to introduce the development process of the plants as the guiding factor to interpret the patterns of the different cannabinoids and terpenes. As it was the case in the PCA analysis, the first regression was carried out with all the data and though the final model with 5 PCs was able to correlate satisfactorily the main variation pattern with time and to select the most significant variables, the uncertainty of the model was too high to provide a robust interpretation. Therefore, most restrictive models were built taking into account only each different types of chemotypes. In the case of the chemotype I plants the final model required 5 PCs but the first three ones explained up to 65 % of the variance in X and 88 % in Y, and in the case of chemotype III 52 % of X and 82 % of Y. The projection of the samples in the PC1-PC2-PC3 space showed the distinction of the three types of plants as seen before in the PCA model with a slightly clearer definition of leaves and flowers as a consequence of the different maturation process. The robustness of each PLS model was estimated by the cross-validation procedure and the statistical features [56]. Among those features we obtained the regression coefficients of each variable with the growing time. In Figure 5 the values of the regression coefficients obtained for chemotyes I and III are plotted including both the significant and non-significant coefficients.

From the regression coefficients it can be observed that neutral cannabinoids show generally higher positive value than acidic cannabinoids, that is in agreement with the increasing concentrations due to decarboxilation as long as the plants are growing. However, these values are clearly influenced by the high content of acidic cannabinoids in mother plant leaves. In the case of terpenes, clear differences between monoterpenes and sesquiterpenes are found again. The monoterpenes show a positive coefficient while the sesquiterpenes are mostly negative, with the clear exception of β-curcumene.

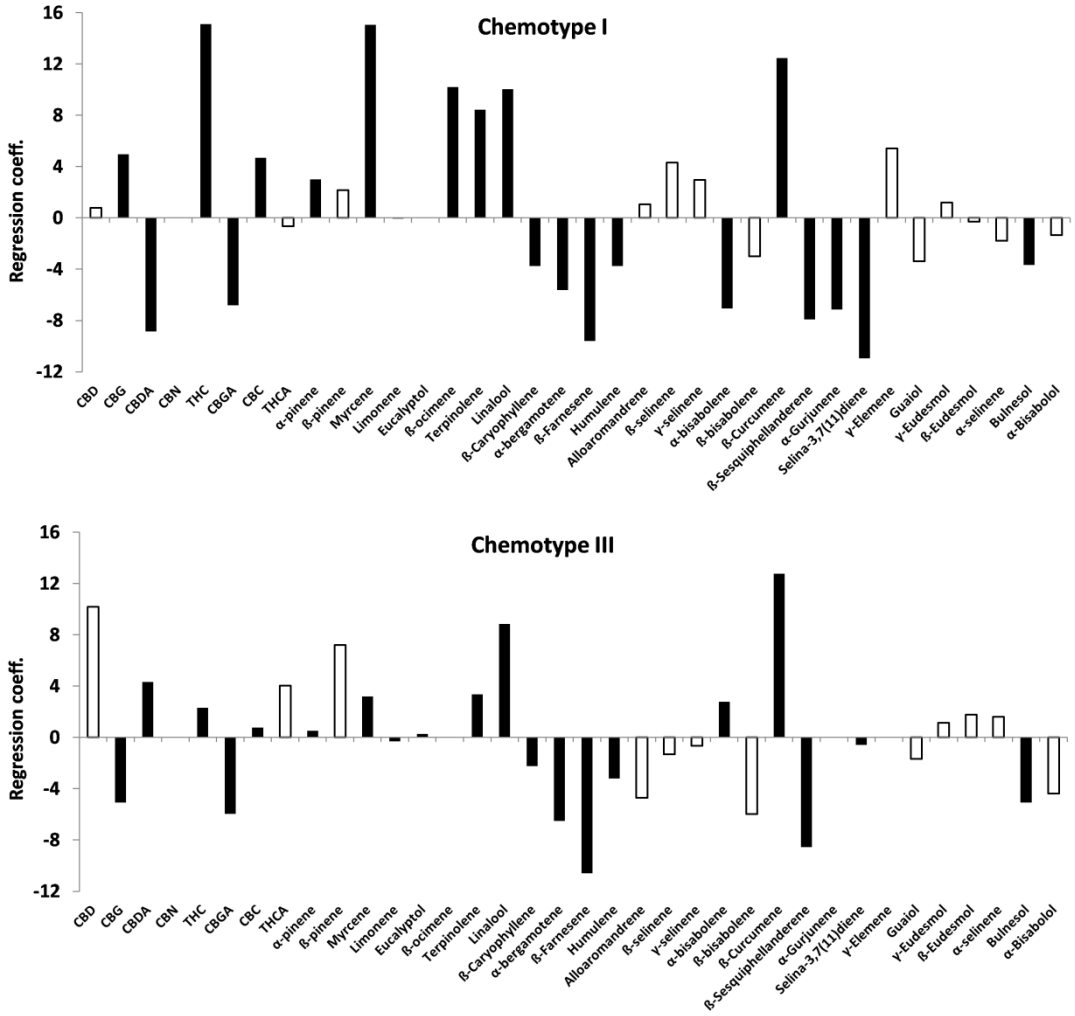

Figure 5: Regression coefficients between growing time and all studied variables in chemotype I and III plants (black filled bars are significant variables and empty bars are non-significant ones).

4 Conclusions

From these results we can select not only the most adequate type of plants to produce the target blend of cannabinoids and terpenes, but also the growing time needed to fulfill these requirements. In addition to this, we have also got important clues about the biosynthesis rate of CBGA in the different chemotypes and the ratios of this particular cannabinoid with CBDA and THCA. Finally, it is also important to point out the relations found with the terpenes due to the synergic effects that show with cannabinoids and the suitability of this combination in certain therapies.

Acknowledgements

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5 References

[1] Abrams DI, Hilton JF, Leiser RJ, Shade SB, Elbeik TA, Aweeka FT, Benowitz NL, Bredt BM, Kosel B, Aberg JA, Deeks SG, Mitchell TF, Mulligan K, Bacchetti P, McCune JM, Schambelan M. 2003. Short-term effects of cannabinoids in patients with HIV-1 infection. A randomized, placebo-controlled clinical trial. Ann Intern Med, 139, 258–266.

[2] Beal JE, Olson R, Laubenstein L, Morales JO, Bellman P, Yangco B, Lefkowitz L, Plasse TF, Shepard KV. 1995. Dronabinol as a treatment for anorexia associated with weight loss in patients with AIDS. J Pain Symptom Manage, 10, 89–97.

[3] Jatoi A, Windschitl HE, Loprinzi CL, Sloan JA, Dakhil SR, Mailliard JA, Pundaleeka S, Kardinal CG, Fitch TR, Krook JE, Novotny PJ, Christensen B. 2002. Dronabinol *versus* megestrol acetate *versus* combination therapy for cancer-associated anorexia: a North central cancer treatment group study. J Clin Oncol, 20, 567–573.

[4] Herman TS, Einhorn LH, Jones SE, Nagy C, Chester MB, Dean J, Furnas B, Williams SD, Leigh SA, Dorr RT, Moon T.E. 1979. Superiority of nabilone over prochlorperazine as an antiemetic in patients receiving cancer chemotherapy. N Engl J Med, 300, 1295–1297.

[5] Hutcheon AW, Palmer JBD, Soukop M, Cunningham D, Mcardle C, Welsh J, Stuart F, Sangster G, Kaye S, Charlton D. 1983. A randomised multicentre single blind comparison of a cannabinoid antiemetic (levonantradol) with chlorpromazine in patients receiving their first cytotoxic chemotherapy. Eur J Cancer Clin Oncol, 19, 1087–1090.

[6] Ungerleider JT, Andrysiak T, Fairbanks L, Goodnight J, Sarna G, Jamison K. 1982. Cannabis and cancer chemotherapy. A comparison of oral delta-9-THC and prochlorperazine. Cancer, 50, 636–645.

[7] Lynch ME, Campbell F. 2011. Cannabinoids for treatment of chronic non-cancer pain; a systematic review of randomized trials. Br J Clin Pharmacol, 72, 735-744.

[8] Wilsey B, Marcotte T, Deutsch R, Gouaux B, Sakai S, Donaghe H. 2013. Low-dose vaporized cannabis significantly improves neuropathic pain. J Pain, 14, 136-148.

[9] Wilsey B, Marcotte T, Tsodikov A, Millman J, Bentley H, Gouaux B, Fishman S. 2008. A randomized, placebo-controlled, crossover trial of cannabis cigarettes in neuropathic pain. J. Pain, 9, 506-521.

[10] Corey-Bloom J, Wolfson T, Gamst A, Jin S, Marcotte TD, Bentley H, Gouaux B. 2012. Smoked cannabis for spasticity in multiple sclerosis: a randomized, placebocontrolled trial. CMAJ, 10, 1143-1150.

[11] Rog DJ, Nurmikko TJ, Friede T, Young CA. 2005. Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. Neurology, 65, 812-819.

[12] Wade DT, Makela P, Robson P, House H, Bateman C. 2004. Do cannabis-based medicinal extracts have general or specific effects on symptoms in multiple sclerosis? A double-blind, randomized, placebo-controlled study on 160 patients. Mult Scler, 10, 434-441.

[13] De Petrocellis L, Vellani V, Schiano-Moriello A, Marini P, Magherini PC, Orlando P, Di Marzo V. 2008. Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. J Pharmacol Exp Ther, 325, 1007-1015.

[14] Galve-Roperh I, Sánchez C, Cortés ML, Gómez Del Pulgar T, Izquierdo M, Guzmán M. 2000. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med, 6, 313-319.

[15] Parolaro D, Massi P. 2008. Cannabinoids as a potential new drug therapy for the treatment of gliomas. Expert Rev Neurother, 8, 37-49.

[16] Velasco G, Sánchez C, Guzmán M. 2012. Towards the use of cannabinoids as antitumour agents. Nat Rev Cancer, 12, 436-444.

[17] Cunha JM, Carlini EA, Pereira AE, Ramos OL, Pimentel C, Gagliardi R, Sanvito WL, Lander N, Mechoulam R. 1980. Chronic administration of cannabidiol to healthy volunteers and epileptic patients. Pharmacol, 21, 175–185.

[18] Porter BE, Jacobson C. 2013. Report of a parnet survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy. Epilepsy Behav, 29, 574-577.

[19] Campbell VA, Gowran A. 2007. Alzheimer′s disease; taking the edge off with cannabinoids? Br J Pharmacol, 152, 655-662.

[20] Eubanks LM, Rogers CJ, Beuscher AE, Koob GF, Olson AJ, Dickerson TJ, Janda KD. 2006. A molecular link between the active component of marijuana and Alzheimer′s disease pathology. Mol Pharm, 3, 773-777.

[21] Luvone T, Esposito G, De Filippis D, Scuderi C, Steardo L. 2009. Cannabidiol: a promising drug for neurodegenerative disorders? CNS Neurosci Ther, 15, 65-75.

[22] Sagredo O, Pazos MR, Valdeolivas S, Fernandez-Ruiz J. 2012. Cannabinoids: Novel Medicines for the Treatment of Huntington′s Disease. Recent Pat CNS Drug Discover, 7, 41-48.

[23] Penner EA, Buettner H, Mittleman MA. 2013. Marijuana use on glucose, insulin, and insulin resistance among US adults. Am J Med, 126, 583-589.

[24] Weiss L, Zeira M, Reich S, Har-Noy M, Mechoulam R, Slavin S, Gallily R. 2006. Cannabidiol lowers incidence of diabetes in non-obese diabetic mice. Autoimmunity, 39, 143-151.

[25] Müller-Vahl KR, Schneider U, Prevedel H, Theloe K, Kolbe H, Daldrup T, Emrich H.M. 2003. Δ9-tetrahydrocannabinol (THC) is effective in the treatment of tics in Tourette syndrome: a 6-week randomized trial. J Clin Psychiatry, 64, 459–465.

[26] Müller-Vahl KR, Schneider U, Koblenz A, Jobges M, Kolbe H, Daldrup T, Emrich H.M. 2002. Treatment of Tourette's syndrome with Δ9-tetrahydrocannabinol (THC): a randomized crossover trial. Pharmacopsychiatry, 35, 57–61.

[27] ElSohly M, Gul W. 2014. Constituents of cannabis sativa, in: Pertwee R (ed.), Handbook of Cannabis. Oxford University Press, Oxford, 3-22.

[28] Ahmed SA, Ross SA, Slade D, Radwan MM, Khan IA, Elsohly MA. 2015. Minor oxygenated cannabinoids from high potency *Cannabis sativa L.* Phytochem, 117, 194-199.

[29] ElSohly M, Slade D. 2005. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. Life Sci, 78, 539–548.

[30] Aizpurua-olaizola O, Omar J, Navarro P, Olivares M, Etxebarria N, Usobiaga A. 2014. Identification and quantification of cannabinoids in *Cannabis sativa L.* plants by high performance liquid chromatography-mass spectrometry. Anal Bioanal Chem, 406, 7549-7560.

[31] Russo EB. 2007. History of Cannabis and Its Preparations in Saga, Science, and Sobriquet. Chem Biodivers, 4, 1614-1648.

[32] Sirikantaramas S, Taura F, Tanaka Y, Ishikawa Y, Morimoto S, Shoyama Y. 2004. The gene controlling marijuana psychoactivity. J Biol Chem, 279, 39767-39774.

[33] Dewick PM. 2002. The biosynthesis of C_5–C_{25} terpenoid compounds. Nat Prod Rep, 19, 181-222.

[34] Grotenhermen F. 2002. Effects of Cannabis and the cannabinoids, in Grotenhermen F, Russo E (ed). Cannabis and Cannabinoids-Pharmacology, Toxicology, and Therapeutic Potential. New York, Haworth Press, 55-65.

[35] Consroe P. 1998. Brain cannabinoid systems as targets for the therapy of neurological disorders. Neurobiol Dis, 5, 534–551.

[36] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci, 30, 515-527.

[37] McPartland JM, Russo EB. 2011. Cannabis and cannabis extracts: greater than the sum of their parts? J Cann Therap, 1, 103-132.

[38] Mechoulam R, Parker LA, Gallity R. 2002. Cannabidiol: an overview of some pharmacological aspects. J Clin Pharmacol, 42, S11-S19.

[39] Morgan CJ, Das RK, Joye A, Vurran HV, Kamboj SK. 2011. Cannabidiol reduces cigarette consumption in tobacco smokers: preliminary findings. Addict Behav, 38, 2433-2436.

[40] Colasanti BK. 2008. A comparison of the ocular and central effects of delta 9-tetrahydrocannabinol and cannabigerol. J Ocul Pharmacol, 6, 259-269.

[41] Borrelli F, Fasolino I, Romano B, Capasso R, Maiello F, Coppola D, Orlando P, Battista G, Pagano E, Di Marzo V, Izzo AA. 2013. Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease. Biochem Pharmacol, 85, 1306-1316.

[42] De Petrocellis L, Vellani V, Schiano-Moriello A, Marini P, Magherini PC, Orlando P, Di Marzo V. 2008. Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. J Pharmacol Exp Ther, 325, 1007-1015.

[43] Maione S, Piscitelli F, Gatta L, Vita D, De Petrocellis L, Palazzo E, de Novellis V, Di Marzo V. 2011. Non-psychoactive cannabinoids modulate the descending pathway of antinociception in anaesthetized rats through several mechanisms of action. Br. J. Pharmacol. 162, 584-596.

[44] Shinjyo N, Di Marzo V. 2013. The effect of cannabichromene on adult neural stem/progenitor cells. Neurochem Int, 63, 432-437.

[45] Izzo AA, Capasso R, Aviello G, Borrelli F, Romano B, Piscitelli F, Gallo L, Capasso F, Orlando P, Di Marzo V. 2012. Inhibitory effect of cannabichromene, a major non-psychotropic cannabinoid extracted from Cannabis sativa, on inflammation-induced hypermotility in mice. Br J Pharmacol, 166, 1444-1460.

[46] Russo EB. 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol, 163, 1344-1364.

[47] Wagner H, Ulrich-Merzenich G. 2009. Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine, 16, 97-110.

[48] Pacifico D, Miselli F, Carboni A, Moschella A, Mandolino G. 2008. Time course of cannabinoid accumulation and chemotype development during the growth of *Cannabis sativa L.* Euphytica, 160, 231-240.

[49] De Meijer EPM, Bagatta M, Carboni A, Prucitti P, Moliterni V, Ranalli P, Mandolino G. 2003. The Inheritance of chemical phenotype in cannabis sativa L. Genetics. 163, 335-346.

[50] Mandolino G, Bagatta M, Carboni A, Ranalli P, De Meijer E. 2003. Qualitative and quantitative aspects of the inheritance of chemical phenotype in cannabis. J Ind Hemp, 8, 51-72.

[51] De Meijer EPM, Hammond KM. 2005. The inheritance of chemical phenotype in *Cannabis sativa L.* (II): cannabigerol predominant plants. Euphytica, 145, 189-198.

[52] Cohen Y. 2014. Cannabis plant named 'avidekel'. US patent 20140259228 A1.

[53] De Backer B, Maebe K, Verstraete AG, Charlier C. 2012. Evolution of the content of THC and other major cannabinoids in drug-type cannabis cuttings and seedlings during growth of plants. J Forensic Sci, 57, 918-922.

[54] Fischedick JT, Hazekamp A, Erkelens T, Choi YH, Verpoorte R. 2010. Metabolic fingerprinting of *Cannabis sativa L.* cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes. Phytochemistry, 71, 2058-2073.

[55] Nagegowda DA. 2010. Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and dubcellar compartmentation. FEBS Letters, 584, 2965-2973.

[56] Eriksson L, Johansson E, Kettaneh-Wold N, Wold S. 2002. Multi- and megavariate data analysis: Principles and applications, I J Chemometrics, 16, 261-262.

Kannabisa: ageriko altxor ezkutua

Ekaiara bidalia

Aizpurua-Olaizola O, Navarro P, Etxebarria N, Usobiaga A

9. ATALA

Laburpena

Etorkizun hurbilean kannabisa sendagai bezala erabili ahal izateko 3 urrats eman dira lan honetan. Alde batetik, landareen kannabinoideen hatz-marka garatzeko metodo bat garatu da fragmentazio bikoitzeko masa-espektometriari akoplaturiko eraginkortasun handiko likido kromatografia bidez (HPLC-MS/MS High Performance Liquid chromatography-tandem mass spectrometry). Bestalde, gernuan eta plasman kannabinoideak eta euren metabolitoak kuantifikatzeko metodo bat garatu da. Horretarako, lehenik analitoek gorputzean osatzen dituzten glukuronido loturak hausteko hidrolisi mota ezberdinak aztertu dira eta hidrolisi entzimatiko-alkalino bikoitzak ehuneko ehunean hausten dituela ikusi da. Ostean, fase solidozko erauzketan (SPE Solid Phase Extraction) eta HPLC-MS/MS bidezko analisian oinarritzen den metodoa garatu eta balioztatu da. Azkenik, kemotipo ezberdineko landareen hazkuntzan zeharreko kannabinoideen eta terpenoen garapena aztertu da. Bertan, konposatu garrantzitsuenen kontzentrazio maximoak noiz agertzen diren ikusteaz gain landare-mota bakoitzaren ezaugarri diren terpenoak zeintzuk diren aurkitu da.

Hitz-gakoak: Kannabisa, hatz-marka, gernu- eta odol-analisia, hazkuntza, kannabinoideak eta terpenoak.

1 Sarrera

Lur azpiko altxorrak ondo ezkutatuta eta mila tranpaz eta amarruz babestuta daudela erakutsi izan digu Hollywood-ek, egon bada ordea lur azpitik bere kabuz irteten den altxorrik ere. Ederki zekiten hori gure arbasoek. Antzinako Txinan duela 6000 urte baino gehiago kalamua sokak, jantziak eta itsasontziak egiteko erabiltzeaz gain, kannabis psikoaktiboa anestesiko moduan eta ekitaldi erlijiosoetan inspirazio-iturri gisa erabiltzen zuten. Ekialde ertainaren inguruetan ere bazuten landarearen berririk, asirioek sendagai, bizigarri eta ekitaldi erlijiosoetarako erabiltzen zuten, greziarrek eta erromatarrek hanturaren aurka, Arabian minari, sukarrari eta epilepsiari aurre egiteko eta Indian aldiz beldurrak eta kezkak uxatzeko; hots, ansiolitiko gisa [1-2].

Zerbait bazekiten jakin antzinako gizakiek; izan ere, erabilera guzti horiek eta gehiago zientifikoki egiaztatuak daude gaur egun. Hala, jakinak dira kannabisak dituen hainbat ezaugarri eta erabilera; esate baterako anorexia, minbizien fase aurreratu edo HIES kasuetan lagungarri den gosea pizteko baliagarria dela [3-5]; minbiziak sendatzeko erabiltzen den kimioterapia errazago jasateko efektu antiemetikoa duela [6-8]; minbiziek, HIESak eta fibromialgia edo erreuma-artritisaren gisako gaixotasunek eragindako min neuropatiko kronikoari aurre egin diezaiokela [9-11] eta esklerosi anizkoitza tratatzeko erabili daitekeela [12-14].

Bestalde, oraindik guztiz frogatuak egon ez arren, geroz eta ikerketa zientifiko gehiagok babesten dute kannabisa beste gaixotasun askotarako irtenbide garrantzitsua izan daitekeela; hala nola hainbat minbizia sendatzeko [15-18], epilepsia tratatzeko [19-20] eta Alzheimerrari [21-22], Hungtintonen gaixotasunari [23-24], diabetes kasuei [25-26] zein Touretten sindromeari [27-28] aurre egiteko. Gizakietan eginiko ikerketa bakanen bat kenduta [19, 27-28], maila zelularrera eta animaliekin eginiko probetara mugatzen dira ordea ikerketa gehienak. Dena den, ikerketa bakan hauetan eta maila zelularrean eta animaliekin egindako probetan lortutako emaitza itxaropentsuek ikerketa kliniko gehiagoren beharra mahai gainean jarri dute.

Landare miragarri honek hainbeste gaixotasunerako balio izateak badu bere azalpen zientifikoa: sistema endokannabinoidea. Sistema hau zenbait lipido neuromodulatzailek eta euren hartzaileek osatzen dute eta gorputzeko hainbat prozesu fisiologikoetan hartzen du parte; hala nola gosean, min sentsazioan, umorean eta memorian. Lipido hauek endokannabinoide gisa ezagutzen dira eta euren hartzaileekiko selektiboak dira; hots, giltza batek sarraila batekin duen funtzio bera betetzen dute hartzaileekin. Kontua da, kannabisean aurkitzen diren kanabinoide ezberdinek ere hartzaile hauekin lotzeko gaitasuna dutela, modu honetan prozesu fisiologiko guzti horietan eraginez [29-30].

Kannabis landarea ez da batere sinplea ordea. Izan ere, 554 konposatutik gora identifikatu dira bertan, horien artean 113 kannabinoide [31] eta 120 terpeno [32]. Kannabinoideak forma azidoan biosintetizatzen dira landarean, baina berotzean edo argiaren presentzian dagokien forma neutrora deskonposatzen dira. Bestalde, degradazioak ere jasan ditzakete eta beste kannabinoideetan bihurtu. Hau da kannabisaren psikoaktibitatearen eragile nagusietako bat den eta tetrahidrokannabinol azidotik (THCA) datorren tetrahidrokannabinolaren (THC) kasua, non kannabinolera (CBN) degradatua izan

daitekeen [33]. 113 kannabinoide horietako bakoitzak aktibitate terapeutiko ezberdina du eta, ondorioz, aplikazio ezberdinetarako erabili daitezke [34]. Hortaz gain, euren artean eragin sinergikoa gertatzen da; esaterako jakina da kannabidiol azidotik (CBDA) datorren kannabidiolak (CBD) THC-ren eragin euforikoa modulatzen duela [35]. Hori gutxi ez eta terpenoek, lurrinaren erantzule izateaz gain, efektu terapeutiko ezberdinak aurkezten dituzte eta kannabinoideekin sinergian joka dezakete baita [36].

Milaka kannabis barietate ezagutzen dira gaur egun, eta kopurua handituz doa egunetik egunera, efektu ezberdinak lortu nahian barietate ezberdinak elkarren artean gurutzatzen baitira berriak sortzeko. Dena den, ia barietate guzti hauek kannabisaren bi azpiespezie nagusien gurutzaketetatik datoz; hots, *sativa*-tik eta *indica*-tik. Hala, gaur egun aurkitzen diren barietateak *sativa* modukoak, batez ere *sativa* modukoak, hibridoak, batez ere *indica* modukoak ala guztiz *indica* modukoak izan daitezke hauen arteko nahastearen ehunekoaren arabera. Orain arte kaleko kulturan, azpiespezie bakoitzari justu elkarren aurkakoa den efektu jakin bat egotzi izan zaie gainera; *sativa*-k THCA/CBDA ratio altuekin lotu izan direnez estimulatzaileak eta bizigarriak direla uste izan da eta *indica*-k aldiz ratio baxuagoekin lotu izan direnez erlaxagarriak. Hala, teorikoki azpiespezie bakoitzari aplikazio jakinak aurkitzeko modua egongo litzateke. Dena den, nahiz eta morfologikoki ezberdinak izan, oraindik eztabaida dago ia kimikoki eta, ondorioz, efektu aldetik ezberdintasun agerikorik duten ala ez [37]. Bestalde, barietate jakin bat leku ezberdinetatik eskuratu ezkero euren konposatuen kontzentrazioetan aldaketa handi xamarra egoten dela jakina da [37]. Kontuak hala, landare jakinak aplikazio jakinekin lotu nahi badira beharrezkoa da batetik landare estandarizatuak izatea eta bestetik konposatuen kontzentrazioen araberako sailkapen berri bat finkatzea.

Laburbilduz, kannabisak bere baitan zein bere osagaia puruek edo hauen nahasteek, medikuntzaren alor ezberdinetan etorkizun handia izan dezaketela esan genezake. Dena den, bide handia dago egiteko oraindik. Aipatu bezala, saiakera kliniko gehiagoren beharra agerikoa da. Bestalde, gaixotasun bakoitzerako landarearen eragin onuragarriak ahalik eta handienak izateko landare ezberdinen profilak zehaztuta izatea, konposatu ezberdinen aktibitateak eta euren arteko sinergiak hobeto ulertzea, eta hauek sistema endokannabinoidearekin duten elkarrekintzak zehazki nola funtzionatzen duen jakitea beharrezkoa da.

Ildo horretan hiru urrats garrantzitsu eman dira lan honetan. Alde batetik, landareen kannabinoideen profila edo hatz-marka deiturikoa zehazteko metodo bat garatu da HPLC-MS/MS bidez.

Bestetik, saiakera klinikoetan lagungarria da kontzentrazio/efektu erlazioak aztertzea; neurri batean kannabinoideak metabolizatu eta glukuronido loturak eratzen dituzte ordea gorputzean. Beraz, lan honetan gernuan eta plasman aurkitzen diren kannabinoideen eta euren metabolitoen kontzentrazio totala neurtu ahal izateko metodo analitiko bat garatu da. Horretarako glukuronido lotura ezberdinak hausteko hidrolisi entzimatiko-alkalino bikoitza erabili da eta konposatuak SPE bidez erauzi ostean analisia HPLC-MS/MS bidez gauzatu da.

Azkenik, saiakera klinikoetan erabiltzeko beharrezkoa da kannabinoide zein terpeno puruak edo hauen kontzentrazio ratio jakinak dituzten landareak eskala handian ekoiztea. Bide horretan, CBDA %

15etik gora eta THCA % 1etik behera duen barietate bat lortu zen 2014an [38]. Dena den, ekoizpena guztiz optimizatzeko beharrezkoa da konposatu hauek landarearen hazkuntzan zehar duten garapena ulertzea. Hori dela eta, lan honetan kannabinoide eta terpeno nagusien garapena aztertu da 7 kannabis barietate ezberdinen (3 THCA altukoak, 3 CBDA altukoak eta 1 bien kontzentrazio parekokoa) hazkuntza-prozesu osoan zehar. Horretarako, 8 kannabinoide nagusi jarraitu dira diodo-segiden detektoreari (DAD Diode Array Detector) akoplaturiko HPLC bidez eta 20 terpenotik gora garraren bidezko ionizazio-detektagailuari loturiko gas kromatografiaren bidez (GC-FID Gas Chromatography – Flame Ionization Detector).

2 Landareetako kannabinoideen hatz-marka

Kannabis landareen kannabinoideen hatz-marka eratu ahal izateko beharrezkoa da ahalik eta kannabinoide gehien identifikatu eta kuantifikatzea. Lan honetan 6 kannabinoide nagusi kuantifikatzeko eta beste 7 identifikatu eta kualitatiboki aztertzeko metodo analitikoa garatu da. Bestalde, metodoa baliagarria dela frogatu da 30 landare ezberdin aztertuz.

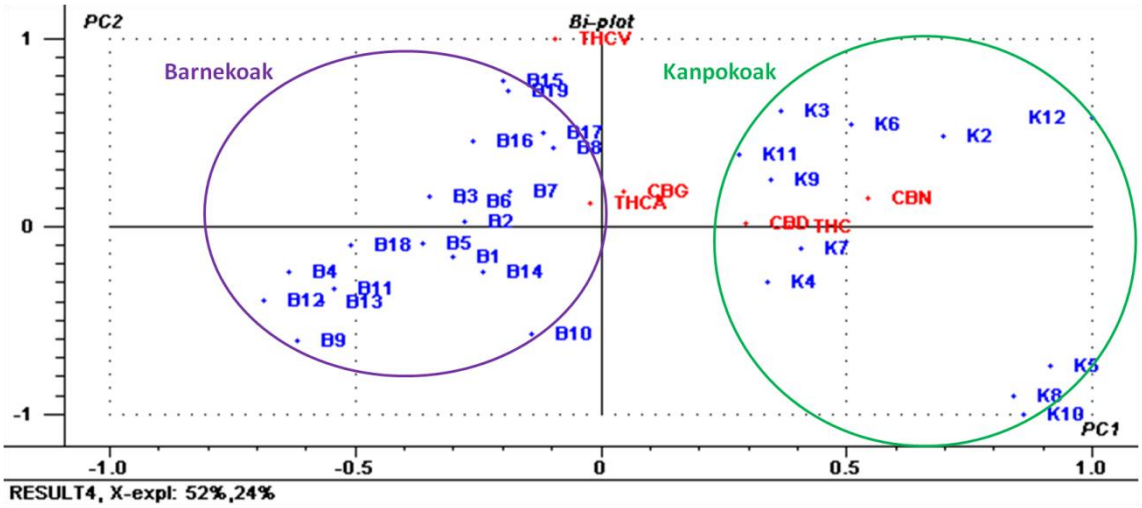
Ildo honetan lehenik eta behin kannabinoideak erauzteko aurrez optimizatutako jariakin gainkritikoen bidezko erauzketa (SFE) erabili da, kannabinoide guztiak eraucteaz gain lortzen diren erauzkinak purutasun altukoak baitira [39].

Bestalde, erauzkin hau aztertu ahal izateko sentikortasun handiko analisi metodo bat garatu da HPLC-MS/MS bidez. Horretarako, alde batetik konposatuen banaketa ziurtatzeko zutabe, disolbatzaile eta gradiente egokia aurkitzeaz gain, MS-eko kuadrupoloetara iritsi aurretik ekipoak dituen aldagai nagusiak optimizatu dira (kapilarreko boltajea, eluzioa lurruntzeko tenperatura, nitrogeno garraiatzailearen fluxua eta tenperatura eta langarreztagailuaren presioa). Bestalde, MS-eko kuadrupoloetan analito bakoitzean gertatzen diren masa-fragmentazio nagusiak identifikatu eta hauen erantzuna ahalik eta handiena izan dadin bakoitzaren fragmentazio-potentzialak eta kolisio-energiak optimizatu dira baita. Modu honetan, 0.02 eta 0.2 ng/mL arteko detekzio mugak erdietsi dira kuantifikatu diren 6 kannabinoideentzat.

Kuantifikatutakoez gain beste 7 kannabinoide identifikatzea lortu da kuadrupoloetan izan dituzten hausturak aztertuz. Euren kolisio-energiak eta fragmentazio-potentzialak optimizatu dira baita, modu honetan analito hauen azterketa kualitatiboa egin ahal izateko. Identifikatutako 7 kannabinoide hauek konposatuen identifikazio zehatzagoa eskeintzen duen hegaldi-denbora bati akoplatutako kuadrupolo (Q-TOF) bidez berretsi dira.

Datuen tratamenduari dagokionez, osagai nagusien analisia (PCA) erabili da datu guztiak bildu eta landareen hatz-marka osatu ahal izateko. Modu honetan, kannabis landare ezberdinak aplikazio terapeutiko ezberdinekin lotzeko baliagarria izan daitekeen kanabinoideen kontzentrazioen araberako banaketa metodo bat garatu da. Metodo honen erabilgarritasunaren adierazgarri, 1. irudian ikus daiteke nola barnean eta kanpoan hazitako landareak elkarrengandik guztiz ezberdindu daitezkeen

kannabinoideen kontzentrazioen arabera. Banaketa honetan gehien eragiten duen kannabinoidea CBN da, THC degradatzen denean sortzen den konposatua hain zuzen ere. Barnean hazitako landareen argi- eta tenperatura-baldintzak kontrolpean egoten dira, ez ordea kanpoan hazitakoenak. Eguraldiak izaten dituen aldaketen ondorioz, zentzuzkoa da, gertatu den bezala, kanpoan hazitako landareetan CBN kontzentrazio altuagoak aurkitzea. THC eta CBD balio handiagoak aurkitzen dira baita landare hauetan, baldintza gogorragoen ondorioz euren homologo azidoak konposatu neutroetara azkarrago degradatzen baitira. THCA-k, kannabigerolak (CBG) eta tetrahidrokannabibarinak (THCV) aldiz ez dute hainbeste eragiten banaketa honetan, aldakortasun oso handia aurkezten baitute landare barietate ezberdinen artean.



1. irudia: *Cannabis sativa* L. landare ezberdinen kannabinoide nagusien kontzentrazioa kontuan hartuz osagai nagusien analisi bidez erdietsitako pisu eta aldagaien irudikapen bikoitza (PC1 vs PC2). (B: barnean hazitako landareei dagokie; K: kanpoan hazitako landareei dagokie).

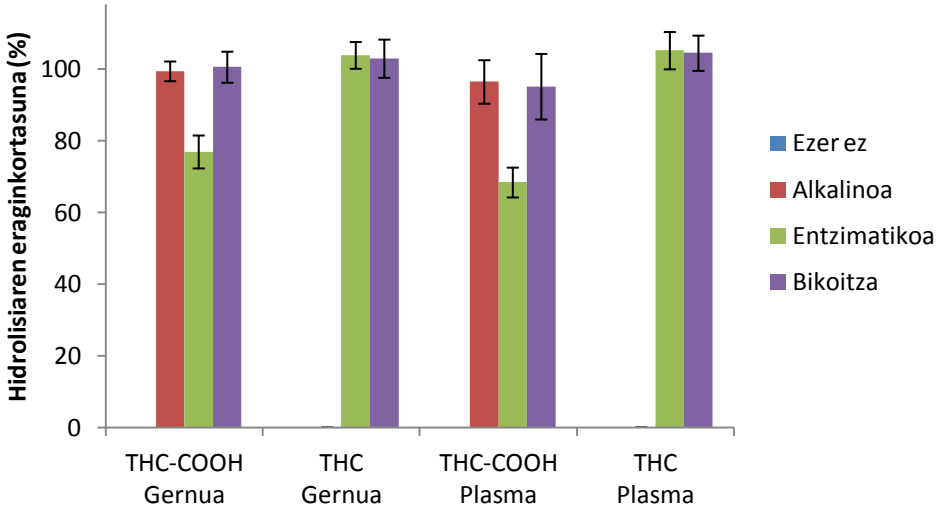
3 Gernuaren eta plasmaren analisia

Arestian aipatu bezala, kannabisak aplikazio ezberdinetarako duen erabilgarritasuna aztertzeko saiakera kliniko gehiagoren beharra agerikoa da. Bestalde, saiakera hauetan oso lagungarria da gorputzak xurgatutako kannabinoideen eta hauen metabolitoen kontzentrazioak ezagutzea gero hauek kannabisa hartzeak eragindako efektuekin erlazionatu ahal izateko. Helburu horrekin, lan honetan THC eta honek gorputzean osatzen dituen bi metabolito nagusiak diren 11-hidroxi- Δ9-tetrahidrokannabinola (11-OH-THC) eta 11-nor-9-karboxi- Δ9-tetrahidrokannabinola (THC-COOH), CBD, CBG, THCV eta CBN kuantifikatzeko HPLC-MS/MS bidezko metodoa garatu eta balioztatu da.

Kannabinoideak, zein hauetatik osatutako metabolitoak, hein batean glukuronizatu egiten dira gorputzean. Kannabinoidearen edo metabolitoaren arabera glukuronido lotura ezberdinak osatzen dituzte gainera, eter eta ester loturak hain zuzen ere [40]. Kannabinoideen eta metabolitoen kontzentrazio totala jakiteko beharrezkoa da beraz lotura glukuronido hauek haustea. Ildo horretatik, lehenik eta behin hidrolisi prozesu ezberdinek bi lotura mota hauek hausteko duten gaitasuna aztertu da, bai gernuan eta baita plasman. Horretarako, plasma- eta gernu-lagin zuriei THC-glukuronido (eter lotura) eta

THCCOOH-glukuronido (ester lotura) estandarrak gehitu zaizkie kontzentrazio ezagun batean. Dopatutako lagin zuri hauei hidrolisi mota ezberdinak aplikatu zaizkie: hidrolisi entzimatikoa β-glukuronidasa erabiliz, hidrolisi alkalinoa sodio hidroxidoa erabiliz eta hidrolisi entzimatiko-alkalino bikoitza hidrolisi metodo bakoitza bata bestearen atzetik aplikatuz. Alderatzeko laginak hidrolisirik gauzatu gabe ere aztertu dira.

Behin hidrolisiak gauzatuta konposatuak SPE bidez erauzi eta aurrez optimizatutako HPLC-MS/MS bidezko metodo baten bidez analizatu dira. Erdietsitako emaitzak 2. irudian daude ikusgai. Bertan ikus daitekeenez hidrolisi alkalinoa ester lotura guztiak hausteko gai da baina ez du gaitasunik eter loturarik hausteko. β-glukuronidasa aldiz eter lotura guztiak hausteko gai da, baita hein batean ester loturak hausteko ere nahiz eta ez ehuneko ehunean. Ondorioz, hidrolisi entzimatiko-alkalino bikoitza hautatu da laginen aurretratamendu gisa, lotura glukuronido guztien % 100 haustea ziurtatzen baitu.



2. irudia: THCCOOH-glukuronidoarekin eta THC-glukuronidoarekin dopatutako gernu eta plasma laginetan glukuronido loturak hausteko aztertutako aurretratamendu ezberdinen eraginkortasuna. Erabilitako aurretratamenduak: hidrolisirik ez, hidrolisi alkalinoa, hidrolisi entzimatikoa eta hidrolisi entzimatiko-alkalino bikoitza.

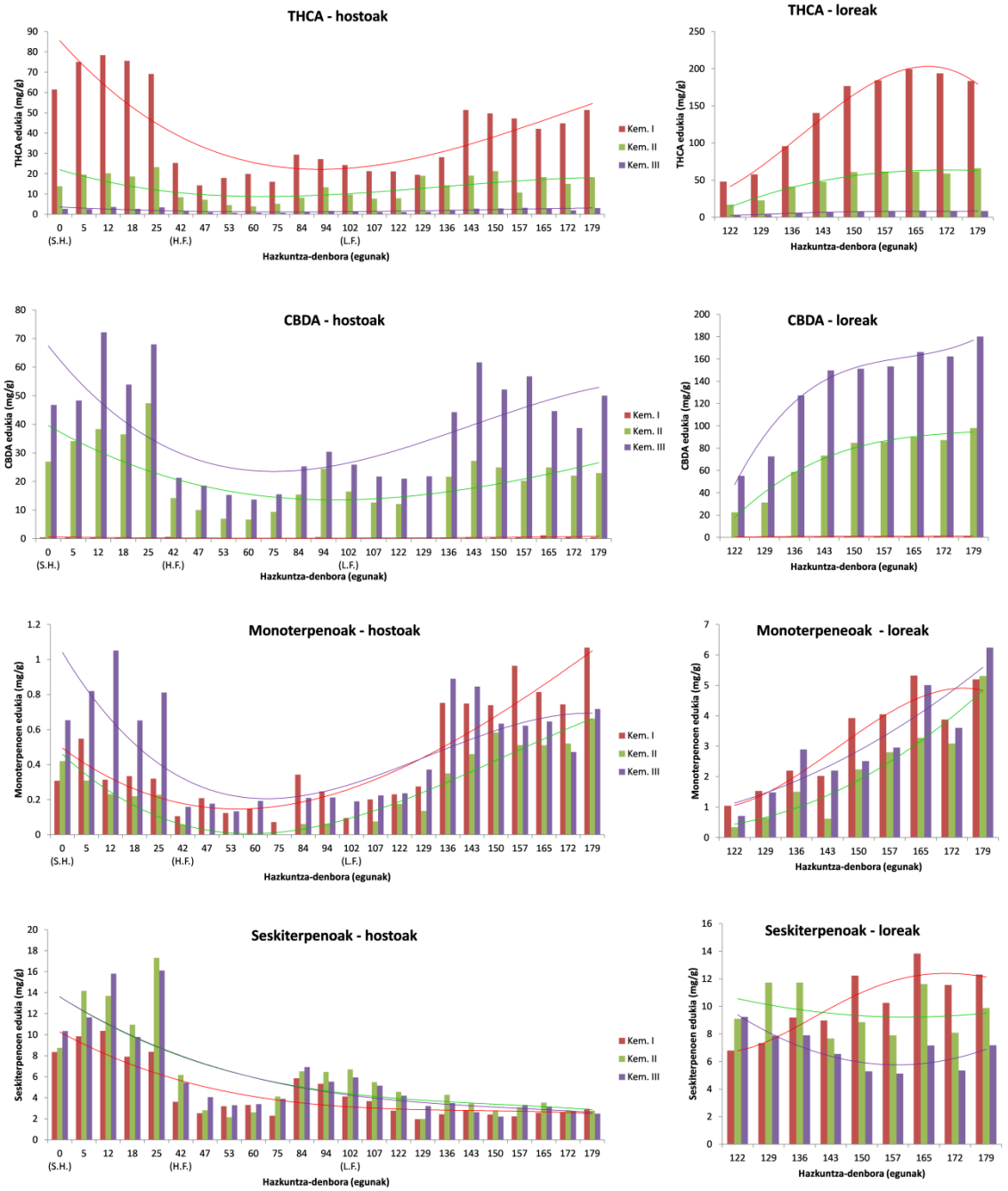
Behin hidrolisi metodoa zehaztuta garatutako teknika gernuan eta plasman balioztatu da. Horretarako THC, 11-OH-THC, THC-COOH, CBD, CBG, THCV eta CBN estandarren kontzentrazio ezagunekin dopatutako lagin zuriei analisi prozesu osoa aplikatu zaie: hidrolisi bikoitza, SPE bidezko erauzketa eta HPLC-MS/MS bidezko analisia. Erdietsitako zehaztasuna % 84 eta % 115 artekoa izan da eta egun barneko eta egunen arteko balioen desbiderapena % 12 baino txikiagoa kasu guztietan. Metodoaren detekzio mugak 1 ng/mL-tik beherakoak izan dira analito guztientzat eta balioztatutako metodoa kannabis erabiltzaileen gernu- eta plasma-laginak aztertzeko erabili da behar bezalak emaitzak erdietsiz.

4 Kannabis landareen hazkuntzaren kimika

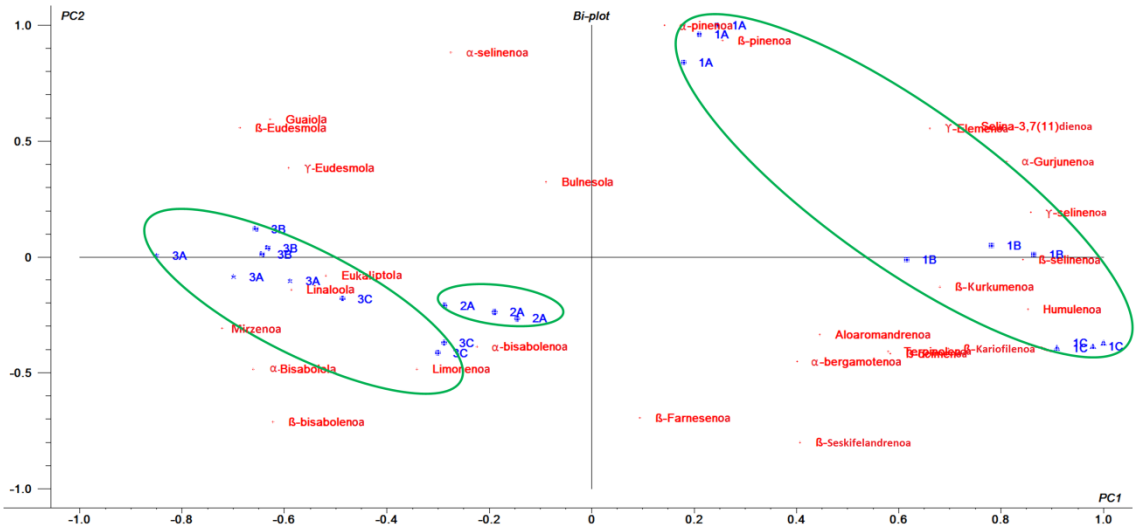
Lehenago esan bezala, lan honetan kannabinoide eta terpeno nagusien garapena aztertu da 7 kannabis barietate ezberdinen hazkuntza-prozesu osoan zehar. 3 barietate THCA altukoak izan dira (I. kemotipoa), 3 CBDA altukoak (III. kemotipoa) eta 1 bien kontzentrazio antzekokoa (II. kemotipoa). Horretarako, ama-landare bakoitzaren 50 klon landatu dira eta barnean hazi dira hiru fase ezberdinetan. Lehen fasean klonak 25 x 25 mm-ko kubotxoetan eduki dira 18 orduko argi-zikloetan sustraiak hazi arte. Orduan 2 L-ko ontzietara pasa eta landareen hazkuntza gauzatu da (bigarren fasea), 18 orduko argi-zikloetan baita. Azken fasean, landareak 10 L-ko ontzietara pasa eta 12 orduko argi-zikloa ezarriz loratzea eragin da. Astero landare bakoitzetik 3 klon moztu eta analizatu dira (kannabinoideak HPLC-DAD bidez eta terpenoak GC-FID bidez).

3. irudian aztertutako bi kannabinoide nagusienak diren THCA-k eta CBDA-k, eta analizatutako monoterpeno eta seskiterpeno guztien baturek, kemotipo ezberdinetako landareen hazkuntzan zehar izandako garapena islatzen da. Bertatik ondorio ezberdinak atera daitezke. Alde batetik, THCA/CBDA erlazioak definitzen duen landarearen kemotipoa haseratik agerikoa eta hazkuntzan zehar egonkorra dela. Bestalde, hostoetako kannabinoideen eta terpenoen kontzentrazioa modu garbian jaisten dela landarearen hazkuntza-fasean zehar eta, ostean, loratze-fasean berriz igo egiten dela, seskiterpenoen salbuespenarekin, hauen kontzentrazioak are gehiago jaisten jarraitzen baitu. Beste ondorio garrantzitsu bat loreetako THCA eta CBDA kontzentrazio maximoak landarearen kemotipoaren arabera aldatu egiten direla da. Hala, I. kemotipoko landareetan maximoa loratze-faseko 9. astean (165. eguna) aurkitzen da, II. eta III. kemotipoetako landareetan aldiz kontzentrazioak gora egiten jarraitzen du ikerketaren bukaera-arte. Monoterpenoen kasuan ere antzerako joera ikusten da, ez ordea seskiterpenoetan, non joerak ez diren hain nabariak.

Azkenik, konposatu bakoitzaren kontzentrazio maximoa noiz gertatzen den jakiteaz gain, etorkizun hurbilean kannabinoideen eta terpenoen sinergiak hobeto ulertu ahal izateko ia kemotipo bakoitzak terpeno bereizgarririk duen aztertu da. 4. irudian argi ikusi daiteke kemotipo ezberdinetako landareen terpenoen profila guztiz ezberdina dela. Modu honetan, esaterako β-bisabolenoa, α-bisabolola edo mirzenoa III. kemotipoko landareetan askoz ugariagoak direla ikusi da, eta, selina-3,7(11)dienoa, ϒ-elemenoa edo α-gurjunenoa aldiz I. kemotipoko landareen bereizgarriak direla. II. taldeko landarea III. taldekoen antzekoa dela ikusi da, ziurrenik bere CBDA edukia THCA edukia baino handiagoa delako.



3. irudia: Landareen hazkuntza prozesuan zeharreko THCA eta CBDA kannabinoide nagusien eta monoterpeno eta seskiterpeno totalen batezbesteko kontzentrazioak ikertutako 3 kemotipo ezberdinetan, hostoetan eta loreetan. ("S.H." sustraien hazkuntza-faseari dagokio, "H.F." landarearen hazkuntza-faseari eta "L.H." loratze-faseari).



4. irudia: Landareen loratze-faseko azken 3 asteetako loreetako terpenoen edukiaren arabera, osagai nagusien analisi bidez erdietsitako pisu eta aldagaien irudikapen bikoitza (PC1 vs PC2). (1: I kemotipoko landareei dagokie; 2: II kemotipoko landareei eta 3: III kemotipoko landareei; A, B eta C ostera kemotipo bakoitzeko landare ezberdinak dira).

5 Ondorioak

Kannabisa medikuntzaren munduan etorkizun handia izango duen landare bat da zalantza izpirik gabe. Oraindik bidearen zati bat egiteke dago ordea. Ildo horretatik, bide hori gauzatzen lagunduko duten hiru urrats eman dira lan honetan.

Alde batetik landare ezberdinak aplikazio ezberdinekin lotzeko lagungarria izan daitekeen landareen kannabinoideen hatz-marka garatzeko metodo bat garatu da. Bestalde, etorkizun hurbilean aurrera eramango diren saiakera klinikoetan kontzentrazio/efektu erlazioak aztertu ahal izateko gernuan eta plasman kannabinoideak eta euren metabolitoak kuantifikatzeko metodo sentikor bat garatu da. Azkenik, saiakera klinikoetarako beharrezkoak diren konposizio jakineko landareen erauzkin aberatsak eta konposatu puruak kopuru handian lortzeko lagungarria izan daitekeen ikerketa bat gauzatu da. Bertan, kannabis landarearen 3 kemotipo ezberdinen hazkuntza-prozesuetan zehar kannabinoide eta terpenoen kontzentrazio maximoak noiz aurkitzen diren ezartzeaz gain, kemotipo bakoitzaren ezaugarri diren terpenoak zeintzuk diren ikusi da. Azken hau kannabinoideen eta terpenoen arteko sinergiak aztertzea helburu duten ikerketetarako lagungarria izan daiteke.

Esker onak

Egileek esker ona adierazi nahi diete Idoki SCF S.L. enpresari jariakin gainkritiko bidezko erauzketa sistema erabiltzen uzteagatik, Aifame GmbH enpresari landareen hazkuntzaren ikerketarako beharrezko material eta baliabide guztiak eskaintzeagatik, Renovatio fundazioari eta Sevillako

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6 Bibliografia

[1] Warf B. 2014. High Points: An Historical Geography of Cannabis. Geograph Rev, 104, 414-438.

[2] Lozano I. 2001. The Therapeutic Use of *Cannabis sativa L.* in Arabic Medicine. J Cannabis Ther, 1, 63-70.

[3] Abrams DI, Hilton JF, Leiser RJ, Shade SB, Elbeik TA, Aweeka FT, Benowitz NL, Bredt BM, Kosel B, Aberg JA, Deeks SG, Mitchell TF, Mulligan K, Bacchetti P, McCune JM, Schambelan M. 2003. Short-term effects of cannabinoids in patients with HIV-1 infection. A randomized, placebo-controlled clinical trial. Ann Intern Med, 139, 258–266.

[4] Beal JE, Olson R, Laubenstein L, Morales JO, Bellman P, Yangco B, Lefkowitz L, Plasse TF, Shepard KV. 1995. Dronabinol as a treatment for anorexia associated with weight loss in patients with AIDS. J Pain Symptom Manage, 10, 89–97.

[5] Jatoi A, Windschitl HE, Loprinzi CL, Sloan JA, Dakhil SR, Mailliard JA, Pundaleeka S, Kardinal CG, Fitch TR, Krook JE, Novotny PJ, Christensen B. 2002. Dronabinol *versus* megestrol acetate *versus* combination therapy for cancer-associated anorexia: a North central cancer treatment group study. J Clin Oncol, 20, 567–573.

[6] Herman TS, Einhorn LH, Jones SE, Nagy C, Chester MB, Dean J, Furnas B, Williams SD, Leigh SA, Dorr RT, Moon T.E. 1979. Superiority of nabilone over prochlorperazine as an antiemetic in patients receiving cancer chemotherapy. N Engl J Med, 300, 1295–1297.

[7] Hutcheon AW, Palmer JBD, Soukop M, Cunningham D, Mcardle C, Welsh J, Stuart F, Sangster G, Kaye S, Charlton D. 1983. A randomised multicentre single blind comparison of a cannabinoid antiemetic (levonantradol) with chlorpromazine in patients receiving their first cytotoxic chemotherapy. Eur J Cancer Clin Oncol, 19, 1087–1090.

[8] Ungerleider JT, Andrysiak T, Fairbanks L, Goodnight J, Sarna G, Jamison K. 1982. Cannabis and cancer chemotherapy. A comparison of oral delta-9-THC and prochlorperazine. Cancer, 50, 636–645.

[9] Lynch ME, Campbell F. 2011. Cannabinoids for treatment of chronic non-cancer pain; a systematic review of randomized trials. Br J Clin Pharmacol, 72, 735-744.

[10] Wilsey B, Marcotte T, Deutsch R, Gouaux B, Sakai S, Donaghe H. 2013. Low-dose vaporized cannabis significantly improves neuropathic pain. J Pain, 14, 136-148.

[11] Wilsey B, Marcotte T, Tsodikov A, Millman J, Bentley H, Gouaux B, Fishman S. 2008. A randomized, placebo-controlled, crossover trial of cannabis cigarettes in neuropathic pain. J. Pain, 9, 506-521.

[12] Corey-Bloom J, Wolfson T, Gamst A, Jin S, Marcotte TD, Bentley H, Gouaux B. 2012. Smoked cannabis for spasticity in multiple sclerosis: a randomized, placebocontrolled trial. CMAJ, 10, 1143-1150.

[13] Rog DJ, Nurmikko TJ, Friede T, Young CA. 2005. Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. Neurology, 65, 812-819.

[14] Wade DT, Makela P, Robson P, House H, Bateman C. 2004. Do cannabis-based medicinal extracts have general or specific effects on symptoms in multiple sclerosis? A double-blind, randomized, placebo-controlled study on 160 patients. Mult Scler, 10, 434-441.

[15] De Petrocellis L, Vellani V, Schiano-Moriello A, Marini P, Magherini PC, Orlando P, Di Marzo V. 2008. Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. J Pharmacol Exp Ther, 325, 1007-1015.

[16] Galve-Roperh I, Sánchez C, Cortés ML, Gómez Del Pulgar T, Izquierdo M, Guzmán M. 2000. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med, 6, 313-319.

[17] Parolaro D, Massi P. 2008. Cannabinoids as a potential new drug therapy for the treatment of gliomas. Expert Rev Neurother, 8, 37-49.

[18] Velasco G, Sánchez C, Guzmán M. 2012. Towards the use of cannabinoids as antitumour agents. Nat Rev Cancer, 12, 436-444.

[19] Cunha JM, Carlini EA, Pereira AE, Ramos OL, Pimentel C, Gagliardi R, Sanvito WL, Lander N, Mechoulam R. 1980. Chronic administration of cannabidiol to healthy volunteers and epileptic patients. Pharmacol, 21, 175–185.

[20] Porter BE, Jacobson C. 2013. Report of a parnet survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy. Epilepsy Behav, 29, 574-577.

[21] Campbell VA, Gowran A. 2007. Alzheimer′s disease; taking the edge off with cannabinoids? Br J Pharmacol, 152, 655-662.

[22] Eubanks LM, Rogers CJ, Beuscher AE, Koob GF, Olson AJ, Dickerson TJ, Janda KD. 2006. A molecular link between the active component of marijuana and Alzheimer′s disease pathology. Mol Pharm, 3, 773-777.

[23] Luvone T, Esposito G, De Filippis D, Scuderi C, Steardo L. 2009. Cannabidiol: a promising drug for neurodegenerative disorders? CNS Neurosci Ther, 15, 65-75.

[24] Sagredo O, Pazos MR, Valdeolivas S, Fernandez-Ruiz J. 2012. Cannabinoids: Novel Medicines for the Treatment of Huntington′s Disease. Recent Pat CNS Drug Discover, 7, 41-48.

[25] Penner EA, Buettner H, Mittleman MA. 2013. Marijuana use on glucose, insulin, and insulin resistance among US adults. Am J Med, 126, 583-589.

[26] Weiss L, Zeira M, Reich S, Har-Noy M, Mechoulam R, Slavin S, Gallily R. 2006. Cannabidiol lowers incidence of diabetes in non-obese diabetic mice. Autoimmunity, 39, 143-151.

[27] Müller-Vahl KR, Schneider U, Prevedel H, Theloe K, Kolbe H, Daldrup T, Emrich H.M. 2003. Δ9-tetrahydrocannabinol (THC) is effective in the treatment of tics in Tourette syndrome: a 6-week randomized trial. J Clin Psychiatry, 64, 459–465.

[28] Müller-Vahl KR, Schneider U, Koblenz A, Jobges M, Kolbe H, Daldrup T, Emrich H.M. 2002. Treatment of Tourette's syndrome with Δ9-tetrahydrocannabinol (THC): a randomized crossover trial. Pharmacopsychiatry, 35, 57–61.

[29] Maccarrone M, Bab I, Bíró T, Cabral GA, Dey SK, Di Marzo V, Konje JC, Kunos G, Mechoulam R, Pacher P, Sharkey KA, Zimmer A. 2015. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci, 36, 277-296.

[30] Di Marzo V, Bifulco M, De Petrocellis L. 2004. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov, 3, 771-784.

[31] Ahmed SA, Ross SA, Slade D, Radwan MM, Khan IA, Elsohly MA. 2015. Minor oxygenated cannabinoids from high potency *Cannabis sativa L*. Phytochem, 117, 194-199.

[32] ElSohly M, Slade D. 2005. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. Life Sci, 78, 539–548.

[33] Aizpurua-olaizola O, Omar J, Navarro P, Olivares M, Etxebarria N, Usobiaga A. 2014. Identification and quantification of cannabinoids in *Cannabis sativa L.* plants by high performance liquid chromatography-mass spectrometry. Anal Bioanal Chem, 406, 7549-7560.

[34] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci, 30, 515-527.

[35] McPartland JM, Russo EB. 2011. Cannabis and cannabis extracts: greater than the sum of their parts? J Cann Therap, 1, 103-132.

[36] Russo EB. 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol, 163, 1344-1364.

[37] Elzinga S, Fischedick J, Podkolinski R, Raber JC. 2015. Cannabinoids and terpenes as chemotaxonomic markers in cannabis. Nat Prod Chem Res, 3: 181. doi:10.4172/2329-6836.1000181.

[38] Cohen Y. 2014. Cannabis plant named 'avidekel'. US patent 20140259228 A1.

[39] Omar J, Olivares M, Alzaga M, Etxebarria N. 2013. Optimisation and characterisation of marihuana extracts obtained by supercritical fluid extraction and focused ultrasound extraction and retention time locking GC-MS. J Sep Sci, 36, 1397-1404.

[40] Abraham TT, Lowe RH, Pirnay SO, Darwin WD, Huestis MA. 2007. Simultaneous GC–EI-MS Determination of Δ9-Tetrahydrocannabinol, 11-Hydroxy-Δ9-Tetrahydrocannabinol, and 11-nor-9-Carboxy-Δ9-Tetrahydrocannabinol in Human Urine Following Tandem Enzyme-Alkaline Hydrolysis. J Anal Toxicol, 31, 477-485.

Ondorioak

10. ATALA

Alde batetik ardo hondakinetatik gantz azido asegabeak eta polifenolak bereizita erauzteko SFE metodoa garatu da, gero hauek dietan aurkitzen diren hutsuneak betetzeko gehigarri gisa erabiltzeko. Garatutako metodoak, azkarra izateaz gain, tenperatura baxuetan gauzatzen denez, konposatu aktiboak degradatzea ekiditen du. Hortaz gain, polifenolak erraz degradatu daitekeenez [1], hauek kapsulatzeko metodo bat garatu da VNM bidez. Jakien industriako aplikazio ezberdinetarako egokiak izan daitezkeen bi tamainatako alginato mikrokapsulen eraketa optimizatu da eta sortutako mikrokapsulek polifenolen egonkortasuna izugarri hobetu dutela ikusi da. Kitosana alginatoaren gehigarri gisa erabiltzea ere aztertu da eta egonkortasuna are gehiago hobetzen dela ikusi da, giro tenperaturan degradazio-abiadura 33 aldiz txikiagoa izatera iritsiz.

Modu honetan, ardo hondakinetatik bi produktu interesgarri lortu dira, batetik gantz azido asegabeetan oso aberatsa den erauzkina eta, bestetik, egonkortasun handiko polifenol mikrokapsulak. Hala, gaur egun ustiapen mugatua duten ardo hondakinen balioa handitzeko urrats garrantzitsu bat eman dela esan daiteke.

Beste alde batetik, kannabisa gaixotasun ezberdinetarako erabilgarria izan daitekeela kontuan hartuz, landare hau sendagai gisa erabiltzeko bidean, lagungarri izan daitezkeen hiru lan gauzatu dira tesi honetan. Lehenengoan landare-mota ezberdinak efektu terapeutiko ezberdinekin lotzeko lagungarria izan daitekeen tresna bat garatu da, landareen kannabinoideen hatz-marka garatzeko HPLC-MS/MS bidezko metodoa hain zuzen ere. Bigarrengoan gernuan eta plasman kannabinoideak eta euren metabolitoak kuantifikatzeko metodo sentikor bat garatu da, hala urte batzuen buruan gauzatuko diren saiakera klinikoetan kontzentrazio/efektu erlazioak ikertu ahal izateko. Kuantifikazio hau fidagarria eta ikerketa ezberdinen artean alderagarria izan dadin, beharrezkoa da lehenik glukuronido-lotura guztiak haustea ordea, eta hidrolisi entzimatiko-alkalino bikoitza erabiliz bi matrizeotan hori ziurtatzen dela ikusi da. Azkenik, saiakera klinikoetarako beharrezkoak izango diren konposizio jakineko landareen erauzkin aberatsak eta konposatu puruak lortzeko prozesua optimizatzeko ikerketa bat gauzatu da. Bertan, kannabis landarearen hiru kemotipo ezberdinen hazkuntza-prozesuetan zehar kannabinoideen eta terpenoenen kontzentrazio maximoak noiz aurkitzen diren ezarri dira. Hala, II. eta III. kemotipoko landareek, kannabinoideen eta monoterpenoen kontzentrazio maximora iristeko I. kemotipokoek baino denbora gehiago behar dutela ikusi da. Bestalde, kemotipo bakoitzaren ezaugarri diren terpenoak zeintzuk diren aurkitu da, hau kannabinoideen eta terpenoen arteko sinergiak aztertu nahi dituzten ikerketetarako lagungarria izan daitekeelarik.

Dena den, mota honetako ikerketa gehienetan gertatzen den bezala, ate bat ixterakoan etorkizunerako helburu eta desafio berriak agerian utzi dituzten ate berriak zabaldu dira lan honetan ere. Besteak beste, hurrengoak aipa genitzake:

- Ardo-hondakinen balioa handitzeko gantz azido asegabeen eta polifenolen erauzketa optimizatu da, optimizazio hau laborategi-mailan eman da ordea. Benetan ardo hondakinen balioa handitu ahal izateko beharrezkoa litzateke laborategi-mailatik eskala industrialera pasatzeko prozesua garatzea.

- Polifenolak erraz degradatzen direnez hauek mikrokapsularatu eta degradatze-prozesu hau izugarri mantsotzea lortu da. Dena den, erraz degradatzeaz gain konposatu hauek bioeskuragarritasun baxua izan ohi dute, gorputzeko jariakin biologikoetako baldintza alkalinoetan ezegonkorrak baitira [2]. Beraz, garatutako mikrokapsulak gehigarri bezala erabili ahal izan aurretik ia arazo hau gainditzeko gai diren aztertzea ezinbestekoa litzateke.

- Kannabis landare ezberdinak efektu terapeutiko ezberdinekin lotu ahal izateko kannabinoideen hatz-marka garatu da lan honetan. Dena den, gaur egun jakin badakigu terpenoek ere kannabinoideekin sinergian joka dezaketela [3]. Ondorioz, komenigarria litzateke hatz-marka osatzeko terpenoak ere kontuan hartzea eta ahal den neurrian kannabinoideen espektroa ere zabaltzea (CBDA, CBC, CBDV...).

- Gernuan eta plasman kannabinoide nagusiak eta THC-ren metabolito nagusiak neurtzeko metodoa garatu da lan honetan. Dena den, gaur arte ikerketa gehienak THC-n oinarritu badira ere, azken urteotan kannabinoide ez-psikoaktiboek (CBD, CBG, CBC...) interes berezia piztu dute, hainbat gaixotasun tratatzeko gaitasuna aurkezteaz gain sendagai gisa erabiltzeko psikoaktibitaterik eza lagungarri baita [4]. Nahiz eta merkatuan eskuragarri ez egon, konposatu hauek ere euren metabolito jakinak sortzen dituzte gorputzean ordea [5]. Beraz, interesgarria litzateke metabolito hauek isolatu/sintetizatu eta kuantifikatzeko metodo analitikoak garatzea.

- Azken lanean hiru kemotipo ezberdinetako landareen hazkuntza-prozesuan zehar kannabinoideek eta terpenoek duten garapena aztertu da. Bertan erabili diren III. kemotipoko landareak droga-motakoak izan dira. Lurralde askotan, Espainian kasu, legeak landareek izan dezaketen THC kopuru maximoa % 0.2-an ezartzen du ordea. Ondorioz, zuntz-motako landareak erabili behar dituzte CBD lortzeko. Landare hauek ere III. kemotipokoak dira, baina aztertu direnetatik oso ezberdinak. Beraz, interesgarria litzateke ikerketa zuntz-motako landare hauetara zabaltzea, eta baita CBG konposatu nagusi duten IV. kemotipokoetara ere.

- Kannabisa hartzeko modurik ohikoena birika-bidezkoa da. Kasu honetan efektua oso azkar gertatzen da, baina bere iraupena laburra izan ohi da [6]. Gaixotasun askotarako egokiagoa da ordea denbora luzeko efektua lortzea. Kasu horietan kannabisa ahotik hartzea eraginkorragoa izan daiteke, nahiz eta efektua berehalakoa ez izan iraunkorragoa izan ohi baita [6]. Polifenolen kasuan gertatzen den bezala, kannabisa ahotik hartzen denean konposatu askoren bioeskuragarritasuna baxua izaten da ordea [7]. Bioeskuragarritasun hau hobetzeko, eta bide batez efektuaren iraupena kontrolatu ahal izateko, aukera egokia izan daiteke mikrokapsularatzea.

- Tesi honetan kannabisaren inguruan gauzatu diren lan guztiek landarea bera edo landaretik eratorritako konposatuak izan dituzte ardatz. Konposatu hauek hainbat gaixotasunetarako erabilgarriak izatearen arrazoia sistema endokannabinoidea da ordea. Azken urteetako ikerketa gehienak noranzko honetan ari dira zuzentzen hain zuzen ere. Helburua gaixotasun ezberdinetan

sistema honek nola jokatzen duen ulertzea da, hala etorkizuneko botikak diseinatu ahal izateko. Beraz, eman beharreko hurrengo urrats bezala defini genezake gorputzeko jariakin eta organo ezberdinetan endokannabinoideak kuantifikatzeko analisi metodo sentikorren garapena.

Bibliografia

[1] Bakowska AM, Kucharska AZ, Oszmianski J. 2003. The effects of heating, UV irradiation and storage on stability of anthocyanin-polyphenol copigment complex. Food Chem, 81, 349-355.

[2] Munin A, Edwards-Lévy F. 2011. Encapsulation of Natural Polyphenolic Compounds; a Review. Pharmaceutics, 3, 793-829.

[3] Russo EB. 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol, 163, 1344-1364.

[4] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci, 30, 515-527.

[5] Mechoulam R, Hanus L. 2002. Cannabidiol: an overview of some chemical and pharmacological aspects. Part I: chemical aspects. Chem Phys Lipids, 121, 35-43.

[6] Wall ME, Perez-Reyes M. 1981. The metabolism of delta-9-tetrahydrocannabinol and related cannabinoids in man. J Clin Pharmacol, 21, 178-189.

[7] Agurell S, Halldin M, Lindgren J, Ohlsson A, Widman M, Gillespie H, Hollister L. 1986. Pharmacokinetics and metabolism of delta-9-THC and other cannabinoids with emphasis on man. Pharmacol Rev, 38, 21-42.

Material gehigarria/Supplementary material

Ondoren aurkezten den material gehigarria 8. eta 9. atalei dagokie. Bertan, I., II. eta III. kemotipoko *cannabis sativa L.* landare ezberdinen hazkuntza-prozesu osoan zehar 8 kannabinoide nagusien eta 28 terpeno nagusien kontzentrazioek izandako bilakaerak adierazten dira. Konposatu bakoitzarentzat taula bat osatu da horretarako. ("t" hazkuntza-denborari dagokio eta egunetan adierazten da; "1" I kemotipoko landareei dagokie, "2" II kemotipoko landareei eta "3" III kemotipoko landareei; "A", "B" eta "C" kemotipo bakoitzaren barruan aztertu diren landare ezberdinak dira; "R.G" ama-landareen klonetan sustraiak hazteko faseari dagokio, "V.P" landarearen hazkuntza-faseari dagokio eta "F.P" loratze faseari; "<LOQ" kontzentrazioa kuantifikazio mugatik beherakoa den kasuetan adierazten da).

The following supplementary material is from chapters 8 and 9. The development of 8 major cannabinoids and 28 major terpenes during the growth of different *Cannabis sativa L.* plants from chemotype I, II, and III is presented. A table has been created for each compound. ("t" refers to growing time in terms of days; "1" refers to chemotype I plants, "2" to chemotype II plants and "3" to chemotype III plants; while "A" "B" and "C" are the different plants of each chemotype; "R.G" refers to root growing phase, "V.P" to vegetative phase and "F.P" to flowering phase; "<LOQ" is written when the values are below the limit of quantification).

Table 1. taula
CBD

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.52±0.01	0.52±0.09
18	<LOQ	<LOQ	<LOQ	<LOQ	0.52±0.09	0.43±0.02	<LOQ
25	<LOQ	<LOQ	<LOQ	0.43±0.04	1.31±0.07	0.54±0.05	0.7±0.2
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	1.1±0.1	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	2.1±0.3	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	0.5±0.2	0.3±0.1	0.32±0.05
94	<LOQ	<LOQ	<LOQ	0.6±0.1	1.40±0.06	0.81±0.01	0.68±0.07
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	0.58±0.01	<LOQ	<LOQ
143	<LOQ	<LOQ	<LOQ	0.6±0.1	2.5±0.1	1.5±0.2	2.9±0.2
150	<LOQ	<LOQ	<LOQ	0.32±0.05	2.9±0.1	0.78±0.01	0.83±0.07
157	<LOQ	<LOQ	<LOQ	1.4±0.4	7±1	2.5±0.9	2.2±0.8
165	<LOQ	<LOQ	<LOQ	0.9±0.1	6.2±0.1	1.4±0.3	0.85±0.01
172	<LOQ	<LOQ	<LOQ	0.6±0.2	5.4±0.1	1.0±0.3	1.3±0.3
179	<LOQ	<LOQ	<LOQ	1.01±0.03	10.9±1.7	1.5±0.3	1.8±0.1
F.P Loreak/Flowers							
122	<LOQ	<LOQ	<LOQ	<LOQ	0.70±0.03	0.45±0.02	0.61±0.04
129	<LOQ	<LOQ	<LOQ	<LOQ	0.79±0.06	0.54±0.01	0.62±0.05
136	<LOQ	<LOQ	<LOQ	0.48±0.02	1.88±0.09	1.4±0.1	1.67±0.01
143	<LOQ	<LOQ	<LOQ	1.9±0.3	6.3±0.2	5.7±0.1	5.4±0.2
150	<LOQ	<LOQ	<LOQ	1.57±0.03	5.12±0.02	2.8±0.1	3.2±0.2
157	<LOQ	<LOQ	<LOQ	1.6±0.1	10±2	5±1	7±2
165	<LOQ	<LOQ	<LOQ	2.8±0.7	14±2	5±1	6±1
172	<LOQ	<LOQ	1.1±0.1	2.7±0.4	11.2±0.4	5.2±0.7	5±1
179	<LOQ	<LOQ	<LOQ	3.1±0.4	11.7±0.2	4.9±0.1	5.3±0.6

Table 2. taula
CBG

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.44±0.01	0.5±0.1
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	1.0±0.2	0.5±0.1	0.52±0.08
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	2.1±0.3	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	0.4±0.1	0.65±0.09	0.52±0.03
143	<LOQ	0.47±0.04	<LOQ	<LOQ	0.58±0.09	0.8±0.1	0.6±0.2
150	<LOQ	0.6±0.2	0.6±0.2	0.35±0.07	0.6±0.1	0.83±0.05	0.7±0.1
157	<LOQ	0.5±0.1	0.80±0.02	<LOQ	0.6±0.2	0.7±0.1	0.8±0.2
165	<LOQ	0.59±0.01	0.5±0.2	0.5±0.1	1.1±0.1	1.0±0.1	1.0±0.3
172	<LOQ	0.53±0.04	0.54±0.08	0.44±0.02	0.81±0.09	1.0±0.1	0.91±0.01
179	<LOQ	0.57±0.02	0.4±0.1	0.53±0.02	0.9±0.1	1.14±0.06	0.97±0.06

Table 3. taula
CBDA

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.36±0.02	0.51±0.01	0.49±0.01	27±2	50±3	42±2	48.3±0.1
5	0.54±0.03	0.5±0.2	0.37±0.05	34.1±0.3	52±6	42.1±0.6	51.2±0.1
12	0.5±0.2	0.28±0.02	0.39±0.05	38±2	70±2	70±2	77±3
18	0.6±0.1	0.35±0.01	0.38±0.06	36±3	58±5	51±1	53±2
25	0.3±0.1	0.29±0.03	0.26±0.05	47±1	81±5	54±2	68.5±0.2
V.P							
42	0.42±0.06	0.4±0.1	1.0±0.3	14.2±0.7	24±2	19±1	21.1±0.5
47	0.5±0.2	<LOQ	<LOQ	10±0.2	20.8±0.4	15.9±0.4	18.9±0.2
53	<LOQ	<LOQ	<LOQ	7.0±0.3	16.3±0.9	10.5±0.2	19±1
60	<LOQ	<LOQ	<LOQ	6.7±0.5	12±2	12.3±0.6	16.7±0.2
75	<LOQ	<LOQ	<LOQ	9.3±0.8	14.6±0.7	12.4±0.5	19.4±0.2
84	0.39±0.04	<LOQ	<LOQ	15.4±0.8	27.2±1.1	18.9±0.8	29.7±0.8
94	0.6±0.0	0.5±0.0	0.5±0.0	24.5±0.5	28.8±0.1	25±1	38±1
F.P Hostoak/Leaves							
102	0.40±0.03	<LOQ	<LOQ	16.44±0.01	20.6±0.5	23.8±0.6	33.1±0.5
107	<LOQ	<LOQ	<LOQ	12.6±0.6	19.8±0.2	19±1	26±1
122	<LOQ	<LOQ	<LOQ	12.1±0.6	24.5±0.1	17.8±0.8	20.6±0.3
129	<LOQ	<LOQ	<LOQ	11.2±0.9	26±1	15.6±0.2	24±1
136	<LOQ	0.42±0.03	<LOQ	21.6±0.5	56.6±0.7	34±2	42±2
143	0.62±0.01	0.44±0.08	0.59±0.01	27±1	74±2	42.4±0.5	69±3
150	0.51±0.01	0.42±0.02	0.46±0.01	25±1	68±3	38±2	50.4±0.3
157	0.30±0.01	0.6±0.1	0.7±0.2	20±3	73.2±0.6	51.1±0.7	46±8
165	1.16±0.01	1.3±0.1	0.90±0.05	25±0	52±4	40.1±0.4	42±2
172	0.82±0.07	<LOQ	0.87±0.09	22±1	39±1	30±2	47.6±0.4
179	0.34±0.02	0.4±0.1	0.57±0.02	22.9±0.3	53±3	44.9±0.3	51.8±0.1
F.P Loreak/Flowers							
122	<LOQ	<LOQ	0.93±0.05	22.4±0.4	64±3	50.7±0.3	50.8±0.7
129	<LOQ	0.4±0.1	0.7±0.1	31±2	76±4	68±2	74±1
136	0.47±0.01	1.14±0.01	0.77±0.05	58.8±0.9	128±1	122±1	132±3
143	0.52±0.01	0.78±0.03	0.8±0.2	73±3.3	155±1.8	128.9±0.4	166±1
150	1.10±0.01	0.55±0.04	0.93±0.05	84.8±0.7	152.0±0.9	139±2	163±5
157	0.89±0.01	1.30±0.04	1.47±0.04	85.6±0.6	163±7	120±20	172±2
165	0.96±0.01	0.88±0.01	0.67±0.03	90±1	184±5	149±5	166±1
172	0.93±0.08	0.57±0.02	1.3±0.2	87±2	164±7	160±3	162±2
179	1.2±0.2	0.81±0.06	1.71±0.03	98±2	180±10	172±1	191±5

Table 4. taula
CBN

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 5. taula
THC

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	0.77±0.03	0.79±0.01	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	0.81±0.07	0.73±0.01	<LOQ	<LOQ	<LOQ	<LOQ
25	0.6±0.2	2.9±0.3	0.59±0.05	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	0.47±0.06	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.49±0.03	1.27±0.04	0.89±0.08	<LOQ	<LOQ	<LOQ	<LOQ
94	0.83±0.04	2.7±0.1	1.50±0.05	1.30±0.03	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	0.59±0.05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	1.24±0.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	1.41±0.01	2.63±0.08	2.14±0.05	1.58±0.01	0.71±0.05	<LOQ	0.52±0.03
150	0.85±0.01	1.0±0.2	1.05±0.01	0.8±0.2	0.63±0.01	<LOQ	<LOQ
157	1.32±0.01	2.4±0.7	1.9±0.5	1.1±0.3	0.9±0.1	<LOQ	<LOQ
165	1.36±0.01	1.0±0.3	1.9±0.5	1.0±0.2	0.45±0.02	<LOQ	<LOQ
172	0.9±0.1	1.77±0.01	1.8±0.2	0.8±0.2	0.39±0.09	<LOQ	<LOQ
179	2.1±0.3	2.00±0.01	3.3±0.2	1.2±0.2	0.95±0.01	<LOQ	<LOQ
F.P Loreak/Flowers							
122	0.62±0.05	0.78±0.06	1.04±0.02	<LOQ	<LOQ	<LOQ	<LOQ
129	0.66±0.01	0.82±0.02	1.5±0.1	<LOQ	<LOQ	<LOQ	<LOQ
136	1.42±0.01	0.93±0.08	2.1±0.1	1.28±0.04	<LOQ	<LOQ	<LOQ
143	4.15±0.01	3.6±0.4	7.6±0.3	3.2±0.1	1.30±0.01	1.13±0.01	1.09±0.03
150	3.84±0.01	2.57±0.04	10±2	2.78±0.05	0.9±0.3	0.92±0.01	0.87±0.08
157	4.73±0.01	5±2	10.2±0.2	2.81±0.09	1.29±0.03	0.86±0.09	1.17±0.02
165	5.08±0.01	2.59±0.01	7±3	3.4±0.5	1.1±0.8	1.1±0.2	1.2±0.1
172	5.0±0.6	4±1	5.6±0.7	3.3±0.2	1.0±0.3	1.2±0.2	1.05±0.07
179	4.0±0.1	4.0±0.9	6.3±0.7	3.7±0.2	1.55±0.07	1.1±0.2	1.09±0.06

Table 6. taula
CBGA

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	5.9±0.3	6.18±0.01	5.6±0.1	2.5±0.2	2.20±0.04	2.8±0.2	2.30±0.01
5	3.2±0.4	5.2±0.2	4.1±0.2	1.26±0.02	0.63±0.09	1.4±0.4	0.98±0.02
12	3.8±0.3	4.4±0.4	4.05±0.01	0.76±0.08	<LOQ	0.9±0.1	0.67±0.07
18	1.59±0.09	3.6±0.1	2.7±0.4	0.74±0.02	0.16±0.09	0.53±0.01	<LOQ
25	1.3±0.1	2.0±0.2	2.1±0.2	0.55±0.07	0.5±0.3	0.42±0.02	<LOQ
V.P							
42	1.23±0.08	0.7±0.1	1.62±0.09	0.504±0.0	0.25±0.03	0.42±0.07	0.31±0.01
47	3.7±0.2	1.1±0.2	1.23±0.04	1.2±0.1	0.56±0.06	0.60±0.04	1.05±0.04
53	2.09±0.08	1.2±0.3	2.0±0.2	0.46±0.05	<LOQ	<LOQ	0.76±0.04
60	1.77±0.08	1.00±0.04	2.05±0.02	<LOQ	<LOQ	<LOQ	0.42±0.06
75	1.21±0.01	<LOQ	1.60±0.07	<LOQ	<LOQ	<LOQ	<LOQ
84	3.28±0.07	1.76±0.05	3.1±0.1	1.7±0.2	1.10±0.07	1.4±0.2	1.52±0.02
94	2.5±0.1	3.07±0.03	2.41±0.01	2.8±0.1	<LOQ	0.50±0.05	0.84±0.04
F.P Hostoak/Leaves							
102	0.77±0.01	1.53±0.02	0.92±0.05	0.92±0.01	<LOQ	<LOQ	<LOQ
107	<LOQ	0.41±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	0.61±0.01	1.52±0.01	0.95±0.04	0.63±0.01	0.44±0.03	0.33±0.01	0.34±0.01
129	0.98±0.01	2.07±0.04	0.64±0.07	0.49±0.08	0.76±0.01	<LOQ	<LOQ
136	1.26±0.01	1.84±0.03	1.40±0.06	1.08±0.03	1.84±0.06	0.91±0.01	0.88±0.04
143	0.90±0.01	2.1±0.3	2.7±0.2	1.00±0.06	0.87±0.07	0.65±0.09	0.76±0.01
150	1.35±0.01	1.19±0.02	2.48±0.06	0.71±0	0.49±0.01	0.4±0.1	0.44±0.04
157	0.55±0.01	0.9±0.1	2.8±0.3	0.33±0.08	0.44±0.04	<LOQ	<LOQ
165	0.66±0.01	0.5±0.2	3.5±0.6	0.38±0.03	0.38±0.01	0.33±0.08	0.34±0.04
172	0.70±0.06	0.8±0.1	2.7±0.2	<LOQ	<LOQ	<LOQ	<LOQ
179	0.7±0.2	0.5±0.1	3.1±0.3	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	2.69±0.06	3.97±0.04	5.44±0.03	2.38±0.04	3.8±0.2	2.7±0.1	2.69±0.06
129	3.40±0.01	6.12±0.08	4.2±0.2	2.8±0.2	3.6±0.2	2.38±0.06	2.17±0.01
136	6.02±0.01	9.3±0.2	7.8±0.6	4.68±0.09	7.3±0.1	5.4±0.1	4.17±0.09
143	3.95±00.1	9.0±0.5	9.3±0.2	3.8±0.3	3.31±0.01	3.28±0.01	2.86±0.05
150	5.95±0.01	7.6±0.8	13.5±0.6	3.0±0.3	2.0±0.1	1.94±0.04	2.81±0.01
157	5.07±0.01	7.3±0.9	16.7±0.4	3.1±0.3	1.8±0.1	1.3±0.2	1.28±0.08
165	5.42±0.01	6.64±0.02	14.7±0.1	2.6±0.1	2.7±0.2	1.60±0.01	1.9±0.2
172	5.3±0.4	6.6±0.2	14.6±0.8	1.5±0.1	1.8±0.4	1.57±0.04	1.10±0.06
179	3.8±0.4	5.5±0.4	12.4±0.7	1.6±0.1	2.53±0.08	1.8±0.1	1.76±0.07

Table 7. taula
CBC

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	1.49±0.08	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	0.48±0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	0.48±0.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	0.6±0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	0.43±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	0.9±0.1	0.4±0.0	<LOQ	<LOQ	<LOQ	<LOQ
143	0.48±0.01	1.2±0.3	0.8±0.3	<LOQ	0.4±0.2	0.5±0.2	<LOQ
150	0.99±0.01	1.8±0.3	0.7±0.3	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	1.3±0.2	0.54±0.02	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	2.11±0.01	0.63±0.04	<LOQ	0.50±0.06	<LOQ	<LOQ
172	0.6±0.3	1.5±0.4	0.4±0.1	<LOQ	<LOQ	<LOQ	<LOQ
179	0.50±0.01	1.4±0.2	0.4±0.1	<LOQ	0.35±0.09	<LOQ	<LOQ

Table 8. taula
THCA

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	33.4±0.3	84.5±0.3	66.4±0.1	14±1	2.8±0.3	2.5±0.1	2.77±0.02
5	57±7	92±5	77±4	19.6±0.2	2.7±0.4	2.6±0.2	2.6±0.1
12	54.8±0.8	90±1	90.0±0.8	20±1	3.4±0.1	3.8±0.1	3.6±0.1
18	45±3	95±3	86.4±0.2	19±1	2.8±0.3	2.59±0.04	2.46±0.01
25	48±1	90±2	70±2	23.2±0.8	4.3±0.4	2.8±0.2	3.0±0.1
V.P							
42	24.3±0.9	20±4	32±1	8.4±0.4	1.8±0.2	1.21±0.08	1.38±0.05
47	19.4±0.7	10.1±0.8	13.3±0.3	7.12±0.05	1.17±0.06	0.81±0.01	1.03±0.04
53	20±1	13±2	21±2	4.5±0.2	0.79±0.05	0.51±0.08	0.85±0.09
60	20.8±0.1	17.6±0.1	21±1	4.0±0.4	0.56±0.09	0.7±0.1	0.73±0.01
75	15.8±0.3	13.4±0.3	19±1	5.2±0.5	0.77±0.07	0.55±0.04	0.75±0.04
84	30.9±0.6	24.6±0.6	32.5±0.1	8.2±0.4	1.56±0.07	1.00±0.06	1.43±0.04
94	18.9±0.1	35.5±0.7	27.1±0.1	13.3±0.3	1.38±0.03	1.6±0.1	1.84±0.03
F.P Hostoak/Leaves							
102	19.1±0.3	28.4±0.4	25.4±0.5	9.76±0.02	1.09±0.05	1.39±0.01	1.47±0.06
107	16.0±0.4	25.9±0.2	21.8±0.2	7.7±0.4	0.95±0.05	1.03±0.01	1.29±0.05
122	15.9±0.8	22.32±0.03	25.36±0.03	7.9±0.4	1.44±0.01	0.92±0.01	1.01±0.04
129	12.8±0.1	26.5±0.3	18.9±0.4	7.7±0.3	1.4±0.2	1.10±0.04	1.21±0.03
136	22.0±0.1	38.4±0.7	24.0±0.5	14.3±0.4	2.7±0.1	1.9±0.1	1.96±0.08
143	27±1	82±12	45±5	19.1±0.7	3.0±0.3	2.7±0.2	2.8±0.2
150	38.4±0.1	65±3	46±1	21±1	3.0±0.4	3.03±0.08	2.65±0.03
157	21.7±0.1	73±8	47±6	11±2	3.5±0.1	3.33±0.07	2.7±0.5
165	21.6±0.1	45±5	60±3	18.3±0.2	3.1±0.8	3.20±0.01	2.6±0.1
172	29±2	56±8	50±3	15.0±0.1	1.61±0.09	1.75±0.08	2.4±0.2
179	34.9±0.1	60±10	60±7	18.2±0.3	2.8±0.3	2.75±0.08	3.65±0.01
F.P Loreak/Flowers							
122	42.2±0.8	41±2	60.0±0.8	16.9±0.4	3.0±0.1	2.64±0.02	2.26±0.01
129	47±2	60±2	66±1	23±1	3.7±0.3	3.5±0.2	3.25±0.03
136	67.7±0.1	122.4±0.4	97±6	41.1±0.6	6.0±0.1	5.54±0.08	6.0±0.2
143	82.3±0.1	206.8±0.2	132±4	48±1	6.48±0.04	5.85±0.09	7.20±0.09
150	121.3±0.1	220±20	190±10	60.9±0.1	6.7±0.2	7.2±0.1	7.4±0.1
157	141.0±0.1	222±7	190±5	62±1	9.4±0.6	6±1	7.4±0.2
165	150.9±0.1	251.4±0.9	196.0±0.8	61±2	11.5±0.6	7.05±0.05	7.6±0.1
172	147±2	242±3	192±7	58.9±0.5	6.8±0.2	8.5±0.5	6.9±0.1
179	118±4	250±10	180±5	66.0±0.6	7.9±0.3	8.9±0.1	8.4±0.2

Table 9. taula
α-pinene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.222±0.003	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	0.46±0.09	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	0.26±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	0.31±0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	0.30±0.08	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	0.11±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	0.09±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	0.11±0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	0.12±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	0.083±0.008	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.324±0.008	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	0.22±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/ Leaves							
102	0.067±0.005	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	0.15±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	0.168±0.005	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	0.21±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	0.45±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	0.3±0.1	<LOQ	<LOQ	<LOQ	0.045±0.007	<LOQ	<LOQ
150	0.25±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	0.26±0.03	0.088±0.001	0.060±0.001	<LOQ	<LOQ	<LOQ	<LOQ
165	0.28±0.04	0.073±0.004	0.064±0.001	<LOQ	<LOQ	<LOQ	<LOQ
172	0.28±0.04	0.064±0.001	0.062±0.001	<LOQ	<LOQ	<LOQ	<LOQ
179	0.38±0.09	0.078±0.004	0.080±0.004	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/ Flowers							
122	1.22±0.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	1.26±0.06	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	1.50±0.08	<LOQ	0.072±0.002	<LOQ	0.051±0.004	<LOQ	0.05±0.01
143	1.6±0.2	<LOQ	<LOQ	<LOQ	0.060±0.005	<LOQ	<LOQ
150	1.03±0.04	0.104±0.001	0.14±0.03	<LOQ	<LOQ	<LOQ	<LOQ
157	1.2±0.3	0.16±0.03	0.182±0.007	0.068±0.001	0.075±0.004	0.060±0.005	0.09±0.01
165	1.60±0.04	0.21±0.03	0.21±0.01	0.08±0.01	0.103±0.008	0.100±0.004	0.106±0.003
172	1.2±0.3	0.17±0.02	0.17±0.02	0.07±0.01	0.084±0.002	0.095±0.001	0.087±0.006
179	1.9±0.2	0.240±0.002	0.14±0.06	0.101±0.004	0.091±0.001	0.09±0.02	0.110±0.001

Table 10. taula
β-pinene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.110±0.005	0.059±0.006	0.058±0.002	<LOQ	<LOQ	<LOQ	0.059±0.002
5	0.26±0.04	0.063±0.003	<LOQ	<LOQ	<LOQ	<LOQ	0.069±0.009
12	0.15±0.01	<LOQ	<LOQ	<LOQ	<LOQ	0.067±0.006	0.074±0.007
18	0.17±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	0.16±0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	0.065±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	0.051±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	0.07±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	0.07±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	0.069±0.002	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.176±0.003	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	0.13±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/ Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	0.081±0.007	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	0.078±0.003	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	0.099±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	0.273±0.001	0.049±0.001	<LOQ	<LOQ	0.063±0.007	0.057±0.002	0.07±0.02
143	0.24±0.03	0.14±0.01	0.087±0.006	0.058±0.001	0.08±0.01	0.10±0.01	0.109±0.007
150	0.16±0.03	0.103±0.002	0.097±0.006	<LOQ	<LOQ	<LOQ	<LOQ
157	0.12±0.01	0.19±0.04	0.074±0.004	<LOQ	0.061±0.003	0.091±0.002	0.070±0.001
165	0.13±0.01	0.16±0.02	0.10±0.03	<LOQ	0.08±0.02	<LOQ	0.075±0.001
172	0.13±0.01	0.093±0.009	0.07±0.01	<LOQ	0.072±0.001	<LOQ	<LOQ
179	0.24±0.07	0.114±0.002	0.14±0.02	0.085±0.001	0.098±0.003	0.100±0.001	0.08±0.03
F.P Loreak/ Flowers							
122	0.59±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	0.62±0.03	<LOQ	0.068±0.003	<LOQ	0.052±0.002	<LOQ	0.049±0.004
136	0.71±0.03	0.052±0.004	0.14±0.01	0.054±0.007	0.092±0.001	0.075±0.002	0.093±0.004
143	0.77±0.07	0.138±0.009	0.12±0.04	<LOQ	0.11±0.01	<LOQ	<LOQ
150	0.61±0.01	0.22±0.01	0.29±0.05	0.096±0.001	0.105±0.006	<LOQ	0.103±0.005
157	0.6±0.1	0.31±0.04	0.345±0.002	0.122±0.007	0.12±0.01	0.09±0.02	0.160±0.003
165	0.78±0.01	0.42±0.06	0.37±0.02	0.14±0.02	0.183±0.005	0.142±0.009	0.169±0.001
172	0.7±0.1	0.34±0.04	0.32±0.02	0.13±0.02	0.12±0.01	0.157±0.004	0.124±0.003
179	1.0±0.1	0.46±0.02	0.3±0.1	0.17±0.02	0.17±0.01	0.14±0.03	0.192±0.005

Table 11. taula
Myrcene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	0.14±0.02	0.11±0.01	0.4±0.1	0.29±0.04	0.22±0.07
5	0.21±0.05	<LOQ	0.07±0.02	0.108±0.009	0.72±0.02	0.28±0.06	0.24±0.04
12	<LOQ	<LOQ	0.21±0.06	0.08±0.02	0.8±0.1	0.39±0.08	0.39±0.08
18	0.13±0.04	<LOQ	<LOQ	0.061±0.001	0.7±0.1	0.223±0.003	0.08±0.02
25	0.067±0.006	<LOQ	0.063±0.001	0.061±0.005	0.65±0.14	0.17±0.01	0.34±0.04
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	0.074±0.007	0.062±0.007	0.062±0.001
47	<LOQ	0.07±0.02	0.13±0.07	<LOQ	0.14±0.09	0.057±0.002	0.12±0.02
53	<LOQ	0.065±0.004	<LOQ	<LOQ	0.08±0.02	0.07±0.02	0.063±0.006
60	<LOQ	0.044±0.002	<LOQ	<LOQ	0.12±0.03	0.09±0.03	0.089±0.002
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.065±0.003	0.057±0.002	0.110±0.005	0.061±0.003	0.15±0.01	0.058±0.003	0.117±0.003
94	<LOQ	0.082±0.006	0.073±0.005	0.062±0.002	<LOQ	0.179±0.009	0.18±0.01
F.P Hostoak/ Leaves							
102	<LOQ	<LOQ	0.070±0.005	<LOQ	<LOQ	0.116±0.002	0.10±0.01
107	<LOQ	0.068±0.003	<LOQ	0.075±0.002	0.084±0.009	0.087±0.004	0.103±0.002
122	<LOQ	0.052±0.002	0.057±0.001	0.06±0.01	0.11±0.01	0.091±0.002	0.071±0.002
129	0.059±0.002	0.18±0.02	<LOQ	0.117±0.002	0.287±0.006	0.138±0.003	0.168±0.001
136	0.107±0.001	0.64±0.01	0.054±0.002	0.227±0.004	0.72±0.02	0.41±0.03	0.43±0.01
143	0.056±0.001	0.6±0.1	0.052±0.008	0.27±0.03	0.7±0.1	0.33±0.04	0.27±0.03
150	0.141±0.009	0.459±0.002	0.097±0.006	0.4±0.1	0.6±0.1	0.261±0.007	0.25±0.08
157	0.13±0.02	0.8±0.2	0.068±0.001	0.32±0.03	0.42±0.05	0.356±0.002	0.151±0.001
165	0.13±0.02	0.39±0.04	0.12±0.06	0.315±0.005	0.58±0.02	0.358±0.002	0.159±0.001
172	0.13±0.02	0.4±0.1	0.10±0.01	0.32±0.01	0.27±0.02	0.288±0.009	0.22±0.01
179	0.16±0.06	0.65±0.08	0.13±0.01	0.4±0.1	0.43±0.07	0.3±0.1	0.30±0.08
F.P Loreak/ Flowers							
122	0.156±0.004	0.193±0.005	0.19±0.01	0.20±0.01	0.41±0.04	0.45±0.02	0.28±0.03
129	0.53±0.02	0.230±0.001	0.210±0.003	0.399±0.002	0.9±0.1	1.11±0.01	0.65±0.03
136	0.57±0.03	0.722±0.001	0.25±0.01	0.94±0.03	1.52±0.01	2.6±0.3	1.77±0.06
143	0.40±0.04	1.10±0.05	0.12±0.04	0.33±0.02	1.7±0.2	0.76±0.01	1.82±0.04
150	0.93±0.03	2.2±0.2	0.8±0.1	1.5±0.3	1.2±0.3	2.2±0.3	1.7±0.2
157	1.2±0.1	1.9±0.4	0.69±0.01	1.8±0.2	1.3±0.2	1.7±0.3	2.6±0.3
165	0.72±0.02	3.7±0.5	0.65±0.03	2.1±0.2	3.7±0.2	4.2±0.1	2.6±0.3
172	1.2±0.1	1.7±0.2	0.42±0.06	2.0±0.2	1.1±0.2	4.5±0.2	1.4±0.1
179	0.7±0.2	3.10±0.07	0.6±0.2	3.6±0.2	3.1±0.3	5.5±0.7	4.34±0.02

Table 12. taula
Limonene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.086±0.004	0.084±0.009	0.16±0.03	0.19±0.02	0.24±0.03	0.21±0.03	0.28±0.03
5	0.41±0.05	0.089±0.006	0.07±0.01	0.125±0.006	0.32±0.02	0.279±0.005	0.294±0.006
12	0.153±0.003	0.058±0.003	0.11±0.01	0.100±0.009	0.37±0.03	0.388±0.005	0.331±0.006
18	0.27±0.04	0.067±0.005	0.065±0.005	0.10±0.01	0.32±0.03	0.258±0.007	0.21±0.02
25	0.261±0.007	0.056±0.003	0.056±0.002	0.108±0.003	0.52±0.14	0.242±0.006	0.31±0.01
V.P							
42	0.138±0.006	<LOQ	<LOQ	0.056±0.005	0.097±0.004	0.093±0.006	0.087±0.005
47	0.064±0.001	<LOQ	0.05±0.02	<LOQ	0.09±0.01	0.07±0.01	0.063±0.001
53	0.075±0.008	<LOQ	<LOQ	<LOQ	0.068±0.003	0.053±0.003	0.06±0.01
60	0.082±0.008	0.043±0.002	<LOQ	<LOQ	0.07±0.01	0.06±0.01	0.062±0.002
75	0.062±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.198±0.002	<LOQ	<LOQ	<LOQ	0.087±0.004	0.061±0.002	0.095±0.003
94	0.082±0.006	<LOQ	<LOQ	<LOQ	<LOQ	0.096±0.001	0.099±0.006
F.P Hostoak/ Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.111±0.001	0.087±0.005
107	0.083±0.007	0.070±0.004	<LOQ	<LOQ	0.08±0.00	0.083±0.005	0.087±0.009
122	0.059±0.001	0.061±0.004	<LOQ	0.062±0.005	0.106±0.007	0.123±0.005	0.085±0.001
129	0.068±0.003	0.076±0.007	<LOQ	0.072±0.006	0.151±0.003	0.098±0.003	0.119±0.008
136	0.074±0.004	0.199±0.007	<LOQ	0.122±0.002	0.28±0.01	0.17±0.01	0.218±0.009
143	0.056±0.001	0.36±0.05	0.059±0.001	0.135±0.009	0.32±0.08	0.15±0.02	0.18±0.02
150	<LOQ	0.322±0.009	0.102±0.006	0.17±0.04	0.34±0.05	0.120±0.003	0.19±0.04
157	0.088±0.008	0.7±0.1	0.057±0.001	0.20±0.01	0.25±0.02	0.188±0.003	0.151±0.001
165	0.094±0.008	0.35±0.03	0.11±0.04	0.195±0.001	0.28±0.01	0.194±0.001	0.127±0.001
172	0.093±0.008	0.36±0.07	0.100±0.009	0.199±0.003	0.211±0.007	0.19±0.01	0.176±0.001
179	0.066±0.009	0.53±0.08	0.138±0.009	0.16±0.04	0.24±0.07	0.17±0.05	0.21±0.04
F.P Loreak/ Flowers							
122	0.245±0.008	0.073±0.007	0.084±0.005	0.079±0.004	0.22±0.02	0.24±0.01	0.148±0.009
129	0.29±0.01	0.122±0.003	0.143±0.003	0.174±0.006	0.44±0.03	0.389±0.004	0.345±0.009
136	0.249±0.008	0.262±0.002	0.208±0.008	0.41±0.02	0.557±0.005	0.59±0.02	0.65±0.01
143	0.18±0.02	0.47±0.02	0.13±0.04	0.218±0.009	0.58±0.05	0.34±0.02	0.66±0.02
150	0.316±0.007	1.08±0.06	0.51±0.08	0.48±0.05	0.51±0.07	0.53±0.04	0.65±0.06
157	0.34±0.02	1.7±0.3	0.50±0.02	0.74±0.07	0.55±0.08	0.47±0.07	0.94±0.03
165	0.239±0.001	2.6±0.5	0.52±0.02	0.84±0.03	1.17±0.04	1.00±0.03	0.98±0.03
172	0.35±0.02	1.8±0.2	0.48±0.05	0.79±0.02	0.61±0.01	1.21±0.01	0.70±0.02
179	0.33±0.4	3.26±0.08	0.6±0.1	1.14±0.04	1.13±0.09	1.4±0.2	1.44±0.04

Table 13. taula
Eucalyptol

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	0.12±0.02	<LOQ	0.163±0.008	0.113±0.002
5	<LOQ	<LOQ	<LOQ	0.077±0.005	<LOQ	0.138±0.006	0.11±0.01
12	<LOQ	<LOQ	<LOQ	0.056±0.003	<LOQ	0.16±0.02	0.14±0.02
18	<LOQ	<LOQ	<LOQ	0.062±0.002	<LOQ	0.126±0.001	<LOQ
25	<LOQ	<LOQ	<LOQ	0.059±0.003	<LOQ	0.089±0.002	0.11±0.01
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.047±0.004	0.041±0.007
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.063±0.005
94	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.077±0.002	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.079±0.005	0.075±0.001
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.076±0.005	0.072±0.003
122	<LOQ	<LOQ	<LOQ	0.053±0.003	<LOQ	0.068±0.004	0.057±0.001
129	<LOQ	<LOQ	0.002±0.000	0.07±0.01	<LOQ	0.082±0.005	0.072±0.005
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.09±0.01	0.077±0.001
143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.067±0.002	0.053±0.001
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.050±0.001
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.051±0.001	<LOQ
F.P Loreak/Flowers							
122	<LOQ	<LOQ	<LOQ	0.062±0.003	<LOQ	0.135±0.007	0.132±0.008
129	<LOQ	<LOQ	<LOQ	0.07±0.01	<LOQ	0.104±0.001	0.115±0.005
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.059±0.009	0.071±0.006
143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.068±0.002
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.052±0.001
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 14. taula
β-ocimene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	0.160±0.005	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	0.055±0.002	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	0.086±0.002	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	0.10±0.01	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	<LOQ	0.156±0.003	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	0.149±0.004	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	0.155±0.003	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	0.217±0.002	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	0.136±0.006	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	0.147±0.003	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	0.098±0.002	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	0.14±0.03	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	0.111±0.001	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	0.192±0.001	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	0.13±0.02	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	0.17±0.05	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	<LOQ	<LOQ	0.392±0.005	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	0.60±0.02	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	0.65±0.02	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	0.29±0.07	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	1.3±0. 2	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	1.238±0.009	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	1.2±0.01	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	0.81±0.09	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	0.9±0.2	<LOQ	<LOQ	<LOQ	<LOQ

Table 15. taula
Terpinolene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/ Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	0.16±0.01	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	0.060±0.001	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	0.118±0.007	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	0.087±0.001	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	0.171±0.001	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	0.13±0.01	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	0.26±0.08	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/ Flowers							
122	<LOQ	<LOQ	<LOQ	<LOQ	0.064±0.002	<LOQ	<LOQ
129	<LOQ	<LOQ	0.38±0.05	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	0.93±0.04	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	0.29±0.07	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	1.5±0.5	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	0.055±0.001	1.0±0.2	<LOQ	0.054±0.003	0.058±0.004	0.03±0.02
165	<LOQ	<LOQ	2.1±0.1	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	1.3±0.4	<LOQ	<LOQ	<LOQ	<LOQ
179	0.104±0.006	0.062±0.001	1.5±0.4	0.056±0.001	0.059±0.002	0.066±0.001	0.071±0.001

Table 16. taula
Linalool

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/ Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	0.108±0.002	<LOQ	<LOQ	0.09±0.01	<LOQ	<LOQ
143	<LOQ	0.13±0.04	<LOQ	<LOQ	0.12±0.01	<LOQ	<LOQ
150	0.099±0.005	0.115±0.009	<LOQ	<LOQ	0.117±0.006	<LOQ	<LOQ
157	<LOQ	0.116±0.009	<LOQ	<LOQ	0.080±0.004	<LOQ	<LOQ
165	<LOQ	0.074±0.004	<LOQ	<LOQ	0.08±0.01	<LOQ	<LOQ
172	<LOQ	0.073±0.007	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	0.08±0.02	<LOQ	0.060±0.008	0.9±0.2	<LOQ	0.051±0.001
F.P Loreak/ Flowers							
122	<LOQ	<LOQ	<LOQ	<LOQ	0.052±0.005	<LOQ	<LOQ
129	0.058±0.003	0.073±0.002	<LOQ	<LOQ	0.125±0.001	0.089±0.002	0.065±0.004
136	0.054±0.001	0.232±0.005	<LOQ	0.102±0.007	0.229±0.005	0.160±0.008	0.143±0.006
143	0.06±0.01	0.35±0.02	<LOQ	0.063±0.001	0.240±0.005	0.113±0.003	0.140±0.003
150	0.21±0.02	0.44±0.02	0.160±0.002	0.132±0.008	0.208±0.005	0.170±0.009	0.160±0.004
157	0.20±0.01	0.31±0.02	0.113±0.005	0.11±0.01	0.16±0.02	0.13±0.02	0.194±0.008
165	0.074±0.002	0.39±0.02	0.105±0.002	0.121±0.009	0.312±0.001	0.167±0.006	0.15±0.02
172	0.21±0.01	0.37±0.02	0.096±0.001	0.115±0.009	0.26±0.05	0.25±0.05	0.11±0.02
179	0.08±0.03	0.34±0.03	0.08±0.01	0.24±0.01	0.361±0.003	0.30±0.01	0.26±0.01

Table 17. taula
β-Caryophyllene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.90±0.03	4.1±0.5	2.25±0.01	3.2±0.2	3.63±0.04	1.7±0.1	4.45±0.05
5	1.6±0.2	4.3±0.3	2.35±0.01	4.72±0.02	3.8±0.5	1.87±0.06	5.39±0.04
12	1.36±0.01	4.31±0.02	3.5±0.3	4.5±0.1	4.46±0.04	3.08±0.09	7.3±0.2
18	0.95±0.07	3.36±0.08	2.6±0.3	3.7±0.3	3.2±0.1	1.34±0.04	4.4±0.3
25	1.26±0.04	4.16±0.01	2.09±0.05	5.77±0.09	5.8±0.5	2.13±0.04	7.03±0.05
V.P							
42	0.642±0.007	1.1±0.1	0.99±0.05	1.50±0.03	1.5±0.1	0.56±0.04	1.9±0.1
47	0.52±0.02	0.79±0.04	0.70±0.04	0.34±0.02	0.67±0.07	0.26±0.01	1.35±0.01
53	0.51±0.03	0.95±0.08	0.87±0.07	0.36±0.01	0.60±0.01	0.171±0.004	1.31±0.06
60	0.57±0.03	0.86±0.02	0.80±0.02	0.56±0.08	0.48±0.06	0.30±0.02	1.49±0.01
75	0.40±0.01	0.69±0.02	0.57±0.02	1.17±0.18	0.80±0.01	0.40±0.03	1.76±0.04
84	1.20±0.02	1.49±0.02	1.27±0.02	1.80±0.08	1.93±0.08	0.65±0.03	3.16±0.07
94	0.93±0.01	1.92±0.05	1.21±0.06	1.8±0.1	0.84±0.05	0.96±0.07	1.26±0.02
F.P Hostoak/Leaves							
102	0.52±0.02	1.34±0.05	1.15±0.05	1.87±0.04	0.68±0.02	1.03±0.02	3.01±0.04
107	0.71±0.01	1.27±0.01	0.779±0.007	1.48±0.07	0.93±0.02	0.68±0.02	2.43±0.07
122	0.53±0.01	0.82±0.04	0.796±0.006	1.37±0.08	1.11±0.05	0.64±0.02	1.72±0.04
129	0.33±0.01	0.98±0.02	0.55±0.01	1.2±0.1	0.82±0.01	0.437±0.001	1.45±0.06
136	0.43±0.01	1.02±0.04	0.673±0.008	1.29±0.08	0.790±0.008	0.55±0.04	1.02±0.01
143	0.42±0.01	1.4±0.3	0.8±0.2	1.04±0.06	0.52±0.02	0.448±0.001	1.07±0.08
150	0.37±0.04	1.36±0.01	0.71±0.06	0.8±0.1	0.47±0.04	0.37±0.04	0.85±0.07
157	0.38±0.01	1.0±0.1	0.56±0.08	1.0±0.2	0.9±0.1	0.39±0.03	1.105±0.002
165	0.400±0.003	0.86±0.04	1.3±0.4	1.04±0.05	0.71±0.04	0.41±0.02	1.41±0.01
172	0.397±0.002	1.2±0.2	0.78±0.04	0.838±0.008	0.607±0.007	0.39±0.03	0.97±0.03
179	0.51±0.06	1.06±0.09	0.97±0.08	0.8±0.2	0.4±0.1	0.4±0.1	1.0±0.3
F.P Loreak/Flowers							
122	1.44±0.08	1.7±0.2	2.03±0.06	2.89±0.03	2.5±0.2	1.71±0.08	3.17±0.05
129	1.31±0.06	2.34±0.04	2.82±0.06	3.9±0.1	2.24±0.03	1.60±0.08	2.40±0.01
136	1.31±0.03	3.91±0.02	3.3±0.2	3.89±0.09	1.63±0.02	1.76±0.03	1.93±0.02
143	1.3±0.1	4.1±0.1	2.43±0.02	2.10±0.04	1.00±0.05	1.10±0.06	1.95±0.05
150	1.61±0.01	5.5±0.3	3.5±0.5	2.3±0.1	0.8±0.1	1.1±0.2	1.6±0.1
157	1.72±0.03	4.7±0.2	2.39±0.04	2.7±0.6	0.95±0.14	0.80±0.07	2.17±0.01
165	1.9±0.1	6.3±0.5	3.96±0.06	3.69±0.04	1.75±0.01	1.27±0.01	1.95±0.02
172	1.79±0.04	5.82±0.07	3.0±0.2	2.6±0.3	0.92±0.05	1.31±0.07	1.19±0.01
179	2.3±0.2	5.6±0.3	4.0±0.8	2.9±0.5	1.2±0.2	1.8±0.3	2.1±0.3

Table 18. taula
α-bergamotene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.243±0.004	0.9±0.1	1.100±0.006	0.68±0.04	0.880±0.004	0.54±0.03	0.23±0.01
5	0.38±0.05	0.95±0.07	1.34±0.01	1.11±0.01	0.9±0.1	0.60±0.02	0.263±0.003
12	0.260±0.009	0.857±0.003	1.6±0.1	1.06±0.03	1.18±0.01	1.00±0.04	0.364±0.008
18	0.21±0.02	0.65±0.02	1.30±0.04	0.87±0.06	0.81±0.05	0.51±0.01	0.25±0.02
25	0.24±0.01	0.825±0.004	1.14±0.01	1.37±0.02	1.53±0.10	0.82±0.02	0.364±0.004
V.P							
42	0.200±0.006	0.36±0.04	0.61±0.04	0.591±0.009	0.58±0.03	0.32±0.02	0.160±0.003
47	0.197±0.005	0.26±0.05	0.39±0.01	0.35±0.02	0.45±0.03	0.28±0.02	0.161±0.002
53	0.25±0.02	0.43±0.04	0.57±0.06	0.255±0.007	0.37±0.02	0.183±0.004	0.14±0.03
60	0.27±0.01	0.44±0.01	0.59±0.01	0.27±0.04	0.27±0.04	0.23±0.02	0.141±0.003
75	0.15±0.01	0.285±0.009	0.44±0.03	0.37±0.05	0.333±0.005	0.22±0.01	0.124±0.002
84	0.49±0.01	0.80±0.02	0.99±0.01	0.62±0.03	0.68±0.03	0.36±0.03	0.271±0.006
94	0.278±0.009	0.91±0.05	0.80±0.04	0.62±0.04	0.32±0.02	0.48±0.04	0.642±0.004
F.P Hostoak/ Leaves							
102	0.17±0.01	0.61±0.02	0.76±0.03	0.62±0.02	0.29±0.01	0.44±0.01	0.269±0.006
107	0.182±0.005	0.60±0.02	0.60±0.01	0.48±0.01	0.38±0.01	0.334±0.004	0.16±0.01
122	0.129±0.004	0.39±0.01	0.584±0.004	0.39±0.02	0.38±0.02	0.27±0.02	0.109±0.006
129	0.078±0.009	0.32±0.01	0.337±0.001	0.28±0.03	0.281±0.001	0.175±0.003	0.100±0.001
136	0.096±0.006	0.323±0.008	0.289±0.004	0.31±0.03	0.262±0.003	0.19±0.01	0.117±0.002
143	0.051±0.001	0.26±0.08	0.26±0.07	0.25±0.03	0.18±0.02	0.154±0.008	0.063±0.001
150	<LOQ	0.25±0.01	0.30±0.03	0.20±0.03	0.17±0.01	0.12±0.01	<LOQ
157	0.067±0.003	0.18±0.02	0.24±0.04	0.25±0.04	0.30±0.05	0.15±0.02	0.075±0.001
165	0.072±0.004	0.17±0.01	0.2±0.1	0.240±0.009	0.23±0.02	0.154±0.001	0.083±0.001
172	0.071±0.004	0.21±0.05	0.33±0.02	0.222±0.005	0.229±0.004	0.153±0.003	0.095±0.003
179	0.062±0.005	0.20±0.01	0.29±0.01	0.20±0.05	0.15±0.04	0.15±0.03	0.062±0.009
F.P Loreak/ Flowers							
122	0.42±0.02	0.72±0.06	1.18±0.03	0.686±0.006	0.68±0.04	0.59±0.04	0.198±0.005
129	0.35±0.03	0.70±0.02	1.08±0.02	0.81±0.03	0.611±0.007	0.46±0.04	0.128±0.004
136	0.31±0.02	0.89±0.02	0.97±0.05	0.72±0.02	0.442±0.001	0.49±0.02	0.219±0.008
143	0.17±0.04	0.69±0.07	0.695±0.005	0.420±0.006	0.35±0.02	0.323±0.007	0.226±0.005
150	0.31±0.05	1. 0±0.1	1.1±0.1	0.61±0.02	0.28±0.02	0.34±0.04	<LOQ
157	0.20±0.05	0.62±0.08	0.822±0.008	0.45±0.07	0.28±0.05	0.23±0.01	0.080±0.002
165	0.259±0.006	1.05±0.07	0.96±0.02	0.636±0.001	0.481±0.006	0.345±0.001	0.075±0.005
172	0.21±0.09	0.8±0.2	0.66±0.09	0.466±0.005	0.27±0.04	0.33±0.03	0.077±0.002
179	0.19±0.03	0.68±0.05	0.7±0.1	0.58±0.04	0.38±0.03	0.48±0.03	0.073±0.001

Table 19. taula
β-Farnesene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.315±0.005	1.4±0.2	2.13±0.04	1.4±0.1	1.82±0.02	1.07±0.07	0.49±0.03
5	0.45±0.06	1.4±0.1	2.88±0.01	2.25±0.01	1.9±0.3	1.4±0.3	0.554±0.006
12	0.335±0.005	1.33±0.01	3.3±0.3	2.16±0.04	2.44±0.03	2.04±0.09	0.75±0.02
18	0.28±0.03	1.07±0.05	2.6±0.3	1.7±0.1	1.7±0.1	1.05±0.03	0.49±0.02
25	0.35±0.01	1.28±0.02	2.18±0.01	2.94±0.08	3.34±0.22	1.62±0.03	0.72±0.01
V.P							
42	0.268±0.005	0.51±0.07	1.10±0.06	1.15±0.02	1.14±0.06	0.61±0.04	0.32±0.02
47	0.23±0.02	0.37±0.02	0.68±0.04	0.67±0.03	0.91±0.08	0.53±0.05	0.302±0.007
53	0.253±0.002	0.50±0.05	1.02±0.08	0.48±0.02	0.69±0.03	0.334±0.007	0.289±0.009
60	0.28±0.01	0.54±0.02	1.06±0.04	0.50±0.07	0.51±0.08	0.42±0.01	0.252±0.001
75	0.186±0.002	0.40±0.01	0.81±0.04	0.69±0.08	0.63±0.01	0.40±0.02	0.237±0.005
84	0.50±0.01	1.03±0.02	1.82±0.02	1.18±0.06	1.33±0.04	0.67±0.04	0.513±0.007
94	0.281±0.003	1.20±0.03	1.44±0.07	1.18±0.08	0.68±0.03	0.90±0.03	1.245±0.005
F.P Hostoak/ Leaves							
102	0.169±0.006	0.79±0.03	1.37±0.06	1.243±0.005	0.60±0.02	0.858±0.006	0.525±0.005
107	0.21±0.01	0.82±0.01	1.13±0.01	0.96±0.06	0.78±0.04	0.63±0.07	0.34±0.02
122	0.13±0.01	0.48±0.04	1.07±0.01	0.75±0.05	0.74±0.05	0.51±0.02	0.213±0.005
129	0.068±0.006	0.33±0.02	0.578±0.002	0.53±0.03	0.545±0.009	0.30±0.01	0.173±0.008
136	0.061±0.002	0.292±0.003	0.45±0.01	0.56±0.06	0.48±0.01	0.35±0.02	0.221±0.003
143	0.053±0.001	0.31±0.09	0.4±0.1	0.42±0.06	0.30±0.02	0.25±0.03	0.12±0.02
150	<LOQ	0.25±0.01	0.44±0.06	0.32±0.05	0.29±0.03	0.19±0.02	<LOQ
157	0.066±0.006	0.23±0.04	0.34±0.07	0.42±0.09	0.5±0.1	0.27±0.05	0.137±0.001
165	0.070±0.007	0.206±0.001	0.4±0.2	0.41±0.02	0.40±0.04	0.27±0.02	0.161±0.001
172	0.070±0.007	0.24±0.04	0.52±0.03	0.37±0.01	0.39±0.01	0.270±0.003	0.180±0.007
179	0.07±0.01	0.268±0.005	0.41±0.04	0.34±0.08	0.24±0.07	0.26±0.05	0.11±0.03
F.P Loreak/ Flowers							
122	0.30±0.02	0.82±0.07	1.96±0.06	1.32±0.02	1.40±0.08	1.10±0.05	0.378±0.002
129	0.22±0.02	0.734±0.001	1.81±0.05	1.60±0.06	1.20±0.01	0.86±0.05	0.273±0.006
136	0.158±0.003	0.83±0.02	1.57±0.09	1.37±0.05	0.82±0.01	0.91±0.03	0.409±0.002
143	0.13±0.01	0.78±0.02	1.08±0.01	0.73±0.02	0.63±0.02	0.58±0.02	0.41±0.01
150	0.161±0.006	0.91±0.05	1.96±0.08	1.10±0.02	0.48±0.02	0.61±0.06	0.129±0.007
157	0.13±0.02	0.66±0.04	1.21±0.01	0.8±0.1	0.46±0.09	0.355±0.003	0.158±0.009
165	0.11±0.01	0.93±0.09	1.48±0.05	1.12±0.03	0.82±0.04	0.604±0.004	0.156±0.002
172	0.13±0.02	0.76±0.03	1.0±0.1	0.76±0.02	0.42±0.09	0.56±0.05	0.145±0.007
179	0.12±0.04	0.79±0.03	1.2±0.1	1.02±0.06	0.67±0.08	0.86±0.05	0.161±0.001

Table 20. taula
Humulene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.34±0.03	1.3±0.2	1.04±0.01	0.79±0.04	0.92±0.02	0.52±0.03	1.26±0.02
5	0.60±0.07	1.4±0.1	1.09±0.01	1.32±0.01	1.0±0.1	0.56±0.01	1.54±0.01
12	0.54±0.01	1.37±0.01	1.50±0.14	1.26±0.02	1.18±0.01	0.86±0.03	2.10±0.04
18	0.36±0.03	1.06±0.02	1.15±0.06	1.00±0.07	0.81±0.04	0.42±0.02	1.27±0.07
25	0.49±0.02	1.32±0.01	0.96±0.03	1.60±0.03	1.5±0.1	0.64±0.01	2.03±0.01
V.P							
42	0.251±0.002	0.38±0.04	0.46±0.02	0.46±0.01	0.43±0.02	0.19±0.01	0.63±0.03
47	0.198±0.008	0.29±0.02	0.34±0.01	0.13±0.01	0.21±0.02	0.098±0.006	0.423±0.004
53	0.20±0.02	0.34±0.04	0.41±0.03	0.110±0.003	0.18±0.01	0.07±0.01	0.40±0.01
60	0.22±0.01	0.31±0.02	0.380±0.008	0.18±0.03	0.14±0.02	0.12±0.01	0.465±0.005
75	0.153±0.000	0.271±0.007	0.27±0.01	0.35±0.04	0.230±0.001	0.142±0.006	0.537±0.009
84	0.446±0.007	0.54±0.01	0.60±0.01	0.53±0.02	0.55±0.02	0.21±0.01	0.89±0.03
94	0.35±0.01	0.67±0.02	0.57±0.03	0.53±0.02	0.269±0.008	0.31±0.02	0.40±0.01
F.P Hostoak/ Leaves							
102	0.213±0.008	0.47±0.03	0.55±0.03	0.561±0.004	0.22±0.01	0.310±0.004	0.84±0.01
107	0.260±0.009	0.46±0.02	0.363±0.009	0.44±0.01	0.27±0.01	0.216±0.006	0.75±0.02
122	0.192±0.008	0.29±0.02	0.369±0.004	0.40±0.02	0.314±0.006	0.199±0.004	0.537±0.007
129	0.119±0.004	0.328±0.006	0.246±0.003	0.33±0.5	0.24±0.01	0.127±0.002	0.44±0.01
136	0.169±0.003	0.35±0.02	0.307±0.001	0.38±0.03	0.229±0.002	0.16±0.01	0.324±0.004
143	0.164±0.001	0.5±0.1	0.36±0.08	0.30±0.01	0.153±0.008	0.130±0.004	0.34±0.02
150	0.146±0.009	0.459±0.001	0.31±0.02	0.24±0.04	0.139±0.001	0.115±0.006	0.27±0.02
157	0.151±0.007	0.33±0.06	0.24±0.04	0.28±0.06	0.26±0.05	0.12±0.02	0.351±0.001
165	0.160±0.008	0.28±0.01	0.47±0.06	0.31±0.02	0.20±0.01	0.133±0.001	0.439±0.001
172	0.159±0.009	0.40±0.08	0.34±0.03	0.239±0.006	0.165±0.003	0.112±0.003	0.30±0.01
179	0.21±0.03	0.37±0.02	0.43±0.04	0.24±0.06	0.12±0.05	0.12±0.02	0.32±0.09
F.P Loreak/ Flowers							
122	0.53±0.03	0.59±0.05	0.96±0.03	0.821±0.003	0.70±0.03	0.52±0.02	0.933±0.003
129	0.50±0.02	0.824±0.004	1.19±0.04	1.09±0.06	0.628±0.007	0.48±0.04	0.760±0.002
136	0.53±0.01	1.34±0.02	1.47±0.09	1.11±0.05	0.468±0.002	0.55±0.01	0.63±0.01
143	0.55±0.05	1.43±0.04	1.18±0.02	0.64±0.01	0.269±0.006	0.363±0.005	0.64±0.02
150	0.708±0.001	1.9±0.1	1.6±0.2	0.72±0.02	0.26±0.03	0.35±0.05	0.57±0.05
157	0.74±0.04	1.60±0.09	1.108±0.001	0.8±0.1	0.27±0.05	0.25±0.02	0.731±0.004
165	0.83±0.06	2.2±0.2	1.78±0.07	1.07±0.02	0.54±0.01	0.41±0.01	0.677±0.004
172	0.77±0.05	1.97±0.06	1.3±0.1	0.738±0.004	0.26±0.04	0.42±0.04	0.409±0.009
179	1.0±0.1	1.9±0.1	1.8±0.4	0.782±0.005	0.36±0.08	0.57±0.09	0.72±0.09

Table 21. taula
Alloaromandrene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.062±0.001
5	0.067±0.007	<LOQ	0.07±0.01	0.09±0.01	0.076±0.005	<LOQ	0.061±0.003
12	0.057±0.001	<LOQ	<LOQ	0.08±0.01	0.089±0.009	0.073±0.007	0.074±0.004
18	<LOQ	<LOQ	<LOQ	0.076±0.006	0.07±0.01	<LOQ	<LOQ
25	<LOQ	<LOQ	<LOQ	0.069±0.008	0.08±0.01	<LOQ	0.064±0.002
V.P							
42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	0.060±0.005	<LOQ	0.053±0.004	0.099±0.006	0.097±0.005	0.083±0.005	0.066±0.001
129	<LOQ	<LOQ	<LOQ	0.081±0.008	0.065±0.002	0.053±0.004	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	<LOQ	0.054±0.001	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	<LOQ	0.060±0.002	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	<LOQ	0.079±0.001	<LOQ	<LOQ	<LOQ	<LOQ

Table 22. taula
β-selinene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	0.14±0.02	0.08±0.01	<LOQ	<LOQ	<LOQ	<LOQ
5	0.09±0.03	0.15±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	0.060±0.002	0.140±0.006	0.097±0.002	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	0.11±0.01	0.093±0.003	<LOQ	<LOQ	<LOQ	<LOQ
25	0.070±0.003	0.135±0.006	<LOQ	0.08±0.01	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	0.079±0.005	0.071±0.005	0.05±0.01	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	0.081±0.005	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	0.076±0.003	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	0.070±0.005	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	0.12±0.03	0.065±0.002	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	0.102±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	0.089±0.001	0.053±0.001	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	0.071±0.004	0.120±0.001	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	0.10±0.02	0.078±0.003	<LOQ	<LOQ	<LOQ	<LOQ
179	0.047±0.001	0.09±0.02	0.100±0.003	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	0.090±0.006	0.10±0.01	0.16±0.01	<LOQ	<LOQ	<LOQ	0.117±0.006
129	0.093±0.005	0.139±0.009	0.23±0.02	0.09±0.01	<LOQ	<LOQ	0.142±0.002
136	0.112±0.007	0.311±0.001	0.29±0.01	0.139±0.004	<LOQ	<LOQ	0.127±0.006
143	0.161±0.007	0.44±0.02	0.32±0.04	0.096±0.002	<LOQ	<LOQ	0.131±0.003
150	0.231±0.004	0.54±0.02	0.50±0.03	0.12±0.02	<LOQ	<LOQ	0.177±0.002
157	0.26±0.01	0.52±0.02	0.33±0.04	0.108±0.001	<LOQ	<LOQ	0.223±0.005
165	0.204±0.005	0.66±0.06	0.48±0.01	0.12±0.02	<LOQ	<LOQ	0.173±0.006
172	0.27±0.01	0.607±0.007	0.37±0.04	0.10±0.02	<LOQ	<LOQ	0.103±0.003
179	0.30±0.01	0.62±0.02	0.49±0.02	0.15±0.02	<LOQ	<LOQ	0.212±0.003

Table 23. taula
ϒ-selinene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	0.16±0.03	0.060±0.001	<LOQ	<LOQ	<LOQ	<LOQ
5	0.10±0.02	0.17±0.02	0.058±0.001	<LOQ	<LOQ	<LOQ	<LOQ
12	0.079±0.005	0.18±0.01	0.069±0.004	<LOQ	<LOQ	<LOQ	<LOQ
18	0.060±0.002	0.140±0.008	0.067±0.001	<LOQ	<LOQ	<LOQ	<LOQ
25	0.076±0.003	0.17±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	<LOQ	0.065±0.006	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.078±0.002	0.086±0.002	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	0.078±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	0.073±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	0.078±0.008	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	0.053±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	0.061±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	0.11±0.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	0.079±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	<LOQ	0.072±0.001	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	0.082±0.009	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	0.07±0.01	0.065±0.001	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	0.10±0.01	0.102±0.003	0.134±0.007	<LOQ	<LOQ	<LOQ	0.07±0.02
129	0.087±0.004	0.14±0.03	0.17±0.02	0.067±0.002	<LOQ	<LOQ	0.091±0.008
136	0.10±0.01	0.300±0.008	0.23±0.01	0.090±0.002	<LOQ	<LOQ	0.08±0.01
143	0.13±0.02	0.418±0.005	0.242±0.001	0.056±0.001	<LOQ	<LOQ	0.088±0.002
150	0.212±0.003	0.506±0.002	0.36±0.02	<LOQ	<LOQ	<LOQ	0.116±0.007
157	0.224±0.008	0.464±0.001	0.203±0.007	0.057±0.001	<LOQ	<LOQ	0.138±0.002
165	0.19±0.03	0.63±0.05	0.40±0.02	0.074±0.003	<LOQ	<LOQ	0.09±0.02
172	0.233±0.009	0.55±0.02	0.21±0.02	0.062±0.004	<LOQ	<LOQ	<LOQ
179	0.25±0.03	0.51±0.05	0.32±0.06	0.08±0.01	<LOQ	<LOQ	0.139±0.005

Table 24. taula
α-bisabolene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.080±0.002	<LOQ	0.24±0.01	0.21±0.04	0.601±0.004	0.21±0.01	0.74±0.04
5	0.17±0.02	<LOQ	0.097±0.007	0.245±0.004	0.53±0.05	0.216±0.009	0.817±0.007
12	0.09±0.02	<LOQ	0.14±0.01	0.200±0.006	0.69±0.01	0.313±0.008	1.00±0.01
18	0.092±0.008	<LOQ	0.083±0.002	0.15±0.01	0.48±0.03	0.103±0.003	0.63±0.05
25	0.07±0.06	<LOQ	0.058±0.001	0.23±0.01	0.78±0.04	0.21±0.01	0.87±0.01
V.P							
42	0.064±0.007	<LOQ	<LOQ	0.095±0.001	0.25±0.02	0.063±0.006	0.238±0.007
47	0.058±0.002	<LOQ	<LOQ	<LOQ	0.133±0.007	<LOQ	0.142±0.004
53	0.064±0.005	<LOQ	<LOQ	<LOQ	0.14±0.01	<LOQ	0.15±0.01
60	0.062±0.001	<LOQ	0.06±0.01	0.06±0.01	0.11±0.02	0.046±0.003	0.224±0.003
75	<LOQ	<LOQ	<LOQ	0.08±0.02	0.160±0.003	0.063±0.006	0.284±0.006
84	0.125±0.001	<LOQ	0.100±0.004	0.13±0.01	0.32±0.02	0.081±0.006	0.355±0.002
94	<LOQ	<LOQ	0.113±0.001	0.13±0.01	0.181±0.003	0.105±0.005	0.101±0.002
F.P Hostoak/Leaves							
102	<LOQ	<LOQ	0.108±0.001	0.124±0.004	0.21±0.01	0.12±0.01	0.39±0.01
107	0.088±0.004	<LOQ	<LOQ	0.10±0.01	0.26±0.01	0.086±0.007	0.40±0.02
122	<LOQ	<LOQ	0.07±0.01	0.079±0.003	0.23±0.02	0.078±0.004	0.27±0.01
129	<LOQ	<LOQ	<LOQ	0.082±0.003	0.23±0.01	0.055±0.006	0.27±0.01
136	<LOQ	<LOQ	0.053±0.002	0.078±0.001	0.216±0.001	0.079±0.007	0.222±0.005
143	<LOQ	<LOQ	0.048±0.001	0.071±0.001	0.12±0.02	0.049±0.002	0.19±0.04
150	<LOQ	<LOQ	<LOQ	<LOQ	0.114±0.008	<LOQ	0.14±0.03
157	<LOQ	<LOQ	0.052±0.001	<LOQ	0.13±0.03	0.068±0.002	0.214±0.001
165	<LOQ	<LOQ	0.17±0.07	<LOQ	0.15±0.01	<LOQ	0.209±0.001
172	<LOQ	<LOQ	0.11±0.01	<LOQ	0.11±0.01	<LOQ	0.141±0.003
179	<LOQ	<LOQ	0.08±0.02	<LOQ	0.09±0.04	<LOQ	0.17±0.08
F.P Loreak/Flowers							
122	0.211±0.007	<LOQ	0.20±0.01	0.19±0.01	0.51±0.02	0.208±0.009	0.56±0.01
129	0.232±0.008	<LOQ	0.256±0.005	0.28±0.02	0.54±0.01	0.21±0.01	0.63±0.02
136	0.19±0.03	<LOQ	0.28±0.01	0.289±0.004	0.398±0.008	0.245±0.005	0.502±0.002
143	0.13±0.03	<LOQ	0.186±0.001	0.14±0.01	0.22±0.02	0.109±0.001	0.50±0.01
150	0.202±0.001	<LOQ	0.4±0.2	0.165±0.001	0.16±0.02	0.114±0.004	0.43±0.05
157	0.23±0.03	<LOQ	0.24±0.01	0.11±0.01	0.15±0.03	0.066±0.005	0.46±0.01
165	0.16±0.02	<LOQ	0.396±0.006	0.165±0.007	0.292±0.001	0.124±0.004	0.48±0.03
172	0.24±0.04	<LOQ	0.36±0.05	0.138±0.006	0.17±0.04	0.11±0.02	0.28±0.02
179	0.18±0.07	<LOQ	0.33±0.05	0.16±0.02	0.24±0.05	0.15±0.03	0.59±0.06

Table 25. taula
β-bisabolene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	0.17±0.02	0.181±0.003	0.37±0.04	0.563±0.009	0.37±0.05	0.88±0.02
5	<LOQ	0.18±0.01	0.209±0.006	0.679±0.004	0.63±0.08	0.38±0.02	1.04±0.01
12	<LOQ	0.163±0.003	0.24±0.02	0.63±0.02	0.772±0.008	0.63±0.02	1.38±0.04
18	<LOQ	0.130±0.005	0.211±0.001	0.51±0.04	0.53±0.03	0.31±0.02	0.92±0.07
25	<LOQ	0.157±0.004	0.176±0.001	0.81±0.03	0.94±0.07	0.517±0.009	1.39±0.03
V.P							
42	<LOQ	0.08±0.01	0.11±0.01	0.37±0.01	0.38±0.02	0.20±0.02	0.59±0.03
47	<LOQ	<LOQ	0.066±0.008	0.20±0.01	0.29±0.03	0.17±0.01	0.56±0.02
53	<LOQ	0.069±0.009	0.093±0.005	0.162±0.004	0.19±0.03	0.118±0.009	0.58±0.03
60	<LOQ	0.072±0.004	0.089±0.006	0.17±0.03	0.17±0.03	0.15±0.01	0.526±0.001
75	<LOQ	0.058±0.005	0.067±0.004	0.24±0.03	0.212±0.009	0.140±0.002	0.49±0.02
84	<LOQ	0.138±0.006	0.149±0.004	0.39±0.03	0.45±0.02	0.22±0.02	0.76±0.02
94	<LOQ	0.159±0.003	0.108±0.003	0.39±0.04	0.262±0.006	0.28±0.01	0.248±0.001
F.P Hostoak/ Leaves							
102	<LOQ	0.10±0.01	0.103±0.003	0.382±0.005	0.221±0.007	0.26±0.02	0.71±0.01
107	<LOQ	0.099±0.004	0.092±0.005	0.29±0.01	0.237±0.008	0.19±0.02	0.60±0.04
122	<LOQ	0.061±0.007	0.080±0.006	0.229±0.009	0.24±0.02	0.152±0.003	0.37±0.02
129	<LOQ	<LOQ	<LOQ	0.176±0.005	0.187±0.008	0.093±0.009	0.320±0.007
136	<LOQ	<LOQ	<LOQ	0.17±0.01	0.160±0.008	0.10±0.01	0.203±0.008
143	<LOQ	<LOQ	0.150±0.004	0.14±0.02	0.11±0.02	0.107±0.004	0.21±0.04
150	<LOQ	<LOQ	<LOQ	0.116±0.019	0.106±0.011	<LOQ	0.142±0.035
157	<LOQ	<LOQ	<LOQ	0.14±0.02	0.17±0.03	0.083±0.003	0.243±0.001
165	<LOQ	<LOQ	0.2±0.1	0.131±0.005	0.141±0.001	0.094±0.006	0.299±0.001
172	<LOQ	<LOQ	<LOQ	0.116±0.002	0.140±0.005	0.090±0.002	0.217±0.009
179	<LOQ	<LOQ	<LOQ	0.12±0.02	0.08±0.02	0.07±0.03	0.19±0.04
F.P Loreak/ Flowers							
122	0.050±0.005	0.11±0.01	0.18±0.01	0.43±0.01	0.46±0.02	0.36±0.02	0.708±0.007
129	<LOQ	0.101±0.001	0.164±0.007	0.51±0.03	0.406±0.007	0.28±0.01	0.50±0.03
136	<LOQ	0.106±0.003	0.153±0.005	0.43±0.01	0.30±0.01	0.31±0.01	0.389±0.007
143	<LOQ	0.106±0.001	0.120±0.009	0.27±0.01	0.223±0.001	0.211±0.002	0.386±0.009
150	<LOQ	0.11±0.01	0.1±0.1	0.365±0.004	0.170±0.005	0.21±0.02	0.25±0.01
157	<LOQ	0.096±0.008	0.14±0.01	0.18±0.08	0.15±0.03	0.12±0.01	0.27±0.01
165	<LOQ	<LOQ	0.165±0.003	0.40±0.09	0.276±0.001	0.198±0.007	0.25±0.01
172	<LOQ	0.093±0.006	0.12±0.02	0.238±0.002	0.16±0.01	0.18±0.01	0.15±0.03
179	<LOQ	0.11±0.02	0.137±0.007	0.31±0.03	0.23±0.01	0.255±0.002	0.26±0.01

Table 26. taula
β-curcumene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	0.067±0.009	<LOQ	0.082±0.005	0.071±0.003	<LOQ	<LOQ
5	<LOQ	0.070±0.006	0.106±0.002	0.097±0.005	0.08±0.01	<LOQ	<LOQ
12	<LOQ	0.066±0.002	0.12±0.01	0.101±0.008	0.100±0.007	0.091±0.007	<LOQ
18	<LOQ	0.065±0.008	0.118±0.008	0.084±0.007	0.073±0.004	<LOQ	<LOQ
25	<LOQ	0.063±0.002	0.111±0.006	0.171±0.005	0.14±0.01	0.098±0.008	<LOQ
V.P							
42	<LOQ	<LOQ	0.09±0.02	0.09±0.03	0.09±0.01	<LOQ	<LOQ
47	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	<LOQ	<LOQ	0.056±0.002	<LOQ	<LOQ	<LOQ	<LOQ
60	<LOQ	<LOQ	0.053±0.003	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	<LOQ	0.063±0.002	0.090±0.005	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	0.114±0.002	0.119±0.006	<LOQ	0.089±0.003	0.099±0.009	0.10±0.01
F.P Hostoak/ Leaves							
102	0.068±0.005	0.09±0.01	0.114±0.007	0.119±0.001	0.08±0.01	0.102±0.003	0.089±0.001
107	<LOQ	0.074±0.003	0.087±0.004	0.09±0.01	0.072±0.006	0.081±0.008	<LOQ
122	<LOQ	<LOQ	0.057±0.009	0.055±0.003	0.058±0.004	<LOQ	<LOQ
129	<LOQ	0.07±0.01	0.080±0.006	0.07±0.01	0.076±0.001	0.063±0.001	<LOQ
136	<LOQ	0.13±0.02	0.068±0.009	0.080±0.007	0.069±0.003	0.061±0.001	<LOQ
143	<LOQ	0.11±0.01	0.056±0.001	0.065±0.001	0.055±0.009	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	0.062±0.001	0.051±0.000	<LOQ	0.059±0.003	<LOQ	0.060±0.001
165	<LOQ	0.065±0.004	0.080±0.001	0.071±0.001	<LOQ	<LOQ	<LOQ
172	<LOQ	0.079±0.001	0.076±0.002	0.062±0.001	0.070±0.001	<LOQ	<LOQ
179	<LOQ	0.063±0.001	0.067±0.002	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/ Flowers							
122	0.053±0.005	0.07±0.02	0.137±0.006	0.098±0.003	0.079±0.001	0.074±0.007	<LOQ
129	0.10±0.04	0.15±0.01	0.146±0.001	0.14±0.01	0.098±0.001	0.091±0.003	0.08±0.02
136	0.072±0.009	0.30±0.05	0.167±0.006	0.160±0.008	0.089±0.003	0.094±0.003	0.088±0.003
143	0.063±0.002	0.3±0.1	0.15±0.01	0.081±0.002	0.067±0.005	<LOQ	0.086±0.002
150	<LOQ	0.438±0.001	0.21±0.02	0.123±0.006	<LOQ	<LOQ	<LOQ
157	0.091±0.001	0.2±0.1	0.164±0.001	0.061±0.001	0.051±0.003	<LOQ	0.054±0.001
165	0.078±0.003	0.61±0.05	0.22±0.02	0.134±0.006	0.086±0.004	0.068±0.004	<LOQ
172	0.094±0.001	0.3±0.1	0.14±0.04	0.10±0.02	<LOQ	0.076±0.001	0.066±0.001
179	0.079±0.001	0.28±0.04	0.18±0.03	0.10±0.02	0.06±0.01	<LOQ	0.078±0.002

Table 27. taula
β-sesquiphellanderene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.084±0.001	0.31±0.04	0.353±0.004	0.182±0.009	0.290±0.008	0.180±0.009	0.131±0.001
5	0.13±0.03	0.33±0.03	0.437±0.001	0.356±0.003	0.31±0.04	0.197±0.005	0.16±0.01
12	0.10±0.01	0.304±0.005	0.51±0.04	0.334±0.006	0.380±0.005	0.32±0.02	0.21±0.01
18	0.091±0.007	0.27±0.01	0.43±0.02	0.27±0.02	0.26±0.01	0.161±0.003	0.141±0.007
25	0.116±0.004	0.293±0.007	0.338±0.003	0.40±0.02	0.48±0.03	0.230±0.004	0.19±0.01
V.P							
42	<LOQ	0.09±0.02	0.14±0.02	0.116±0.007	0.124±0.003	0.07±0.01	0.063±0.001
47	0.08±0.02	0.080±0.008	0.120±0.003	0.102±0.001	0.14±0.01	0.08±0.01	0.085±0.006
53	0.071±0.004	0.10±0.01	0.17±0.01	0.076±0.005	0.115±0.006	0.053±0.004	0.078±0.005
60	0.076±0.006	0.111±0.006	0.17±0.01	0.084±0.008	0.087±0.008	0.071±0.006	0.068±0.005
75	<LOQ	0.084±0.007	0.13±0.01	0.12±0.02	0.108±0.003	0.071±0.009	0.073±0.003
84	0.12±0.01	0.19±0.01	0.292±0.002	0.195±0.006	0.23±0.01	0.114±0.004	0.132±0.005
94	<LOQ	0.238±0.002	0.249±0.005	0.196±0.007	0.130±0.005	0.15±0.01	0.206±0.003
F.P Hostoak/ Leaves							
102	<LOQ	0.162±0.009	0.237±0.006	0.204±0.003	0.112±0.001	0.146±0.008	0.130±0.009
107	<LOQ	0.15±0.01	0.184±0.006	0.15±0.02	0.126±0.007	0.096±0.005	0.097±0.002
122	<LOQ	0.103±0.005	0.18±0.01	0.123±0.009	0.120±0.009	0.089±0.001	0.064±0.004
129	<LOQ	0.061±0.003	0.107±0.003	0.087±0.006	0.101±0.005	0.054±0.002	<LOQ
136	<LOQ	0.093±0.004	0.093±0.004	0.120±0.009	0.090±0.006	0.066±0.008	0.062±0.001
143	<LOQ	0.11±0.01	0.091±0.002	0.090±0.001	0.047±0.007	0.048±0.002	<LOQ
150	<LOQ	<LOQ	0.099±0.006	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	0.058±0.001	0.074±0.001	<LOQ	0.094±0.005	<LOQ	0.060±0.001
165	<LOQ	<LOQ	0.118±0.001	0.064±0.001	0.066±0.003	<LOQ	<LOQ
172	<LOQ	0.069±0.001	0.099±0.009	<LOQ	0.069±0.005	<LOQ	<LOQ
179	<LOQ	0.07±0.02	0.09±0.02	0.070±0.001	<LOQ	<LOQ	<LOQ
F.P Loreak/ Flowers							
122	0.094±0.009	0.17±0.01	0.39±0.01	0.242±0.005	0.23±0.01	0.194±0.006	0.17±0.02
129	<LOQ	0.198±0.006	0.37±0.02	0.29±0.03	0.20±0.01	0.14±0.01	0.13±0.03
136	<LOQ	0.25±0.02	0.39±0.01	0.28±0.01	0.150±0.008	0.164±0.003	0.138±0.002
143	<LOQ	0.26±0.02	0.30±0.04	0.144±0.003	0.120±0.004	0.12±0.01	0.140±0.003
150	<LOQ	0.276±0.009	0.47±0.05	0.224±0.008	0.10±0.01	0.11±0.02	<LOQ
157	<LOQ	0.19±0.02	0.31±0.03	0.150±0.004	0.094±0.003	0.074±0.006	0.078±0.002
165	<LOQ	0.314±0.007	0.48±0.03	0.225±0.003	0.147±0.002	0.106±0.004	0.102±0.004
172	<LOQ	0.23±0.04	0.28±0.05	0.157±0.009	0.08±0.01	0.093±0.004	<LOQ
179	<LOQ	0.21±0.04	0.33±0.04	0.19±0.03	0.12±0.02	0.13±0.02	0.084±0.002

Table 28. taula
α-Gurjunene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	<LOQ	0.28±0.05	0.099±0.001	<LOQ	<LOQ	<LOQ	<LOQ
5	0.17±0.02	0.29±0.04	0.103±0.004	<LOQ	<LOQ	<LOQ	<LOQ
12	0.168±0.007	0.317±0.004	0.13±0.01	<LOQ	<LOQ	<LOQ	<LOQ
18	0.14±0.01	0.317±0.003	0.111±0.007	<LOQ	<LOQ	<LOQ	<LOQ
25	0.21±0.01	0.305±0.002	0.105±0.002	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	0.105±0.009	0.11±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	0.071±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
53	0.068±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
60	0.077±0.005	0.055±0.002	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.11±0.01	0.10±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
94	<LOQ	0.121±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	<LOQ	0.098±0.003	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
107	<LOQ	0.092±0.007	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	<LOQ	0.068±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
143	<LOQ	0.13±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	0.096±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	0.083±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	0.076±0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	0.094±0.029	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	0.053±0.001	0.097±0.007	0.054±0.001	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	0.12±0.01	0.12±0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
129	0.10±0.01	0.148±0.005	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	0.10±0.02	0.219±0.001	0.13±0.01	<LOQ	<LOQ	<LOQ	<LOQ
143	0.13±0.01	0.32±0.02	0.17±0.03	<LOQ	<LOQ	<LOQ	<LOQ
150	0.16±0.02	0.38±0.02	0.119±0.004	<LOQ	<LOQ	<LOQ	<LOQ
157	0.16±0.04	0.35±0.04	0.14±0.03	<LOQ	<LOQ	<LOQ	<LOQ
165	0.17±0.03	0.46±0.03	0.211±0.006	<LOQ	<LOQ	<LOQ	<LOQ
172	0.17±0.04	0.37±0.06	0.14±0.03	<LOQ	<LOQ	<LOQ	<LOQ
179	0.16±0.02	0.4±0.1	0.20±0.02	<LOQ	<LOQ	<LOQ	<LOQ

Table 29. taula
Selina-3,7(11)diene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.607±0.009	1.9±0.3	0.67±0.02	<LOQ	<LOQ	<LOQ	<LOQ
5	1.1±0.2	2.0±0.3	0.751±0.006	<LOQ	<LOQ	<LOQ	<LOQ
12	1.02±0.01	2.04±0.01	0.90±0.08	<LOQ	<LOQ	<LOQ	<LOQ
18	0.87±0.03	1.935±0.006	0.71±0.03	<LOQ	<LOQ	<LOQ	0.261±0.008
25	1.24±0.06	1.96±0.01	0.53±0.05	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	0.49±0.02	0.53±0.08	0.24±0.02	<LOQ	<LOQ	<LOQ	<LOQ
47	0.41±0.02	0.21±0.01	0.12±0.02	<LOQ	<LOQ	<LOQ	<LOQ
53	0.40±0.02	0.27±0.03	0.19±0.02	<LOQ	<LOQ	<LOQ	<LOQ
60	0.48±0.02	0.34±0.02	0.273±0.006	<LOQ	<LOQ	<LOQ	<LOQ
75	0.32±0.01	0.34±0.02	0.145±0.005	<LOQ	<LOQ	<LOQ	<LOQ
84	0.77±0.01	0.57±0.02	0.32±0.02	<LOQ	<LOQ	<LOQ	<LOQ
94	0.605±0.006	0.71±0.05	0.31±0.02	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	0.37±0.02	0.629±0.007	0.29±0.02	<LOQ	<LOQ	<LOQ	<LOQ
107	0.415±0.001	0.64±0.04	0.246±0.004	<LOQ	<LOQ	<LOQ	<LOQ
122	0.29±0.02	0.41±0.03	0.202±0.008	<LOQ	<LOQ	<LOQ	<LOQ
129	0.122±0.006	0.23±0.04	0.084±0.009	<LOQ	<LOQ	<LOQ	<LOQ
136	0.24±0.02	0.385±0.006	0.148±0.009	<LOQ	<LOQ	<LOQ	<LOQ
143	0.096±0.008	0.23±0.02	0.072±0.002	<LOQ	<LOQ	<LOQ	<LOQ
150	0.2±0.1	0.29±0.05	0.122±0.007	<LOQ	<LOQ	<LOQ	<LOQ
157	0.107±0.001	0.27±0.06	0.102±0.001	<LOQ	<LOQ	<LOQ	<LOQ
165	0.114±0.002	0.2±0.1	0.235±0.001	<LOQ	<LOQ	<LOQ	<LOQ
172	0.113±0.003	0.305±0.003	0.167±0.004	<LOQ	<LOQ	<LOQ	<LOQ
179	0.18±0.05	0.32±0.03	0.14±0.03	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	0.70±0.04	0.66±0.03	0.47±0.03	<LOQ	<LOQ	<LOQ	0.073±0.008
129	0.58±0.03	0.75±0.02	0.46±0.07	0.052±0.006	<LOQ	<LOQ	0.085±0.002
136	0.7±0.1	0.9±0.1	0.56±0.03	0.066±0.003	<LOQ	<LOQ	0.06±0.02
143	0.45±0.02	1.3±0.3	0.53±0.07	<LOQ	<LOQ	<LOQ	0.078±0.002
150	0.676±0.001	1.52±0.09	0.5±0.1	<LOQ	<LOQ	<LOQ	<LOQ
157	0.79±0.03	1.0±0.2	0.3±0.1	<LOQ	<LOQ	<LOQ	<LOQ
165	1.0±0.2	1.89±0.03	0.88±0.02	0.065±0.003	<LOQ	<LOQ	0.092±0.005
172	0.82±0.03	1.1±0.2	0.4±0.1	<LOQ	<LOQ	<LOQ	<LOQ
179	0.55±0.07	1.2±0.2	0.51±0.05	<LOQ	<LOQ	<LOQ	<LOQ

Table 30. taula
ϒ-Elemene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.323±0.007	1.0±0.1	0.273±0.005	<LOQ	<LOQ	<LOQ	<LOQ
5	0.6±0.1	1.09±0.09	0.202±0.008	<LOQ	<LOQ	<LOQ	<LOQ
12	0.25±0.01	0.71±0.04	0.331±0.001	<LOQ	<LOQ	<LOQ	<LOQ
18	0.105±0.004	0.16±0.01	0.082±0.007	<LOQ	<LOQ	<LOQ	<LOQ
25	<LOQ	0.68±0.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
V.P							
42	0.11±0.01	0.16±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	0.15±0.01	0.22±0.03	0.066±0.008	<LOQ	<LOQ	<LOQ	<LOQ
53	0.18±0.02	0.32±0.04	0.08±0.01	<LOQ	<LOQ	<LOQ	<LOQ
60	0.14±0.01	0.31±0.02	0.063±0.006	<LOQ	<LOQ	<LOQ	<LOQ
75	<LOQ	0.090±0.001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
84	0.37±0.01	0.61±0.02	0.14±0.01	<LOQ	<LOQ	<LOQ	<LOQ
94	0.22±0.02	0.58±0.04	0.124±0.003	<LOQ	<LOQ	<LOQ	<LOQ
F.P Hostoak/Leaves							
102	0.139±0.003	0.45±0.02	0.118±0.002	<LOQ	<LOQ	<LOQ	<LOQ
107	0.074±0.001	0.37±0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
122	0.073±0.004	0.27±0.02	0.077±0.007	<LOQ	<LOQ	<LOQ	<LOQ
129	0.074±0.002	0.25±0.03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
136	0.174±0.002	0.362±0.005	0.08±0.02	<LOQ	<LOQ	<LOQ	<LOQ
143	0.084±0.001	0.22±0.06	0.049±0.001	<LOQ	<LOQ	<LOQ	<LOQ
150	0.111±0.006	0.184±0.007	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
157	<LOQ	0.128±0.002	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
165	<LOQ	0.10±0.02	0.085±0.001	<LOQ	<LOQ	<LOQ	<LOQ
172	<LOQ	0.11±0.04	0.066±0.001	<LOQ	<LOQ	<LOQ	<LOQ
179	0.104±0.001	0.071±0.001	0.080±0.001	<LOQ	<LOQ	<LOQ	<LOQ
F.P Loreak/Flowers							
122	0.51±0.03	0.55±0.02	0.335±0.004	<LOQ	<LOQ	<LOQ	<LOQ
129	0.57±0.03	0.77±0.02	0.41±0.05	<LOQ	<LOQ	<LOQ	<LOQ
136	0.64±0.06	1.41±0.07	0.58±0.07	<LOQ	<LOQ	<LOQ	<LOQ
143	0.36±0.06	0.96±0.05	0.189±0.001	<LOQ	<LOQ	<LOQ	<LOQ
150	0.7±0.1	1.8±0.4	0.4±0. 2	<LOQ	<LOQ	<LOQ	<LOQ
157	0.782±0.005	0.9±0.3	0.21±0.04	<LOQ	<LOQ	<LOQ	<LOQ
165	0. 9±0.1	2.46±0.03	0.61±0.07	<LOQ	<LOQ	<LOQ	<LOQ
172	0.814±0.009	1.89±0.07	0.3±0.1	<LOQ	<LOQ	<LOQ	<LOQ
179	0.6±0.2	1.24±0.02	0.33±0.08	<LOQ	<LOQ	<LOQ	<LOQ

Table 31. taula
Guaiol

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.25±0.01	<LOQ	<LOQ	0.37±0.04	0.76±0.01	0.78±0.01	<LOQ
5	0.44±0.07	<LOQ	<LOQ	0.785±0.001	1.10±0.04	0.76±0.07	<LOQ
12	0.9±0.1	<LOQ	<LOQ	0.8±0.1	1.19±0.02	1.16±0.06	<LOQ
18	0.46±0.06	<LOQ	<LOQ	0.41±0.02	0.82±0.04	0.72±0.05	<LOQ
25	0.295±0.009	<LOQ	<LOQ	0.6±0.1	1.4±0.1	0.73±0.01	<LOQ
V.P							
42	0.45±0.02	<LOQ	<LOQ	0.41±0.08	0.44±0.01	0.40±0.09	<LOQ
47	0.222±0.005	<LOQ	<LOQ	0.08±0.02	0.35±0.01	0.32±0.08	<LOQ
53	0.15±0.01	<LOQ	<LOQ	0.098±0.003	0.26±0.03	0.06±0.01	<LOQ
60	0.16±0.03	<LOQ	<LOQ	0.10±0.02	0.22±0.02	0.183±0.004	<LOQ
75	0.111±0.004	<LOQ	<LOQ	0.138±0.007	0.302±0.007	0.146±0.008	<LOQ
84	0.264±0.002	<LOQ	<LOQ	0.216±0.009	0.52±0.02	0.28±0.01	<LOQ
94	0.17±0.01	<LOQ	<LOQ	0.21±0.01	0.411±0.004	0.374±0.004	0.278±0.005
F.P Hostoak/ Leaves							
102	0.08±0.01	<LOQ	<LOQ	0.086±0.008	0.351±0.007	0.40±0.05	<LOQ
107	0.106±0.007	<LOQ	<LOQ	0.25±0.01	0.33±0.04	0.37±0.03	<LOQ
122	0.113±0.009	<LOQ	<LOQ	0.134±0.005	0.30±0.02	0.19±0.02	<LOQ
129	0.09±0.02	<LOQ	<LOQ	0.107±0.008	0.268±0.003	0.152±0.002	<LOQ
136	0.18±0.02	<LOQ	<LOQ	0.211±0.009	0.33±0.03	0.287±0.002	0.138±0.008
143	0.185±0.004	<LOQ	<LOQ	0.198±0.001	0.24±0.02	0.26±0.08	<LOQ
150	0.22±0.02	<LOQ	<LOQ	0.18±0.02	0.29±0.04	0.24±0.03	<LOQ
157	0.155±0.002	<LOQ	<LOQ	0.16±0.06	0.3±0.1	0.27±0.08	<LOQ
165	0.164±0.003	<LOQ	<LOQ	0.23±0.03	0.32±0.05	0.31±0.02	<LOQ
172	0.163±0.004	<LOQ	<LOQ	0.17±0.03	0.25±0.05	0.21±0.04	0.10±0.02
179	0.27±0.04	<LOQ	<LOQ	0.18±0.03	0.25±0.08	0.30±0.04	<LOQ
F.P Loreak/ Flowers							
122	0.31±0.01	<LOQ	<LOQ	0.323±0.002	0.70±0.05	0.69±0.02	<LOQ
129	0.329±0.006	<LOQ	<LOQ	0.42±0.06	0.69±0.03	0.71±0.05	<LOQ
136	0.469±0.008	<LOQ	<LOQ	0.583±0.008	0.72±0.02	1.04±0.03	0.35±0.01
143	0.59±0.04	<LOQ	<LOQ	0.57±0.05	0.58±0.02	0.94±0.07	0.356±0.009
150	0.75±0.03	<LOQ	0.128±0.007	0.60±0.01	0.55±0.01	0.91±0.07	<LOQ
157	0.8±0.2	0.11±0.01	0.073±0.001	0.51±0.08	0.6±0.2	0.63±0.03	<LOQ
165	0.98±0.07	0.11±0.01	0.095±0.005	0.87±0.03	0.97±0.03	1.08±0.04	<LOQ
172	0.8±0.2	0.126±0.001	0.08±0.03	0.60±0.07	0.72±0.07	0.9±0.3	0.24±0.05
179	1.09±0.06	0.14±0.05	0.12±0.05	0.81±0.03	0.749±0.006	1.21±0.03	<LOQ

Table 32. taula
ϒ-eudesmol

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.170±0.006	<LOQ	0.08±0.02	0.28±0.05	0.67±0.03	0.47±0.02	<LOQ
5	0.29±0.05	<LOQ	<LOQ	0.388±0.008	0.67±0.08	0.51±0.01	<LOQ
12	0.28±0.01	<LOQ	<LOQ	0.393±0.006	0.85±0.02	0.80±0.04	<LOQ
18	0.20±0.02	<LOQ	<LOQ	0.36±0.01	0.60±0.05	0.45±0.03	<LOQ
25	0.263±0.003	<LOQ	<LOQ	0.516±0.007	1.08±0.08	0.66±0.02	<LOQ
V.P							
42	0.134±0.006	0.09±0.01	<LOQ	0.17±0.06	0.41±0.02	0.27±0.03	<LOQ
47	0.124±0.005	<LOQ	<LOQ	0.147±0.006	0.33±0.02	0.21±0.05	<LOQ
53	0.14±0.02	<LOQ	<LOQ	0.11±0.01	0.28±0.05	0.144±0.003	<LOQ
60	0.147±0.003	<LOQ	<LOQ	0.096±0.009	0.22±0.04	0.19±0.02	<LOQ
75	0.108±0.008	<LOQ	<LOQ	0.16±0.01	0.29±0.01	0.21±0.01	<LOQ
84	0.20±0.02	<LOQ	<LOQ	0.188±0.001	0.453±0.009	0.239±0.008	<LOQ
94	0.137±0.003	<LOQ	<LOQ	0.187±0.001	0.390±0.004	0.346±0.008	0.257±0.008
F.P Hostoak/ Leaves							
102	0.099±0.004	<LOQ	<LOQ	0.17±0.01	0.28±0.01	0.30±0.02	0.08±0.01
107	0.103±0.003	<LOQ	<LOQ	0.160±0.002	0.260±0.002	0.23±0.02	<LOQ
122	0.09±0.01	<LOQ	<LOQ	0.133±0.008	0.27±0.02	0.198±0.003	<LOQ
129	0.065±0.002	<LOQ	<LOQ	0.099±0.014	0.218±0.007	0.119±0.000	<LOQ
136	0.138±0.009	<LOQ	<LOQ	0.171±0.004	0.301±0.002	0.256±0.007	0.106±0.006
143	0.12±0.02	0.247±0.001	0.100±0.002	0.140±0.005	0.19±0.04	0.18±0.03	<LOQ
150	0.17±0.03	0.14±0.02	<LOQ	0.16±0.03	0.25±0.05	0.191±0.006	<LOQ
157	0.08±0.02	0.12±0.07	0.057±0.001	0.179±0.008	0.3±0.1	0.18±0.09	0.065±0.001
165	0.09±0.03	0.119±0.007	0.101±0.004	0.19±0.06	0.26±0.08	0.25±0.01	0.092±0.001
172	0.09±0.03	0.17±0.02	0.10±0.03	0.14±0.05	0.2±0.1	0.15±0.06	0.083±0.004
179	0.17±0.01	0.09±0.01	0.13±0.02	0.15±0.03	0.21±0.05	0.25±0.03	0.08±0.02
F.P Loreak/ Flowers							
122	0.27±0.03	<LOQ	<LOQ	0.30±0.03	0.66±0.05	0.60±0.01	<LOQ
129	0.273±0.002	<LOQ	<LOQ	0.37±0.01	0.64±0.02	0.60±0.04	<LOQ
136	0.43±0.04	<LOQ	<LOQ	0.52±0.01	0.65±0.01	0.93±0.03	0.30±0.02
143	0.5±0.1	0.26±0.06	0.4±0.1	0.48±0.05	0.46±0.03	0.80±0.09	0.312±0.007
150	0.54±0.05	0.30±0.04	0.5±0.2	0.509±0.007	0.48±0.02	0.82±0.08	0.187±0.007
157	0.62±0.02	0.8±0.4	0.34±0.02	0.4±0.1	0.5±0.2	0.50±0.08	0.19±0.04
165	0.75±0.04	0.32±0.06	0.51±0.03	0.781±0.002	0.84±0.03	0.97±0.04	0.17±0.03
172	0.64±0.01	0.39±0.02	0.40±0.03	0.5±0.1	0.59±0.05	0. 7±0. 2	0.17±0.07
179	0.6±0.1	0.33±0.02	0.53±0.07	0.666±0.006	0.70±0.03	1.03±0.03	0.192±0.009

Table 33. taula
β-eudesmol

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.137±0.008	<LOQ	<LOQ	0.18±0.02	0.393±0.005	0.33±0.02	<LOQ
5	0.23±0.03	<LOQ	<LOQ	0.28±0.01	0.44±0.05	0.37±0.01	0.059±0.002
12	0.246±0.006	<LOQ	<LOQ	0.32±0.02	0.57±0.01	0.59±0.02	0.09±0.01
18	0.17±0.02	<LOQ	<LOQ	0.255±0.009	0.41±0.02	0.36±0.02	0.073±0.003
25	0.25±0.02	<LOQ	<LOQ	0.423±0.007	0.72±0.04	0.502±0.007	0.079±0.007
V.P							
42	0.20±0.02	<LOQ	<LOQ	0.17±0.02	0.32±0.01	0.23±0.03	0.070±0.003
47	0.105±0.002	<LOQ	0.07±0.01	0.133±0.008	0.26±0.01	0.22±0.01	<LOQ
53	0.13±0.01	0.077±0.004	0.073±0.007	0.098±0.005	0.20±0.03	0.123±0.009	<LOQ
60	0.131±0.003	0.071±0.004	0.059±0.003	0.086±0.005	0.17±0.03	0.17±0.01	0.048±0.004
75	0.139±0.002	0.167±0.008	0.072±0.008	0.130±0.001	0.228±0.003	0.171±0.003	0.074±0.007
84	0.20±0.01	<LOQ	<LOQ	0.15±0.01	0.32±0.02	0.19±0.02	0.071±0.007
94	0.134±0.001	<LOQ	<LOQ	0.14±0.01	0.310±0.003	0.26±0.01	0.200±0.001
F.P Hostoak/ Leaves							
102	0.139±0.006	<LOQ	<LOQ	0.177±0.003	0.257±0.004	0.29±0.02	0.11±0.01
107	0.109±0.007	<LOQ	<LOQ	0.144±0.002	0.215±0.006	0.21±0.01	<LOQ
122	0.083±0.009	<LOQ	<LOQ	0.131±0.002	0.211±0.008	0.173±0.001	<LOQ
129	0.085±0.003	<LOQ	<LOQ	0.100±0.005	0.19±0.02	0.141±0.006	<LOQ
136	0.12±0.01	<LOQ	<LOQ	0.169±0.008	0.26±0.01	0.22±0.01	0.103±0.005
143	0.18±0.01	<LOQ	<LOQ	0.196±0.001	0.23±0.03	0.277±0.007	0.079±0.001
150	0.207±0.003	<LOQ	0.108±0.009	0.19±0.01	0.25±0.01	0.247±0.001	<LOQ
157	0.19±0.03	0.272±0.004	0.107±0.001	0.22±0.05	0.41±0.04	0.31±0.01	0.119±0.001
165	0.20±0.03	0.21±0.05	0.12±0.01	0.23±0.02	0.28±0.04	0.29±0.01	0.084±0.001
172	0.20±0.03	0.23±0.07	0.151±0.003	0.21±0.02	0.32±0.03	0.28±0.03	0.12±0.02
179	0.33±0.04	0.24±0.04	0.19±0.04	0.22±0.05	0.3±0.1	0.37±0.08	0.12±0.03
F.P Loreak/ Flowers							
122	0.21±0.02	<LOQ	<LOQ	0.23±0.01	0.48±0.03	0.461±0.008	<LOQ
129	0.21±0.01	<LOQ	<LOQ	0.297±0.006	0.45±0.01	0.45±0.02	<LOQ
136	0.29±0.01	<LOQ	<LOQ	0.380±0.007	0.502±0.006	0.70±0.01	0.234±0.008
143	0.428±0.006	<LOQ	<LOQ	0.480±0.007	0.524±0.002	0.81±0.06	0.229±0.005
150	0.6±0.1	<LOQ	<LOQ	0.47±0.02	0.47±0.04	0.73±0.04	<LOQ
157	0. 7±0.1	<LOQ	<LOQ	0.46±0.02	0.53±0.05	0.63±0.08	<LOQ
165	0.72±0.04	<LOQ	<LOQ	0.65±0.02	0.72±0.02	0.79±0.02	0.235±0.009
172	0. 7±0.1	<LOQ	<LOQ	0.52±0.04	0.644±0.009	0.85±0.02	0.220±0.002
179	0.92±0.05	<LOQ	<LOQ	0.65±0.05	0.69±0.04	1.01±0.05	<LOQ

Table 34. taula
α-selinene

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.157±0.003	0.107±0.003	0.065±0.005	0.163±0.009	0.452±0.007	0.35±0.04	<LOQ
5	0.25±0.04	0.113±0.008	<LOQ	0.28±0.01	0.50±0.07	0.35±0.02	<LOQ
12	0.27±0.01	<LOQ	<LOQ	0.29±0.03	0.58±0.04	0.60±0.02	<LOQ
18	0.16±0.01	<LOQ	<LOQ	0.25±0.01	0.41±0.03	0.344±0.004	<LOQ
25	0.25±0.02	<LOQ	<LOQ	0.36±0.02	0.73±0.05	0.505±0.009	<LOQ
V.P							
42	0.12±0.02	<LOQ	<LOQ	0.14±0.01	0.292±0.009	0.19±0.01	<LOQ
47	0.091±0.006	<LOQ	<LOQ	0.110±0.008	0.25±0.03	0.24±0.08	<LOQ
53	0.10±0.02	<LOQ	<LOQ	0.073±0.009	0.20±0.04	0.10±0.01	<LOQ
60	0.121±0.003	<LOQ	<LOQ	0.067±0.009	0.15±0.03	0.14±0.02	<LOQ
75	0.09±0.01	<LOQ	<LOQ	0.077±0.006	0.172±0.003	0.114±0.007	<LOQ
84	0.24±0.02	<LOQ	<LOQ	0.16±0.01	0.38±0.02	0.20±0.03	0.060±0.001
94	0.146±0.007	<LOQ	<LOQ	0.16±0.01	0.33±0.01	0.292±0.009	0.22±0.01
F.P Hostoak/ Leaves							
102	0.140±0.002	<LOQ	<LOQ	0.161±0.003	0.26±0.01	0.289±0.006	<LOQ
107	0.097±0.006	<LOQ	<LOQ	0.135±0.004	0.22±0.01	0.220±0.002	<LOQ
122	0.091±0.009	<LOQ	<LOQ	0.129±0.008	0.232±0.008	0.18±0.02	<LOQ
129	0.079±0.004	<LOQ	<LOQ	0.09±0.01	0.187±0.003	0.117±0.008	<LOQ
136	0.10±0.01	<LOQ	<LOQ	0.15±0.02	0.24±0.02	0.20±0.01	0.08±0.01
143	0.14±0.02	0.277±0.006	0.11±0.02	0.17±0.01	0.21±0.04	0.22±0.02	<LOQ
150	0.17±0.03	0.20±0.04	<LOQ	0.159±0.008	0.24±0.01	0.21±0.01	<LOQ
157	0.23±0.03	0.203±0.003	0.132±0.001	0.14±0.03	0.33±0.01	0.235±0.006	<LOQ
165	0.24±0.03	<LOQ	<LOQ	0.190±0.005	0.25±0.02	0.233±0.004	<LOQ
172	0.24±0.03	0.21±0.07	<LOQ	0.153±0.007	0.238±0.006	0.19±0.01	0.075±0.003
179	0.265±0.007	0.22±0.02	<LOQ	0.153±0.002	0.21±0.04	0.25±0.02	<LOQ
F.P Loreak/ Flowers							
122	0.29±0.03	<LOQ	<LOQ	0.271±0.006	0.57±0.04	0.568±0.007	<LOQ
129	0.26±0.03	<LOQ	<LOQ	0.33±0.01	0.50±0.04	0.49±0.03	<LOQ
136	0.40±0.05	<LOQ	<LOQ	0.46±0.05	0.538±0.008	0.75±0.01	0.230±0.006
143	0.82±0.03	0.5±0.2	0.3±0.1	0.498±0.003	0.51±0.03	0.79±0.09	0.277±0.005
150	0.8±0.1	0.55±0.02	0.43±0.06	0.52±0.01	0.48±0.06	0.68±0.02	<LOQ
157	1.1±0.1	0.8±0.1	0.36±0.04	0.46±0.02	0.49±0.02	0.6±0.1	<LOQ
165	0.92±0.01	0.57±0.09	0.280±0.008	0.66±0.02	0.699±0.006	0.82±0.01	<LOQ
172	1.2±0.1	0.8±0.2	0.38±0.05	0.507±0.005	0.60±0.08	0.753±0.005	0.22±0.01
179	1.36±0.05	0.8±0.1	0.46±0.01	0.65±0.02	0.62±0.01	1.0±0.1	<LOQ

Table 35. taula
Bulnesol

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.159±0.006	<LOQ	0.073±0.001	0.19±0.02	0.54±0.06	0.28±0.01	<LOQ
5	0.24±0.05	<LOQ	<LOQ	0.246±0.005	0.43±0.05	0.381±0.009	<LOQ
12	0.20±0.01	<LOQ	<LOQ	0.21±0.02	0.480±0.005	0.57±0.09	<LOQ
18	0.172±0.009	<LOQ	<LOQ	0.30±0.03	0.41±0.02	0.31±0.03	<LOQ
25	0.22±0.02	<LOQ	<LOQ	0.36±0.01	0.81±0.06	0.37±0.02	<LOQ
V.P							
42	0.108±0.008	<LOQ	<LOQ	0.10±0.01	0.313±0.006	0.21±0.03	<LOQ
47	0.104±0.009	<LOQ	<LOQ	0.090±0.003	0.252±0.009	<LOQ	<LOQ
53	0.11±0.02	<LOQ	<LOQ	<LOQ	0.25±0.03	0.09±0.01	<LOQ
60	0.139±0.006	<LOQ	<LOQ	0.071±0.002	0.18±0.02	0.13±0.02	<LOQ
75	0.083±0.003	<LOQ	<LOQ	0.091±0.001	0.17±0.05	0.12±0.01	<LOQ
84	0.19±0.01	<LOQ	<LOQ	0.12±0.02	0.364±0.002	0.10±0.01	<LOQ
94	0.12±0.02	<LOQ	<LOQ	0.12±0.02	0.31±0.03	0.19±0.02	0.13±0.01
F.P Hostoak/ Leaves							
102	0.076±0.001	<LOQ	<LOQ	0.11±0.03	0.131±0.007	0.16±0.01	<LOQ
107	0.096±0.009	<LOQ	<LOQ	0.12±0.02	0.207±0.005	0.165±0.008	<LOQ
122	0.08±0.02	<LOQ	<LOQ	0.09±0.02	0.22±0.05	0.139±0.007	<LOQ
129	<LOQ	<LOQ	<LOQ	<LOQ	0.086±0.007	<LOQ	<LOQ
136	0.126±0.002	<LOQ	<LOQ	0.141±0.007	0.27±0.02	0.178±0.005	0.092±0.004
143	<LOQ	<LOQ	0.058±0.001	<LOQ	<LOQ	<LOQ	<LOQ
150	<LOQ	<LOQ	<LOQ	<LOQ	0.114±0.008	<LOQ	<LOQ
157	<LOQ	<LOQ	<LOQ	<LOQ	0.066±0.003	<LOQ	<LOQ
165	<LOQ	<LOQ	<LOQ	0.079±0.001	0.068±0.004	<LOQ	<LOQ
172	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
179	<LOQ	0.056±0.001	0.061±0.002	<LOQ	<LOQ	<LOQ	0.066±0.001
F.P Loreak/ Flowers							
122	0.18±0.03	<LOQ	<LOQ	0.188±0.003	0.49±0.03	0.27±0.05	<LOQ
129	0.128±0.001	<LOQ	<LOQ	0.145±0.005	0.25±0.08	0.2±0.1	<LOQ
136	0.19±0.05	<LOQ	<LOQ	0.21±0.03	0.16±0.01	0.32±0.06	0.10±0.03
143	0.078±0.002	0.108±0.001	0.13±0.02	0.106±0.004	<LOQ	0.135±0.005	0.120±0.003
150	0.123±0.004	0.12±0.02	0.17±0.03	0.091±0.000	<LOQ	0.11±0.02	0.092±0.003
157	0.10±0.03	0.14±0.02	0.129±0.001	0.099±0.001	0.096±0.005	<LOQ	0.088±0.008
165	0.23±0.07	0.10±0.01	0.119±0.003	0.13±0.08	0.14±0.03	0.20±0.06	0.070±0.004
172	0.11±0.04	0.13±0.03	0.114±0.006	0.09±0.01	<LOQ	0.103±0.001	0.070±0.005
179	0.105±0.007	0.13±0.02	0.16±0.03	0.11±0.03	0.119±0.004	0.12±0.02	0.11±0.01

Table 36. taula
α-Bisabolol

t	1A	1B	1C	2A	3A	3B	3C
R.G							
0	0.069±0.003	0.63±0.09	0.057±0.002	0.76±0.02	1.27±0.01	0.73±0.04	2.41±0.05
5	0.11±0.03	0.67±0.07	<LOQ	1.34±0.06	1.3±0.2	0.790±0.007	2.98±0.03
12	0.08±0.02	0.59±0.01	<LOQ	1.28±0.08	1.63±0.03	1.32±0.07	4.1±0.2
18	0.07±0.01	0.465±0.005	<LOQ	1.04±0.06	1.12±0.07	0.58±0.03	2.6±0.3
25	0.086±0.009	0.57±0.01	<LOQ	1.60±0.07	2.1±0.1	1.07±0.03	4.0±0.1
V.P							
42	0.057±0.004	0.30±0.05	<LOQ	0.74±0.07	0.77±0.04	0.40±0.05	1.6±0.1
47	0.058±0.003	0.21±0.02	<LOQ	0.44±0.04	0.61±0.04	0.35±0.02	1.56±0.07
53	0.062±0.005	0.29±0.03	<LOQ	0.322±0.008	0.52±0.08	0.21±0.02	1.46±0.08
60	0.063±0.005	0.31±0.02	<LOQ	0.37±0.04	0.39±0.05	0.32±0.02	1.392±0.006
75	<LOQ	0.229±0.004	<LOQ	0.40±0.04	0.47±0.02	0.31±0.02	1.49±0.04
84	0.13±0.01	0.673±0.008	<LOQ	0.84±0.05	1.04±0.04	0.49±0.05	2.17±0.07
94	0.08±0.01	0.704±0.004	<LOQ	0.82±0.05	0.86±0.03	0.65±0.04	0.496±0.008
F.P Hostoak Leaves							
102	0.075±0.004	0.463±0.005	<LOQ	0.862±0.004	0.66±0.01	0.58±0.03	2.02±0.01
107	<LOQ	0.442±0.002	<LOQ	0.687±0.005	0.562±0.008	0.42±0.03	1.85±0.05
122	<LOQ	0.27±0.02	<LOQ	0.55±0.04	0.55±0.04	0.33±0.01	1.17±0.01
129	<LOQ	0.21±0.02	<LOQ	0.40±0.04	0.44±0.01	0.206±0.001	0.97±0.07
136	<LOQ	0.20±0.01	<LOQ	0.45±0.02	0.43±0.02	0.301±0.006	0.67±0.01
143	<LOQ	0.23±0.05	<LOQ	0.39±0.01	0.28±0.05	0.261±0.003	0.81±0.03
150	<LOQ	0.19±0.01	<LOQ	0.37±0.03	0.28±0.02	0.203±0.001	0.59±0.02
157	<LOQ	0.14±0.01	<LOQ	0.35±0.08	0.48±0.07	0.22±0.02	0.868±0.001
165	<LOQ	0.122±0.002	<LOQ	0.34±0.02	0.33±0.04	0.22±0.02	0.940±0.003
172	<LOQ	0.16±0.04	<LOQ	0.30±0.01	0.324±0.009	0.209±0.001	0.70±0.01
179	<LOQ	0.155±0.003	<LOQ	0.29±0.05	0.23±0.04	0.21±0.04	0.68±0.08
F.P Loreak/ Flowers							
122	0.10±0.01	0.48±0.04	<LOQ	1.09±0.02	1.16±0.06	0.82±0.02	2.31±0.02
129	0.084±0.008	0.46±0.01	<LOQ	1.22±0.03	0.94±0.02	0.64±0.04	1.60±0.01
136	0.080±0.005	0.58±0.01	<LOQ	1.02±0.01	0.817±0.008	0.793±0.003	1.19±0.01
143	0.084±0.002	0.52±0.07	<LOQ	0.87±0.01	0.665±0.003	0.67±0.05	1.19±0.03
150	0.113±0.004	0.56±0.05	<LOQ	0.92±0.02	0.50±0.02	0.56±0.04	0.87±0.01
157	0.09±0.01	0.35±0.01	<LOQ	0.67±0.05	0.52±0.06	0.38±0.04	1.00±0.03
165	<LOQ	0.41±0.03	<LOQ	0.826±0.002	0.773±0.003	0.493±0.006	0.94±0.01
172	0.09±0.01	0.41±0.02	<LOQ	0.567±0.005	0.45±0.06	0.46±0.01	0.60±0.03
179	0.068±0.003	0.34±0.02	<LOQ	0.759±0.004	0.65±0.03	0.587±0.009	0.87±0.03