Utjecaj tretmana visokonaponskim električnim pražnjenjem na svojstva kakaove ljuske

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SVEUČILIŠTE JOSIPA JURJA STROSSMAYERA U OSIJEKU
PREHRAMBENO-TEHNOLOŠKI FAKULTET OSIJEK

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UTJECAJ TRETMANA VISOKONAPONSKIM ELEKTRIČNIM PRAŽNJENJEM NA SVOJSTVA KAKAOVE LJUSKE

DOKTORSKA DISERTACIJA

Osijek, travanj, 2022.

TEMELJNA DOKUMENTACIJSKA KARTICA

DOKTORSKA DISERTACIJA

Sveučilište Josipa Jurja Strossmayera u Osijeku
Prehrambeno-tehnološki fakultet Osijek
Poslijediplomski sveučilišni studij Prehrambena tehnologija i nutricionizam
Smjer: Prehrambena tehnologija

Zavod za prehrambene tehnologije
Katedra za tehnologiju ugljikohidrata
Franje Kuhača 18, 31000 Osijek, Hrvatska
UDK: 663.918.3(043.3)
613.2+641.1(043.3)
Znanstveno područje: Biotehničke znanosti
Znanstveno polje: Prehrambena tehnologija
Znanstvena grana Inženjerstvo
Tema rada je prihvaćena na XI. sjednici Fakultetskog vijeća Prehrambeno-tehnološkog fakulteta Osijek održanoj 24. 6. 2021.
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UTJECAJ TRETMANA VISOKONAPONSKIM ELEKTRIČNIM PRAŽNJENJEM NA SVOJSTVA KAKAOVE LJUSKE

Veronika Barišić, 01131359088

Sažetak: Kakaova ljuska je nusproizvod prehrambene industrije koji je još uvijek nedovoljno iskorišten. Zbog svog zanimljivog sastava (vlakna i polifenoli) sve više se istražuje za primjenu u proizvodnji funkcionalne hrane. Osim bioaktivnih spojeva i komponenti koje mogu imati pozitivan utjecaj na ljudsko zdravlje, kakaova ljuska može sadržavati i različite kontaminante, neželjene organske spojeve koji se moraju ukloniti prije korištenja ljuske u proizvodnji hrane. Osim toga, kakaova se ljuska, zbog visokog udjela netopivih vlakana, teško usitnjava. Cilj ove disertacije bio je utvrditi utjecaj visokonaponskog električnog pražnjenja (HVED) na svojstva kakaove ljuske i mogućnost rješavanja navedenih problema. HVED je netermička metoda koja se bazira na stvaranju električnih izboja direktno u otopini između elektrode izboja i elektrode uzemljenja. Provedeno je ispitivanje različitih frekvencija, vremena i koncentracija kakaove ljuske u vodenoj suspenziji prilikom tretmana. Nakon toga su provedena dva različita postupka sušenja (u sušioniku pri 60 °C i liofilizacija) na HVED tretiranoj ljusci (pri odabranim uvjetima). Dobiveni rezultati pokazuju kako je HVED utjecao na bolje zadržavanje metilksantina i određenih polifenola u kakaovoj ljusci u usporedbi s kontrolnim uzorcima. Također, došlo je do značajnih promjena na vlaknima i kemijskom sastavu kakaove ljuske, što je dokazano FTIR-ATR analizom i diferencijalnom motridbenom kalorimetrijom. Nadalje, došlo je i do smanjenja udjela nepoželjnih komponenti i enterobakterija. Ispitivanjem različitih postupaka sušenja ljuske (u sušioniku pri 60 °C i liofilizacija) utvrđeno je da je liofilizacija imala povoljniji utjecaj na svojstva kakaove ljuske.

Ključne riječi: kakaova ljuska, HVED, dekontaminacija, polifenoli, vlakna

Rad sadrži: 103 stranice
27 slika
18 tablica
6 priloga
207 literaturnih referenci

Jezik izvornika: hrvatski

Sastav Povjerenstva za obranu:

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Datum obrane: 8. travnja 2022.

BASIC DOCUMENTATION CARD

DOCTORAL THESIS

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Scientific area: Biotechnical sciences
Scientific field: Food technology
Thesis subject was approved by the Faculty Council of the Faculty of Food Technology at its session no. XI. held on June 24, 2021.
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EFFECT OF HIGH VOLTAGE ELECTRICAL DISCHARGE TREATMENT ON PROPERTIES OF COCOA BEAN SHELL

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Summary: Cocoa bean shell is a by-product of the food industry that is still underutilized. Due to its characteristic composition (fibers and polyphenols), it is increasingly being researched for use in the production of functional foods. In addition to bioactive compounds and components that can have a positive impact on human health, cocoa shells can also contain various contaminants, unwanted organic compounds that must be removed before using the shell in food production. In addition, cocoa shells, due to the high content of insoluble fiber, are difficult to mill. The aim of this dissertation was to determine the influence of high voltage electrical discharge (HVED) on the properties of cocoa shell and the possibility of solving these problems. HVED is a non-thermal technology based on the generation of electrical discharges directly in the solution between the discharge and ground electrode. Different frequencies, times and concetrations of cocoa shell in water suspension during treatment were studied. Subsequently, two different drying procedures were performed (in an oven at 60 °C and lyophilization) on HVED-treated shell (under selected conditions). The obtained results show that HVED had an improved retention of methylxanthines and certain polyphenols in cocoa shell compared to control samples. Also, there were significant changes of the fibers and chemical composition of the cocoa shell, as evidenced by FTIR-ATR analysis and differential scanning calorimetry. Furthermore, there was a decrease in the content of undesirable components and enterobacteria. Examination of various shell drying procedures (in an oven at 60 °C and lyophilization) showed that lyophilization had a more favorable effect on the properties of cocoa shell.

Key words: cocoa bean shell, HVED, decontamination, polyphenols, fibers

Thesis contains: 103 pages
27 figures
18 tables
6 supplements
207 references
Original in: Croatian

Defense committee:

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Defense date: April 8th 2022

Zahvaljujem mentorici prof. dr. sc. Đurđici Ačkar i komentorici izv. prof. dr. sc. Ivani Flanjak na savjetima, strpljenju, vođenju, pomoći i prenesenom znanju tijekom izrade ove doktorske disertacije. Hvala Danieli, Antunu, Anti i svim ostalim profesorima, asistentima i zaposlenicima Prehrambeno-tehnološkog fakulteta Osijek na pomoći i savjetima.

Hvala kolegicama i prijateljicama s PTF-a na uspomenama, druženjima i pomoći. Veliko hvala obitelji na bezuvjetnoj pomoći i strpljenju tijekom mog školovanja.

Ovu doktorsku disertaciju je sufinancirala Hrvatska zaklada za znanost projektom UIP-2017-05-8709.

Sadržaj

Popis oznaka, kratica i simbola

5-HMF	5-hidroksimetilfurfural
ANOVA	analiza varijance (engl. *analysis of variance*)
a_w	aktivitet vode
DNK	deoksiribonukleinska kiselina
DSC	diferencijalna motridbena kalorimetrija (engl. *differential scanning calorimetry*)
FTIR-ATR	infracrvena spektroskopija s Fourierovom transformacijom – prigušena totalna refleksija (engl. *Fourier transform infrared spectroscopy – attenuated total reflectance*)
GA	galna kiselina (engl. *gallic acid*)
HDCS	kakaova ljuska tretirana HVED-om i sušena u sušioniku (engl. *HVED treated, oven-dried cocoa shell*)
HFCS	kakaova ljuska tretirana HVED-om i sušena liofilizacijom (engl. *HVED treated, freeze-dried cocoa shell*)
HVED	visokonaponsko električno pražnjenje (engl. *high voltage electrical discharge*)
IDF	netopiva prehrambena vlakna (engl. *insoluble dietary fibers*)
OBC	kapacitet vezanja ulja (engl. *oil binding capacity*)
PAH	policiklički aromatski ugljikovodik (engl. *polycyclic aromatic hydrocarbon*)
SDF	topiva prehrambena vlakna (engl. *soluble dietary fibers*)
TA	taninska kiselina (engl. *tannic acid*)
TPC	udio ukupnih fenola (engl. *total phenolic content*)
UCS	netretirana kakaova ljuska (engl. *untreated cocoa shell*)
UV	ultraljubičasto (engl. *ultraviolet*)
WBC	kapacitet vezanja vode (engl. *water binding capacity*)
WDCS	kakaova ljuska miješana u vodi i sušena u sušioniku (engl. *water treated, oven-dried cocoa shell*)
WFCS	kakaova ljuska miješana u vodi i sušena liofilizacijom (engl. *water treated, freeze-dried cocoa shell*)

1. UVOD

Kakaova ljuska je nusproizvod industrije prerade kakaovca koja se još uvijek nedovoljno iskorištava. Sastoji se uglavnom od vlakana i to većim dijelom netopivih, a sadrži i značajne količine proteina (Martin-Cabrejas i sur., 1994; Arlorio i sur., 2001). Istraživanja su pokazala da kakaova ljuska sadrži masti koje su po sastavu vrlo slične kakaovom maslacu (Okiyama i sur., 2019). Osim toga, tijekom procesa prerade kakaovih zrna dio bioaktivnih komponenti karakterističnih za kakaova zrna iz endosperma migriraju u kakaovu ljusku. Najviše su zastupljeni metilksantini i fenolne komponente (Okiyama i sur., 2017).

Industrija prerade kakaovca generira ogromne količine nusproizvoda među kojima je i kakaova ljuska koja je nutritivno bogata i ima potencijal za primjenu u ljudskoj prehrani (Panak Balentić i sur., 2018). Budući da se u posljednje vrijeme sve više teži k održivoj proizvodnji i iskorištavanju nusproizvoda prehrambene industrije, ispitivanje mogućnosti korištenja kakaove ljuske je vrlo bitno za ostvarivanje ovih ciljeva.

U literaturi su navedene neke mogućnosti korištenja kakaove ljuske u prehrambenim proizvodima, na primjer za povećanje udjela vlakana i bioaktivnih komponenti. Korištena je u proizvodnji kruha (Collar i sur., 2009), muffina (Martínez-Cervera i sur., 2011), sira (Brandstetter i sur., 2010), kobasica (Choi i sur., 2019), ekstrudiranih snack proizvoda (Jozinović i sur., 2019), itd. Iako su rezultati pokazali da je kakaovu ljusku moguće koristiti za proizvodnju različitih prehrambenih proizvoda, treba uzeti u obzir da kakaova ljuska sadrži neke nepoželjne komponente koje se moraju ukloniti prije upotrebe (Nascimento i sur., 2015; Copetti i sur., 2013; Assa i sur., 2018).

Visokonaponsko električno pražnjenje (HVED) je netermička metoda za obradu različitih sirovina. Primjenjuje se za ekstrakciju bioaktivnih komponenti, ali i za inaktivaciju mikroorganizama i razgradnju štetnih organskih spojeva (Boussetta i Vorobiev, 2014). Tijekom ovog procesa dolazi do stvaranja električnih izboja izravno u vodi. Uređaj se sastoji od elektrode izboja i elektrode uzemljenja koje su uronjene u tekućinu koja se tretira. Tijekom stvaranja izboja izravno u otopini dolazi do ionizacije, stvaranja ultraljubičastog svjetla, generiranja različitih radikala i sl. (Boussetta i sur., 2011).

Cilj ovoga rada bio je ispitati utjecaj HVED tretmana pri dvije različite frekvencije (40 i 80 Hz) i tri različita vremena (15, 30 i 45 min) na svojstva kakakove ljuske. Uzorci su osušeni u sušioniku pri 40 °C te im je određen: aktivitet vode, kapacitet vezanja vode i ulja, udio bioaktivnih komponenti, udio vlakana, karakteristike mljevenja, udio 5-hidroksimetilfurfurala i akrilamida i FTIR-ATR spektar. Rezultati su uspoređeni s netretiranim uzorkom i kontrolnim uzorcima dobivenim miješanjem u vodi bez HVED tretmana. Drugi dio istraživanja imao je za cilj utvrditi utjecaj različitih postupaka sušenja (sušenje u sušioniku pri 60 °C i liofilizacija) odabranog uzorka kakaove ljuske tretirane HVED-om na: udio vode, aktivitet vode, boju,

kapacitet vezanja vode i ulja, specifični volumen, prividnu i nasipnu gustoću, udio bioaktivnih komponenti, udio Klason lignina i termofizikalna svojstva kakaove ljuske. Rezultati proistekli iz ovog istraživanja objavljeni su u znanstvenim radovima (u prilogu).

2. TEORIJSKI DIO

2.1. KAKAOVAC: IZAZOVI I MOGUĆNOSTI

Iz godine u godinu raste potražnja za čokoladom i srodnim proizvodima, a samim time i za kakaovim zrnima. Najveći proizvođači kakaovih zrna su Obala Bjelokosti, Gana i Indonezija. Preradom kakaovca dolazi do generiranja velike količine nusproizvoda jer kakaovo zrno čini samo 20-30 % cijelog ploda. Tri su glavna nusproizvoda koja nastaju tijekom procesa prerade kakaovca: ljuska ploda, sok pulpe i ljuska kakaovih zrna (Figueroa i sur., 2019).

Mnoga istraživanja su se u posljednjih nekoliko godina fokusirala na iskorištavanje nusproizvoda prehrambene industrije zbog ekoloških razloga, ali i vrijednosti nusproizvoda koja nije bila dovoljno prepoznata. Većina ovih sirovina sadrži visok udio komponenti koje bi mogle imati pozitivan utjecaj na ljudsko zdravlje (Figueroa i sur., 2019). Također, zbog klimatskih promjena i sve većeg broja ljudi na Zemlji suočeni smo s nedostatkom sirovina i hrane. Iskorištavanje nusproizvoda prehrambene industrije riješilo bi jedan dio problema s kojima se prehrambena industrija suočava (Martínez i sur., 2012). Samim time, iskorištavanje kakaove ljuske u proizvodnji prehrambenih proizvoda imalo bi pozitivan utjecaj na okoliš i ljudsko zdravlje, te ekonomski razvitak zemalja koje uzgajaju kakaovac.

Industrija prerade kakaovca suočava se s fluktuacijom cijena kakaovih zrna i društvenim i političkim nestabilnostima u zemljama u kojima se kakaovac uzgaja. Industrija prerade kakaovca suočava se s nekim relevantnim problemima, a jedan od njih je i ekološki aspekt koji se tiče upravljanja nusproizvodima što nastaju tijekom proizvodnje (ICO, 2012; Ntiamoah i Afrane, 2008). Tržište funkcionalne hrane konstantno se širi i raste te su potrošači sve više zainteresirani za ovaj tip proizvoda pa ne čudi što se nusproizvodi prehrambene industrije doživljavaju kao novi sastojci za ovu vrstu proizvoda (Gullón i sur., 2011). Ekonomska, društvena i ekološka održivost cilj je svake prehrambene industrije, uključujući i industriju čokolade. Mnoge institucije žele osigurati pravilno gospodarenje otpadom kako bi postigle ovaj cilj (Puértolas i Barba, 2016).

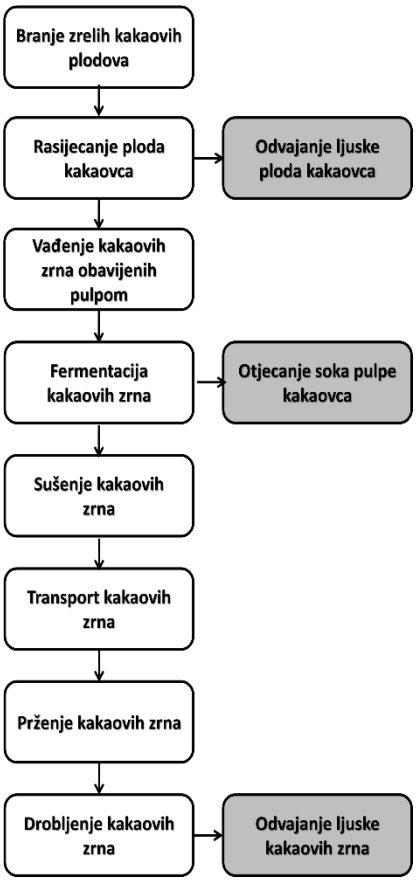
Kakaova ljuska (Slika 1) je nusproizvod industrije prerade kakaovca, a ima visoku nutritivnu vrijednost. Može se koristiti u prehrambenoj industriji, ali i u farmaceutskoj, kozmetičkoj i poljoprivrednoj industriji. Proizvodnja kakaovih zrna može se podijeliti u tri faze:

- Uzgoj, berba i prethodna obrada;
- Primarna prerada kakaovca i proizvodnja poluproizvoda; i
- Proizvodnja gotovih proizvoda (Da Silva do Nascimento i sur., 2010).

Slika 1 Kakaova ljuska

Cijeli proces prerade kakaovca prikazan je na Slici 2, a započinje ubiranjem zrelih plodova. U jednom plodu kakaovca nalazi se 20-40 kakaovih zrna (Prabhakaran Nair, 2010; Minifie, 1989). Zrna se vade iz ploda i zajedno s pulpom koja ih obavija idu na fermentaciju koja traje oko 8 dana, pri čemu dolazi do rasta kvasaca i bakterija te različitih fizikalno-kemijskih promjena (Vásquez i sur., 2019). Faze fermentacije kakaovih zrna su:

1. Prva faza – Dominiraju anaerobni kvasci, smanjen je dotok kisika i nizak je pH;
2. Druga faza – Dominiraju bakterije mliječne kiseline;
3. Treća faza – Dominiraju bakterije octene kiseline, povećan je dotok kisika i dolazi do egzotermne reakcije (Beckett i sur., 2017).



Slika 2 Proces prerade kakaovca (Barišić i sur., 2021a)

Nakon fermentacije kakaova zrna se suše da bi se smanjio udio vlage i zaustavili oksidativni procesi koji se odvijaju u prethodnom procesu. Također, smanjenjem udjela vlage sprječava se rast plijesni što čini zrna stabilnijima tijekom skladištenja i transporta (Gutiérrez, 2017; Beckett i sur., 2017).

Industrijska obrada kakaovih zrna započinje prženjem. Ovaj proces se provodi pri visokim temperaturama, uobičajeno između 120 i 140 °C. Prženje je vrlo bitno za smanjenje udjela nepoželjnih sastojaka, lakše odvajanje kakaove ljuske, dekontaminaciju te razvoj arome i okusa koji su specifični za proizvode na bazi kakaovog zrna. Komponente koje su zaslužne za odgovarajuću aromu kakaovih zrna uglavnom su rezultat Maillardovih reakcija (Gutiérrez, 2017; Beckett i sur., 2017; Afoakwa i sur., 2008).

2.2. KAKAOVA LJUSKA

Preradom kakaovca nastaju velike količine otpada koji se sastoji od ljuske kakaovog ploda, pulpe i ljuske kakaovih zrna. Naime, kakaova zrna, koja su glavni sastojak u proizvodnji čokolade, vade se iz kakaovog ploda (mahune) nakon čega prolaze proces fermentacije i

sušenja. Ljuska kakaovih zrna se odvaja od samog zrna prije ili nakon prženja kakaovih zrna (Vásquez i sur., 2019).

Nakon odvajanja ljuske od kotiledona ona se obično baca ili prodaje kao poljoprivredni malč. Budući da ljuska čini 12 % - 20 % zrna, očito je da je to najveći otpad koji nastaje tijekom prerade zrna (Vásquez i sur., 2019; Okiyama i sur., 2017; Djali i sur., 2018). Prema Međunarodnoj organizaciji za kakao (ICO, 2016) svjetska proizvodnja otpada iz industrije prerade kakaovca može se procijeniti na 700 tisuća tona godišnje.

Ljuska kakaovih zrna bogata je prehrambenim vlaknima, proteinima, polifenolima (Martínez i sur., 2012), metilksantinima (Rusconi i Conti, 2010), itd. Prehrambena vlakna se općenito dijele na topiva i netopiva vlakna, a njihov udio u ljusci je 50 do 60 %. Topiva vlakna kakaove ljuske pokazala su pozitivan utjecaj na razinu glukoze, kolesterola i inzulina u krvnoj plazmi, a budući da mogu utjecati na dulji osjećaj sitosti (a time i na smanjenje unosa hrane) postoji mogući utjecaj na održavanje, čak i smanjenje tjelesne mase (Sánchez i sur., 2010; Ramos i sur., 2008). Omjer topiva/netopiva vlakna vrlo je važan u ljudskoj prehrani, a ljuska kakaovih zrna ima omjer blizu poželjnog što joj daje mogućnost za izravnu primjenu u hrani (Vojvodić i sur., 2016). Prehrambena vlakna kakaove ljuske uglavnom se sastoje od pektina, lignina, celuloze i hemiceluloze (Redgwell i sur., 2003). Također, kakaova ljuska sadrži i visok udio proteina (Tablica 1), ali su prethodna istraživanja pokazala da je jako mali postotak tih proteina u slobodnom obliku (Okiyama i sur., 2017), što utječe na njihovu biodostupnost.

Osim toga, kakaova ljuska bogata je flavanolima (katehin i epikatehin) koji imaju antioksidativno djelovanje te metilksantinima (teobromin i kafein) koji djeluju na živčani sustav (Grillo i sur., 2019a; Mazzutti i sur., 2018). Ovi spojevi su prisutni i u endospermu kakaovih zrna, a u kakaovu ljusku uglavnom migriraju tijekom procesa fermentacije. Okiyama i sur. (2019) istraživali su lipidni profil kakaove ljuske i zaključili da je sličan onome kakaovog maslaca, što bi moglo dovesti do njene primjene kao djelomične zamjene za kakaov maslac. Iz svega navedenog vidljivo je da je ljuska iskoristiva u prehrambenoj industriji za povećanje nutritivne vrijednosti hrane u kojoj se koristi.

Tablica 1 Kemijski sastav ljuske kakaovih zrna (Barišić i sur., 2021a)

Kemijski sastav (%)	Nepržena kakaova ljuska (Fakhlaei i sur., 2020)	Kakaova ljuska pržena na 150 °C (Fakhlaei i sur., 2020)	Pržena kakaova ljuska (Gomez Hoyos i sur., 2020)	Sterilizirana kakaova ljuska (Lecumberri i sur., 2007)
Vlažnost	11,11 ± 0,19	4,89 ± 0,19	2,60 ± 0,30	-
Vlakna	64,35 ± 0,71	61,21 ± 0,81	-	60,54 ± 0,32
Proteini	18,00 ± 0,22	10,93 ± 0,01	-	16,71 ± 0,18
Masti	4,09 ± 0,16	6,82 ± 0,26	3,60 ± 0,90	6,62 ± 0,38
Lignin	-	-	15,60	-
Celuloza	-	-	27,70	-
Hemiceluloza	-	-	11,10	-
Pepeo	10,22 ± 0,38	10,89 ± 0,38	-	-

2.2.1. Primjena kakaove ljuske u prehrambenoj industriji

Sastav kakaove ljuske zainteresirao je mnoge znanstvenike i usmjerio ih na implementaciju kakaove ljuske izravno u prehrambene proizvode i istraživanje svojstava i senzorske prihvatljivosti dobivenih proizvoda. Osim toga, određeni dio istraživanja bavio se i primjenom različitih komponenti kakaove ljuske u hrani.

Martínez-Cervera i sur. (2011) koristili su topiva prehrambena vlakna ekstrahirana iz kakaove ljuske u proizvodnji čokoladnih muffina. Vlakna su korištena kao zamjena za mast, a rezultati su pokazali smanjeno sušenje (povećanje tvrdoće) tijekom skladištenja, dobru teksturu, veću vlažnost i ugodnu boju obogaćenih muffina. Topiva prehrambena vlakna iz kakaove ljuske također su korištena u proizvodnji pšeničnog kruha, pokazujući učinak omekšavanja u ovim proizvodima (Collar i sur., 2009). Zaključeno je da se ova vlakna mogu koristiti do udjela 6 % i da nemaju negativan učinak na senzorsku prihvatljivost i skladištenje kruha. Obogaćivanje proizvoda koji se često konzumiraju (poput muffina i kruha) prehrambenim vlaknima iz kakaove

ljuske moglo bi imati povoljan učinak na adsorpciju glukoze i kapacitet vezanja kolesterola u ljudskom organizmu, kao što je pokazala *in vitro* studija Nsor-Atindana i sur. (2012). Također, u istom istraživanju pokazalo se i da su vlakna izolirana iz ljuske bogata polifenolima. Mazzutti i sur. (2018) koristili su kakaovu ljusku za dobivanje ekstrakta bogatog lipidima i ekstrakta bogatog fenolima. Ovi ekstrakti su pokazali veliki potencijal za korištenje u proizvodnji prehrambenih proizvoda. Nakon toga provedeno je istraživanje s ciljem zaštitite polifenola u ekstraktima (sušenjem raspršivanjem s maltodekstrinima) (Papilo i sur., 2019). Rezultati su pokazali da su polifenoli zaštićeni tijekom pečenja i skladištenja čime se može postići veći unos u ljudski organizam.

Alkalizirana kakaova ljuska također je ispitana za izravnu primjenu u proizvodnji hrane. Bernaert i Ruysscher (2016) koristili su je za proizvodnju kakaovog napitka bogatog prehrambenim vlaknima. U drugom istraživanju zaključili su da se prah kakaove ljuske može koristiti u različitim prehrambenim proizvodima kao zamjena za kakaov prah (Bernaert i Ruysscher, 2013). Provedeno istraživanje u svrhu ispitivanja senzorskih svojstava funkcionalnih napitaka s dodatkom kakaove ljuske (Rojo-Poveda i sur., 2019) pokazalo je da su napitci s najvećim udjelom bioaktivnih spojeva bili najmanje prihvaćeni od strane potrošača. Razlog tomu je vjerojatno veći udio polifenola i metilksantina koji su nosioci oporog okusa.

Alkalizirana kakaova ljuska korištena je i u proizvodnji keksa, a dobiveni proizvod pokazao je veću otpornost na lomljenje u odnosu na pšenične kekse (Handojo i sur., 2019). Istraživanje Osundahunsi i sur. (2007) pokazalo je da pepeo dobiven iz ljuske može služiti za alkalizaciju kakaovog loma i da nema negativan utjecaj na krajnji proizvod.

Izravna primjena kakaove ljuske u prehrambenim proizvodima bez prethodne obrade uključuje proizvodnju svinjskih kobasica (Choi i sur., 2019) i ekstrudiranih snack proizvoda (Jozinović i sur., 2019). Svinjske kobasice s udjelom kakaove ljuske od 1 % ili manje imale su poboljšanu boju, viskoznost, sadržaj vlage i stabilnu emulziju. Također je zanimljivo otkriće da dodavanje kakaove ljuske može inhibirati oksidaciju lipida u ovim vrstama proizvoda, vjerojatno zbog visokog udjela antioksidanasa. Jozinović i sur. (2019) dodali su kakaovu ljusku u ekstrudirane snack proizvode u količinama od 5 %, 10 % i 15 %. Ovo obogaćivanje povećalo je sadržaj rezistentnog škroba, udio polifenola i antioksidativnu aktivnost. Iako su fizikalna svojstva bila nešto lošija u usporedbi s konvencionalnim proizvodima, ipak su bila prihvatljiva. Korištenje kakaove ljuske u nutritivno siromašnim proizvodima ima velik potencijal za rješavanje problema njenog zbrinjavanja, ali i obogaćivanja prehrambenih proizvoda s jeftinom i bogatom sirovinom.

Ispitana je i mogućnost korištenja kakaove ljuske u proizvodnji biskvita (Rojo-Poveda i sur., 2020), keksa (de Barros i sur., 2020) i bezglutenskog kruha (Rinaldi i sur., 2020). Keksi i

bezglutenski kruh su dodatkom kakaove ljuske postali nutritivno bogatiji jer su imali veći udio proteina i vlakana. Proizvedeni keksi u istraživanju de Barros i sur. (2020) pokazali su dobra senzorska svojstva, dok je bezglutenski kruh (Rinaldi i sur., 2020) pokazao lošiju kvalitetu koja se očituje u smanjenju specifičnog volumena i povećanju pukotina. Biskviti koji su proizvedeni s dodatkom kakaove ljuske pokazali su lošiju biodostupnost bioaktivnih spojeva iz ljuske (Rojo-Poveda i sur., 2020). Kao i u slučaju proteina koji su u kakaovoj ljusci uglavnom u vezanom obliku, i polifenoli bi mogli biti vezani na vlakna koja su prisutna u kakaovoj ljusci i samim time manje biodostupna.

Kakaova ljuska je korištena u proizvodnji različitih vrsta sireva. Usporedbom s dodatkom kakaovog praha u iste proizvode pokazalo se da kakaova ljuska ima bolji utjecaj na viskoznost, mazivost, čvrstoću i konzistenciju sireva u usporedbi s dodatkom kakaovog praha. Također, dodatak kakaove ljuske imao je pozitivan utjecaj na stabilnost teksture sireva nakon zamrzavanja i odmrzavanja (Brandstetter i sur., 2010).

Osim u izravnoj primjeni u prehrambenim proizvodima, kakaova ljuska se koristila i za proizvodnju aktivne biorazgradnje ambalaže za hranu. Njezin sastav, odnosno antioksidativna aktivnost i udio vlakana utječu na dobra mehanička svojstva i duži rok trajanja hrane (Papadopoulou i sur., 2019; Tran i sur., 2017).

U posljednjih nekoliko godina brojni se istraživači bave obogaćivanjem čokolade vlaknima i polifenolima jer je to konditorski proizvod koji se konzumira u cijelom svijetu i među svim generacijama (Barišić i sur., 2021b). Budući da je kakaova ljuska bogata i jednim i drugim spojevima, njezina primjena u proizvodnji čokolade ima veliki potencijal. Isto tako, kakaova ljuska se generira u industriji čokolade pa bi samim time njezina primjena u proizvodnji čokolade imala pozitivan ekonomski učinak.

2.2.2. Problemi s upotrebom kakaove ljuske u proizvodnji hrane

Budući da je očito da ljuska kakaovih zrna ima veliki potencijal i da je bogata mnogim bioaktivnim komponentama koje mogu biti od koristi za ljudsko zdravlje, postavlja se pitanje zašto se u značajnijoj mjeri još ne koristi u komercijalnoj proizvodnji hrane. Jedan od razloga je taj što kakaova ljuska sadrži nepoželjne komponente koje je potrebno ukloniti prije nego se iskoristi u proizvodnji prehrambenih proizvoda. Neke od tih komponenti su mikotoksini, teški metali, policiklički aromatski ugljikovodici (PAH) i različiti mikroorganizmi.

Kakaova zrna prolaze proces fermentacije, sušenja i najčešće se čuvaju u nehigijenskim uvjetima. To je vidljivo iz činjenice da su zrna često kontaminirana vrstama *Aspergillus*, *Eurotium* i *Absidia* (Okiyama i sur., 2017). Također, neke vrste plijesni mogu producirati mikotoksine koji se teško uklanjaju u procesima proizvodnje hrane (Bonvehi, 2004). Copetti i

sur. (2013) izvijestili su da je okratoksin A, koji proizvode plijesni rodova *Aspergillus* i *Penicillium*, koncentriran u kakaovoj ljusci. Ovaj toksin je prisutan u širokom rasponu namirnica kao što su zrna kave, suho voće i žitarice (Brera i sur., 2011). Samo mali dio ovog toksina nalazi se u kakaovim zrnima. Također, aflatoksini B1, B2, G1 i G2 pronađeni su u kakaovoj ljusci. Zaključeno je da se u usporedbi s ostalim dijelovima zrna češće pojavljuju u kakaovoj ljusci baš iz razloga što je kakaova ljuska tijekom prerade zrna u kontaktu s vanjskim utjecajima, te da se pojavljuju u 11 % ispitivanih uzoraka (Copetti i sur., 2012). Ove komponente su vrlo stabilne i ne mogu se potpuno uništiti tijekom procesa proizvodnje čokolade (Bonvehi, 2004).

Kakaova zrna mogu biti kontaminirana i teškim metalima zbog okolišnih i vanjskih utjecaja (Amézqueta i sur., 2005). Veliku zabrinutost predstavlja prisutnost nikla (Ni), kadmija (Cd), kroma (Cr) i olova (Pb) (Assa i sur., 2018). Dosadašnja istraživanja uglavnom su provedena kako bi se ispitao sadržaj teških metala u čokoladi i proizvodima od kakaa (Rankin i sur., 2005; Dahiya i sur., 2005). Povećana kontaminacija uglavnom je uzrokovana upotrebom gnojiva, pesticida, insekticida itd. Ako se te aktivnosti ne kontroliraju i procesom ne upravlja u skladu s dobrom poljoprivrednom praksom i dobrom proizvođačkom praksom, to može dovesti do povećanog sadržaja teških metala (Aikpopodion i sur., 2013). Fermentacija, sušenje, drobljenje i kontakt s metalnim uređajima tijekom obrade dodatno mogu utjecati na sadržaj teških metala (Kruszewski i sur., 2018). Kakaova ljuska u većini slučajeva ima veći sadržaj ovih elemenata zbog svoje visoke sposobnosti adsorpcije teških metala. Zahvaljujući ovoj karakteristici kakaova ljuska korištena je u nekoliko istraživanja kao novi adsorbens za uklanjanje teških metala iz onečišćene vode (Meunier i sur., 2003; Meunier i sur., 2004).

Policiklički aromatski ugljikovodici (PAH-ovi) su poznati kao genotoksični karcinogeni, a u kakaovim zrnima mogu nastati tijekom procesa sušenja i prženja (SCF, 2002; Ciecierska, 2020). Nastaju u pečenoj hrani bogatoj ugljikohidratima kroz dva procesa: pirolizom i pirosintezom (Cheng i sur., 2015; Singh, 2013). Povećani sadržaj PAH-ova u kakaovim zrnima najčešće je posljedica neodgovarajućeg sušenja zrna. Najveći rizik od kontaminacije prisutan je kod umjetnog sušenja kada proizvođači koriste drva za ogrjev ili fosilna goriva (Misnawi, 2012). Osim toga, Ciecierska (2020) je zaključila da čak i niske temperature tijekom procesa prženja pogoduju razvoju PAH-ova. Budući da se kakaova zrna najčešće prže s kakaovom ljuskom, postoji velika mogućnost da je i ona onečišćena PAH-ovima. Agus i sur. (2020) utvrdili su da pržena kakaova ljuska ima manje količine PAH-ova od sušene. To bi moglo biti posljedica migracije ovih spojeva u kakaova zrna tijekom prženja.

Tijekom sušenja kakaovih zrna na plantažama, ptice i kukci često dolaze u dodir sa zrnima kakaovca. Oni su prenosioci bakterija *Escherichia coli* i *Salmonella* (Da Silva do Nascimento i sur., 2010). Iako su kakaova zrna podvrgnuta procesu prženja, istraživanje Izurieta i

Komitopoulou (2012) pokazalo je da su sojevi *Salmonella* prisutni na kakaovoj ljusci otporni na toplinu. Da bi se rizik od onečišćenja kakaovih zrna sveo na najmanju moguću mjeru, na plantažama kakaovca treba provoditi dobru higijensku, poljoprivrednu i proizvođačku praksu. Treba izbjegavati ostavljanje nezaštićenih kakaovih zrna kako bi se smanjio kontakt s prenosiocima kontaminacije (Da Silva do Nascimento i sur., 2013).

Osim kontaminacije, jedan od problema korištenja kakaove ljuske u proizvodnji prehrambenih proizvoda je i njezino mljevenje. Kao što je napomenuto, kakaova ljuska sadrži visok udio netopivih vlakana koja se teško usitnjavaju i potrebno je uložiti velike količine energije za taj proces. Dvije skupine istraživača bavile su se ovim pitanjem (Kopp i sur., 2007; Bernaert i Ruysscer, 2018), pri čemu su izrađena dva patenta koja prikazuju rješenja za proces mljevenja ljuske. Zaključeno je da bi se dobiveni prah mogao koristiti kao bojilo za hranu, inhibitor sivljenja proizvoda baziranih na kakau ili kao zamjena za kakaov prah.

2.3. VISOKONAPONSKO ELEKTRIČNO PRAŽNJENJE

Visokonaponsko električno pražnjenje (engl. *high voltage electrical discharge* - HVED) postalo je vrlo zanimljivo mnogim istraživačima jer može razgraditi organske spojeve i inaktivirati bakterije, viruse i kvasce (Grymonpré i sur., 2001). Ovaj tretman dovodi do niza kemijskih i fizikalnih procesa: proizvodnje ultraljubičastog svjetla, udarnih valova, proizvodnje različitih reaktivnih spojeva itd. (Chen i sur., 2009). Sve ove promjene zaslužne su za sposobnost HVED-a da bude tehnologija dezinfekcije i ekstrakcije. Također, visokonaponsko električno pražnjenje je niskoenergetska i netermička tehnologija koja ima veliki potencijal za korištenje u tretmanu nusproizvoda u prehrambenoj industriji.

HVED se temelji na stvaranju električnih pražnjenja izravno u vodi. Kontakt izboja i vode stvara fizikalno-kemijske promjene i različite kemijske procese u vodi (Barba i sur., 2015a). Primjena kratkotrajnih impulsa visokog napona i intenziteta između dvije elektrode uronjene u tekućinu dovodi do ionizacije. Proces se sastoji od tri faze: stvaranje električnih impulsa, strujno pražnjenje i stvaranje električnog luka (Boussetta i Vorobiev, 2014). Proces se ubrzava mjehurićima plina prisutnim u otopini ili nastalim tijekom lokalnog zagrijavanja. Lavina elektrona od visokonaponske elektrode do elektrode uzemljenja dogodit će se ako je razlika potencijala između elektroda dovoljna. Tijekom tog procesa, zbog velike količine utrošene energije, nastaje niz oksidacijskih spojeva (Puértolas i Barba, 2016).

HVED uređaji se mogu podijeliti na šaržne, kontinuirane i cirkulacijske sustave. Osnovni mehanizam ova tri sustava je isti, ali su načini koncentriranja lokalnog električnog polja različiti (Li i sur., 2019). Za korištenje HVED-a u ekstrakciji ili za bilo koju drugu svrhu postoje dva

važna parametra: ukupno trajanje HVED tretmana (t_{HVED}) (Formula 1) i energetski unos HVED-a (w_{HVED}) (Formula 2) (Li i sur., 2019):

$$t_{HVED}(s) = n \times t_i \quad (1)$$

gdje je n broj izboja i t_i trajanje izboja (s).

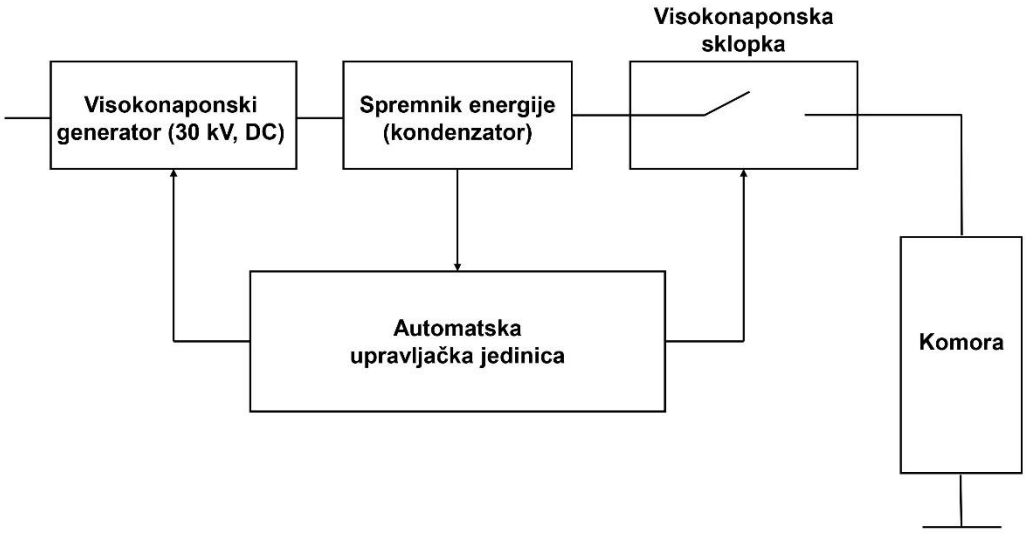
$$w_{HVED}\left(\frac{kJ}{kg}\right) = \frac{E_p \times n}{m} \quad (2)$$

gdje je E_p energija jednog impulsa (kJ), n broj pražnjenja, a m masa suspenzije (kg).

Tijekom HVED tretmana dolazi do fotonske disocijacije vode, što dovodi do emisije UV-svjetla i generiranja $OH^{\cdot}$ radikala. UV-svjetlo može inaktivirati stanice oštećujući DNK, a stvoreni udarni valovi mogu fragmentirati tkivo proizvoda koji se tretira. Električna jakost polja izravno je proporcionalna poraciji stanične membrane tretiranog materijala, a ta se pojava naziva elektroporacija (Li i sur., 2019; El Kantar i sur., 2018).

Budući da postoji mnogo uređaja izrađenih po narudžbi koji se koriste u istraživanju, a neke od njih dobro su opisali Takaki i sur. (2019), u nadolazećem tekstu kao primjer je opisan visokonaponski uređaj za električno pražnjenje (Slika 3) koji je za Prehrambeno-tehnološki fakultet Osijek izradio Ingeniare CPTS1. Generator impulsa visokog napona (Slika 4) sastoji se od visokonaponskog istosmjernog generatora (30 kV) s promjenjivom frekvencijom impulsa od 20 Hz do 100 Hz, spremnika energije (kondenzatora), visokonaponske sklopke, komore i automatske upravljačke jedinice. Automatska upravljačka jedinica omogućuje kontrolu vremena tretmana, frekvencije impulsa i brzine miješanja.

Slika 3 Uređaj za visokonaponsko električno pražnjenje



Slika 4 Shematski prikaz generatora HVED-a

Visokonaponske elektrode smještene su u komori za obradu i pričvršćene su na nosač elektroda s mogućnošću podešavanja razmaka između elektroda. Visokonaponska volframova elektroda i pločica ili elektroda uzemljenja (promjera 45 mm) su tijekom tretmana uronjene u otopinu. Visina elektroda također se može podesiti. Udaljenost između elektroda podešava se prema vodljivosti uzorka koji će se tretirati, što ovisi i o vrsti uzorka i o koncentraciji otopine. Prije tretmana potrebno je izmjeriti vodljivost otopine. Utjecaj vodljivosti vode na stvaranje udarnih valova proučavali su Cathignol i sur. (1991) gdje je zaključeno da se povećanjem vodljivosti smanjuje generacija udarnih valova.

2.3.1. Mogućnosti primjene HVED-a

HVED se trenutno najviše koristi za ekstrakciju polifenola, proteina i pektina iz otpadnih materijala (Puértolas i Barba, 2016; Boussetta i sur., 2009; Barba i sur., 2015a). U ovoj disertaciji fokus je stavljen na druge mogućnosti ove tehnologije koje će otvoriti potencijal korištenja HVED-a kao tehnologije za dekontaminaciju i ekstrakciju u isto vrijeme.

HVED se može koristiti za inaktivaciju bakterija kao što su *Sallmonella* spp., *Escherichia coli*, *Listeria*, itd. (Niemira, 2012). Zhao i sur. (2020) dizajnirali su uređaj za pražnjenje dielektrične barijere atmosferskog zraka, koji su koristili za dekontaminaciju površine voća. U tom istraživanju su prikazali da ovaj tretman pokazuje zadovoljavajuće rezultate vezane za uklanjanje *Staphylococcus aureus* bez oštećenja površine ploda. Osim toga, postoji mnogo

primjera učinkovite primjene hladne plazme u uklanjanju mikotoksina i plijesni, čak i štetnih spora (Gavahian i Cullen, 2020). To implicira da bi HVED također mogao imati takav učinak u tekućim medijima. Anpilov i sur. (2002) izvijestili su da je HVED učinkovit u uništavanju bakterija *Escherichia coli* u vodi, vjerojatno zbog stvaranja različitih radikala i UV zračenja. Reaktivni kisikovi spojevi i vodikov peroksid koji nastaju tijekom tretmana izazivaju oksidativni stres koji ima veliki učinak na inaktivaciju mikroorganizama. Ovi produkti oksidiraju membranske komponente mikroorganizama (Stulić i sur., 2019). Učinkovitost HVED-a posljedica je kombinacije fizičkih, kemijskih i električnih učinaka, a ne samo jednog čimbenika (Moisan i sur., 2001). Tijekom tretmana dolazi do stvaranja vodikovog peroksida, što dalje dovodi do stvaranja H_3O^+, koji je najviše odgovoran za smanjenje pH. Bakterije poput *Escherichia coli* posebno su osjetljive na nizak pH, što dovodi do njihovog uništenja (Chen i sur., 2008). Međutim, samo pH ne može biti odgovoran za inaktivaciju ovih razmjera (Chen i sur., 2009). Kombinacija UV svjetla i H_2O_2 također može dovesti do mutacije i oštećenja DNK mikroorganizama. Ozon je dodatni čimbenik koji doprinosi dezinfekcijskim učincima HVED-a, budući da je poznati dezinficijens (Nguyen i sur., 2019).

Tessier i sur. (2001) ispitivali su uklanjanje PAH-ova primjenom električnog pražnjenja. Taj se proces odvijao u čvrstoj fazi pa nije sigurno na koji način će se ti spojevi ponašati u vodi tijekom stvaranja izboja. Potrebna su daljnja istraživanja kako bi se vidjelo može li ovaj proces uspješno ukloniti PAH-ove iz ljuske kakaovih zrna. Poznato je da se ova tehnologija može koristiti i za uklanjanje različitih organskih nečistoća iz vode, vjerojatno iz istih razloga (Puértolas i Barba, 2016). Potencijal HVED-a za dekontaminaciju materijala i tekućina leži u činjenici da su produkti (O^-, $^{\bullet}OH$, O_3), koji nastaju tijekom pražnjenja u tekućini, vrlo aktivni. Radikali koji nastaju disocijacijom vode mogu oksidirati organske spojeve koji su prisutni u materijalu ili tekućini koja se tretira, te ih se na takav način može ukloniti (Wang i Hsu, 2019; Ceccato, 2009). Poznato je da tijekom električne obrade napredni oksidacijski procesi razgrađuju niz organskih spojeva (Grymonpré i sur., 1999). Hidroksil radikal, ozon i vodikov peroksid su spojevi koji izravno reagiraju s organskim spojevima (Sun i sur., 1999). Du i sur. (2006) također su uspjeli smanjiti udio PAH-ova za 74,4 % tijekom lučnog pražnjenja. Naveli su da je $OH^{\bullet}$ vjerojatno reagirao s aromatskim prstenom, gdje je daljnja reakcija s kisikom rezultirala produktima cijepanja prstena.

Pražnjenja plazme poznata su po svojoj mogućnosti uklanjanja plijesni i mikotoksina (Gavahian i sur., 2020). Ouf i sur. (2015) eliminirali su okratoksin A tretmanom hladnom plazmom u trajanju od 7,5 min. Reaktivne čestice formirane plazmom uglavnom su odgovorne za dekontaminaciju, ali UV svjetlost također igra važnu ulogu. Park i sur. (2007) uspjeli su potpuno razgraditi okratoksine i deoksinivalenol tretmanom hladnom plazmom. Međutim, mikotoksine u matriksu hrane moglo bi biti malo teže razgraditi jer bi matriks mogao usporiti

učinak plazme i reagirati s dijelom reaktivnih vrsta (Bosch i sur., 2017). Također je utvrđeno da ovaj tretman uništava integritet stanične strukture spora *Aspergillus* vrste (Dasan i sur., 2016).

Električno pražnjenje pokazalo se učinkovitim i u smanjenju sadržaja Pb, Cd, Fe i Mn u otpadnim vodama, vjerojatno zbog stvaranja netopivih oksida i hidroksida (Grinevich i sur., 2011). Rincón i Motta (2014) također su uspjeli ukloniti cink, bakar i nikal iz otpadnih voda metodom elektrokoagulacije. To implicira da bi ovaj proces mogao biti učinkovit i u uklanjanju metala iz kakaove ljuske.

Budući da se HVED tehnologija uglavnom koristi za ekstrakciju, to može biti dodatni razlog za korištenje ove metode u tretiranju nusproizvoda prehrambene industrije. Bilo bi ekonomski isplativo ekstrakt dobiven kao nusproizvod u obradi kakaove ljuske dalje koristiti za proizvodnju bioaktivnih komponenti, proteina ili pektina. Jokić i sur. (2019) koristili su ovu metodu za ekstrakciju polifenola i metilksantina. U drugim istraživanjima je provedena HVED ekstrakcija proteina iz pogače *Camellia olcifera* (Li i sur., 2020) i pektina iz pulpe šećerne repe (Almohammed i sur., 2017). U usporedbi s drugim konvencionalnim tehnikama ekstrakcije, HVED se pokazao kao izvrstan proces za dobivanje većeg sadržaja fenola u ekstraktima iz lišća masline (Žuntar i sur., 2019) i sjemenki grožđa (Boussetta i sur., 2012). U slučaju sjemenki grožđa, HVED je također utjecao i na veličinu sjemenki grožđa zbog generiranih izboja koji mogu poremetiti tkivne i stanične strukture (Barba i sur., 2015a). Istodobno, bio je manje selektivan u pogledu količine antocijana dobivenih tijekom ekstrakcije. Zbog velikog potencijala za ekstrakciju različitih biokomponenti, kraće obrade i manjeg termičkog utjecaja u odnosu na druge tehnike, HVED se već pokazao kao izvrsna tehnika ekstrakcije. Uzme li se u obzir i potencijal za dekontaminaciju vode i različitih biomaterijala, ova bi tehnologija mogla zamijeniti nekoliko tehnologija koje su trenutno prisutne u industriji, ne samo za obradu kakaove ljuske već i za druge nusproizvode i otpadne tokove prehrambene industrije.

3. EKSPERIMENTALNI DIO

3.1. ZADATAK

Zadatak ove doktorske disertacije bio je:

- Tretirati kakaovu ljusku suspendiranu u vodi pri dvije različite koncentracije (1,5 % i 3 %, w/v) HVED-om, pri dvije različite frekvencije (40 i 80 Hz) i tijekom tri različita vremena (15, 30 i 45 minuta) uz negativnu kontrolu: miješanje vodenih suspenzija istih koncentracija tijekom istog vremena bez HVED tretmana;
- Utvrditi udio fenolnih komponenti i metilksantina HPLC-PDA metodom u netretiranoj, kontrolnoj i tretiranoj kakaovoj ljusci;
- Utvrditi udio 5-hidroksimetilfurfurala (HPLC-PDA) i akrilamida (LC-MS/MS) u netretiranoj, kontrolnoj i tretiranoj kakaovoj ljusci;
- Utvrditi udio i sastav prehrambenih vlakana u netretiranoj, kontrolnoj i tretiranoj kakaovoj ljusci;
- Utvrditi udio vode, aktivitet vode, boju, kapacitet vezanja vode i ulja i meljivost netretirane, kontrolne i tretirane kakaove ljuske;
- Spektrofotometrijski odrediti udio ukupnih fenola i tanina Folin-Ciocalteu metodom u netretiranoj, kontrolnoj i tretiranoj kakaovoj ljusci;
- Snimiti FTIR-ATR spektar netretirane, kontrolne i tretirane kakaove ljuske;
- Tretirati kakaovu ljusku HVED-om pri odabranom vremenu i frekvenciji (na osnovi prethodno dobivenih rezultata) te osušiti na 60 °C i liofilizacijom;
- Ispitati svojstva kakaove ljuske nakon različitih postupaka sušenja: udio vode, aktivitet vode, boja, kapacitet vezanja vode i ulja, specifični volumen, prividna i nasipna gustoća;
- Spektrofotometrijski odrediti udio ukupnih tanina i fenola u kakaovoj ljusci dobivenoj nakon tretmana HVED-om i različitim postupcima sušenja;
- Utvrditi udio fenolnih komponenti i metilksantina HPLC-PDA metodom u kakaovoj ljusci dobivenoj nakon tretmana HVED-om i s različitim postupcima sušenja;
- Odrediti termofizikalna svojstava (DSC) kakaove ljuske dobivene nakon tretmana HVED-om i različitim postupcima sušenja.

3.2. MATERIJAL I METODE

3.2.1. Priprema uzoraka kakaove ljuske

Fermentirana kakaova zrna (West Afrika mix, Huyser, Möller B.V. Nizozemska) su pržena pri 135 °C 55 min, a nakon toga je odvojena kakaova ljuska od kotiledona. Pržena kakaova ljuska je samljevena u laboratorijskom mlinu (IKA, M20) (25 g tijekom 2 min) i pohranjena u zamrzivaču do provedbe analiza (uzorak netretirane kakaove ljuske, UCS).

Slika 5 Tretiranje kakaove ljuske HVED-om

Pripremljene su vodene suspenzije neusitnjene kakaove ljuske (u koncentracijama 1,5 i 3 %) i tretirane HVED-om (Slika 5) na 40 i 80 Hz tijekom 15, 30 i 45 minuta. HVED uređaj se sastoji od komore spojene na visokonaponski impulsni generator od 30 kV (uređaj je izradio Inganiare CPTS1 za Prehrambeno-tehnološki fakultet Osijek). Komora za tretiranje je opremljena cilindričnom volframovom iglom (izbojnom elektrodom) (promjera 2,5 mm) i elektrodom uzemljenja u obliku pločice (promjera 45 mm). Razmak između elektroda tijekom svih tretmana bio je 2 cm. Miješanje uzoraka je postignuto magnetskom miješalicom. Za procjenu utjecaja HVED-a na svojstva kakaove ljuske pripremljeni su i kontrolni uzorci. Kontrolni uzorci bili su vodene suspenzije neusitnjene kakaove ljuske, u jednakim koncentracijama, miješane na magnetskoj mješalici 15, 30 i 45 minuta (Slika 6). Nakon tretmana kakaova ljuska je osušena na 40 °C u laboratorijskom sušioniku (Memmert, UFE 500), mljevena u laboratorijskom mlinu s hlađenjem (IKA, M20) (25 g tijekom 2 min) i pohranjena u zamrzivaču do analiza.

Slika 6 Miješanje kakaove ljuske u vodi

3.2.2. Određivanje fenolnih komponenti i metilksantina HPLC metodom

HPLC (visoko djelotvorna tekućinska kromatografija) s apsorpcijskim detektorom je korištena za određivanje šest fenolnih komponenti (galna kiselina, kafeinska kiselina, *p*-kumarinska kiselina, (+)-katehin, (−)-epikatehin i (−)-epikatehin galat) i dva metilksantina (teobromin i kafein) u kakaovoj ljusci. Svi standardi fenola i metilksantina bili su prikladni za HPLC analizu i kupljeni od Sigma-Aldrich (St. Louis, MO). Metanol (J.T. Baker, Nizozemska), n-heksan (Carlo Erba Reagents, Španjolska) i mravlja kiselina (Scharlau Chemie, Španjolska) bili su prikladni za HPLC analizu.

Uzorci kakaove ljuske pripremljeni su prema metodi koju su opisali Adamson i sur. (1999). Dva grama (± 0,01 g) kakaove ljuske ekstrahirana su tri puta sa po 10 mL n-heksana HPLC čistoće radi uklanjanja lipida. Uzorci odmašćene kakaove ljuske nakon toga su osušeni na zraku. Odmašćenom uzorku dodane je 5 mL 70 %-tnog metanola, nakon čega je provedena

ekstracija potpomognuta ultrazvukom tijekom 30 min i centrifugiranje (10 min pri 3000 o/min). Supernatant je dekantiran u odmjernu tikvicu od 10 mL. Postupak ekstrakcije je ponovljen još jednom, prikupljeni supernatanti su sjedinjeni, a tikvica je nadopunjena 70 %-tnim metanolom do 10 mL. Ekstrakti su pohranjeni u zamrzivaču do analize.

Prije injektiranja ekstrakti su filtrirani kroz 0,45-μm najlonski membranski filter. Izvorna metoda korištena u ovom istraživanju bila je HPLC metoda koju su opisali Belščak i sur. (2009). Kromatografski uvjeti su modificirani i optimizirani za primijenjenu kromatografsku kolonu i instrument. Analiza je provedena na sustavu za tekućinsku kromatografiju koji se sastoji od Shimadzu LC-20AD pumpe, Shimadzu CTO-20AC komore za kolonu, Shimadzu autosamplera SIL-10AF i Shimadzu SPD-M20A detektora s nizom fotodioda spojenog na računalo sa softverom LabSolution Lite 5.52. Za odjeljivanje komponenti od interesa korištena je HPLC kolona Inertsil ODS-3V (GL Sciences, 250 mm × 4,6 mm, veličina čestica 5 μm). Mobilna faza se sastojala od 1 %-tne mravlje kiseline (otapalo A) i metanola (otapalo B) HPLC čistoće. Brzina protoka mobilne faze bila je 0,8 mL/min, a provedeno je gradijentno eluiranje. Početni postotak otapala B u mobilnoj fazi bio je 10 %, nakon čega slijedi linearni porast na 32 % B u 15 min, 40 % B od 20 min do 25 min i 60 % B na 30 min. Volumen injektiranja bio je 20 μL. Temperatura kolone i detektora postavljena je na 30 °C. Raspon valnih duljina praćenja bio je 200-400 nm, dok je valna duljina detekcije postavljena na 278 nm. Identifikacija fenolnih komponenti i metilksantina postignuta je na temelju vremena zadržavanja i usporedbe spektra apsorbancije s onima čistih komponenti. Kvantifikacija identificiranih komponenti provedena je metodom vanjske kalibracije. Sve analize su rađene u tri ponavljanja. Rezultati su izraženi kao mg specifične komponente po g kakaove ljuske (mg/g).

3.2.3. Određivanje udjela akrilamida

Akrilamid (AA) stupnja čistoće 99 % nabavljen je od Dr. Ehrenstorfer GmbH (Augsburg, Njemačka). $^{13}C_3$ Akrilamid (AA-IS) (izotopska čistoća 99 %) u metanolu, koji je korišten kao interni standard, kupljen je od Cambridge Isotop Laboratories (Andover, MA, SAD). n-Heksan (za GC ECD i FID analize) nabavljen je od firme Merck (Kenilworth, SAD). Acetonitril (za UHPLC) je od ITW Reagents (Barcelona, Španjolska). Metanol (za UV, IR i HPLC analize) je nabavljen od Panreac AppliChem (Darmstadt, Njemačka). Mravlja kiselina (98 %) je nabavljena od firme Scharlau (Barcelona, Španjolska). Tijekom analiza je korištena ultračista voda (Nirosta VV System, Nirosta Water Technologies, Osijek, Hrvatska). Soli za QueEChERS: $MgSO_4$ (4 g) + NaCl (0,5 g); i d-SPE soli (ekstrakcija disperzivne čvrste faze): $MgSO_4$ (150 g)+ PSA (50 mg) kupljeni su od Bekoluta (Haupstuhl, Njemačka).

Priprema uzoraka provedena je prema Agilent aplikaciji "Analize akrilamida u pomfritu koristeći Agilent Bond Elut QuECHERS AOAC kit i LC-MS/MS" (Al-Taher, 2012) uz određene modifikacije. Približno 1,00 ± 0,05 g homogeniziranog uzorka izvagano je u kivete za centrifugiranje od 50 mL. U svaku vialu je dodano 20 µL otopine internog standarda (10 µg/mL AA-IS). Dodano je 5 mL n-heksana i miješano 1 min. Dodano je 20 mL smjese voda:acetonitril (1:1) i miješano 1 min. Viale su miješane kroz 5 min (IKA-WERKE, Staufen, Njemačka) (250–300 o/min). QueChERS ($MgSO_4$ (4 g) + NaCl (0,5 g)) soli su dodane u smjesu i miješani 1 min. Uzorci su centrifugirani 15 minuta na 4600 o/min. Heksanski sloj je odbačen, a alikvot acetonitrilnog ekstrakta od 5 mL prebačen je pipetom u vialu sa solima (d-SPE, $MgSO_4$ (150 g) + PSA (50 mg)). Sadržaj viale je pomiješan i zatim centrifugiran 5 minuta na 4600 o/min. 2 mL alikvota acetonitrilnog ekstrakta prebačeno je u staklenu epruvetu i ispareno u struji dušika na 45 °C. Dobiveni ekstrakti su otopljeni u 500 µL vode i filtrirani kroz membranski najlon filter (0,2 µm, Pall Life Sciences, New York, SAD) u staklenu vialu za autosampler. Pripremljeni ekstrakti čuvani su u hladnjaku do analize.

Za analizu uzoraka korišten je tekućinski kromatograf Waters Xevo spojen s Waters Triple Quadrupole MS detektorom (Waters Corp., Milford, MA) s pozitivnom elektrosprej ionizacijom i softverom MassLynx V4.1. Komponente su odijeljene na koloni Luna C-18 (150 × 2 mm, 3 µm; Phenomenex, Kalifornija, SAD) na 30 °C. Korištena mobilna faza sastojala se od otapala (A), 0,1 % (v/v) vodene otopine mravlje kiseline i otapala (B) metanola. Primijenjen je sljedeći režim gradijenta: od 0 do 0,5 min 2 % B, od 0,5 do 6 min 2–90 % B, od 6 do 8 min 90 % B, od 8 do 8,5 min 90-2 % B, od 8,5 do 13,5 min 2 % B. Volumen injektiranja bio je 10 µL, vrijeme analize 15 min, protok mobilne faze 0,2 mL/min. Detektor je bio tandemski maseni spektrometar s temperaturom isparavanja 550 °C, protokom plina isparavanja 800 L/h, temperaturom ionskog izvora 150 °C, ionizacijom ESI (+) i kapilarnim naponom: 2,8 kV. Prijelazi m/z 72,1 → 55,1; m/z 72,1 → 44,1 i m/z 72,1 → 27,1 praćeni su za akrilamid, a prijelazi m/z 75,1 → 58,0 za $^{13}C_3$-akrilamid. Napon konusa postavljen je na 22 V, a energija sudara na 10 V. Sadržaj akrilamida u uzorcima izračunat je korištenjem kalibracijske krivulje koja je izrađena na temelju omjera površine pika akrilamida i $^{13}C_3$ označenog internog standarda. Također, uz svaku seriju uzoraka provjerena je i učinkovitost postupka ekstrakcije (iskorištenje) na LOQ. Rezultati su izraženi kao µg/kg.

3.2.4. Određivanje udjela 5-hidroksimetilfurfurala

Sadržaj 5-hidroksimetilfurfurala (5-HMF) određen je metodom za određivanje 5-HMF u medu opisanom u Swiss Food Book (SL, 2006). Ukratko, 2,5 g uzorka je izvagano u kivetu od 50 mL i pomiješano s vodom na miješalici. Uzorak je centrifugiran 15 minuta na 4500 o/min. Ekstrakt

je filtriran kroz 0,45 μm membranski najlon filter (Pall Life Sciences, New York, SAD) u vijalu autosamplera. Uzorak pripremljen za kromatografsku analizu potom je stavljen u autosampler za tekućinsku kromatografiju. Sadržaj 5-hidroksimetilfurfurala određen je na Shimadzu kromatografu s detektorom s nizom fotodioda (PDA) korištenjem kolone ODS C18 (HyperClone, 250 × 4,6 mm, 5 μm) koja je bila termostatirana na 25 °C. Mobilna faza bila je voda:metanol (90:10) s brzinom protoka od 1 mL/min i volumenom injektiranja 20 μL. Detektor je postavljen na 285 nm. Prisutnost 5-HMF određena je usporedbom vremena zadržavanja u uzorku s vremenom zadržavanja standarda. Količina prisutnog 5-HMF izračunata je prema kalibracijskoj krivulji korištenjem metode vanjske kalibracije. LOD je iznosila 0,25 mg/kg, a LOQ 1,25 mg/kg s koeficijentom determinacije od 0,999991.

3.2.5. Određivanje udjela vode i aktiviteta vode

Aktivitet vode je određen pri 25 °C pomoću HygroLab 3 (Rotronic, Switzerland). Udio vode određen je prema ISO 6540:1980 (ISO, 1980), a izračunat je sljedećom jednadžbom (3):

$$Udio\ vode\ (\%) = \frac{masa\ uzorka - masa\ uzorka\ nakon\ sušenja}{masa\ uzorka} \times 100 \qquad (3)$$

3.2.6. Određivanje boje

Za određivanje boje usitnjene kakaove ljuske korišten je kromametar Konica Minolta CR-400 s nastavkom za praškaste uzorke (Zyzelewicz i sur., 2014). Mjerenja su provedena u CIEL*a*b* i L*Ch° sustavima u 5 ponavljanja, pri čemu L* vrijednost predstavlja svjetlinu (0 je crna, a 100 bijela), a* vrijednosti crvenu (pozitivne vrijednosti) ili zelenu boju (negativne vrijednosti) i b* žutu (pozitivne vrijednosti) ili plavu boju (negativne vrijednosti). Ukupna promjena boje (ΔE) izračunata je pomoću sljedeće jednadžbe (4), gdje L*, a*, b* predstavljaju vrijednosti za tretirane uzorke i L_0*, a_0*, b_0* vrijednosti za netretiranu kakaovu ljusku:

$$\Delta E = \sqrt{(L - L_0)^2 + (b - b_0)^2 + (a - a_0)^2} \qquad (4)$$

3.2.7. Određivanje udjela tanina

Ekstrakcija

Svaki uzorak je izvagan (2 g) i ekstrahiran tri puta s 10 mL n-heksana (Carlo Erba Reagents, Val de Reuil, Francuska) kako bi se uklonili lipidi. Uzorci su sušeni na zraku preko noći pa je

provedena ekstrakcija s 5 mL 70 %-tnog metanola (J. T. Baker, Deventer, Nizozemska) u ultrazvučnoj kupelji. Nakon toga uzorci su centrifugirani 10 min na 3000 o/min (Sigma 2-16, Sigma, Osterode, Njemačka). Supernatant je dekantiran u odmjernu tikvicu od 10 mL. Taj je postupak ponovljen još jednom nakon čega je tikvica sa supernatantom napunjena do oznake 70 %-tnim metanolom.

Spektrofotometrijska analiza

Sadržaj tanina određen je metodom koju su opisali Amorim i sur. (2008). Metoda se temelji na kompleksiranju tanina s kazeinom. Kalibracijska krivulja izrađena je sa standardnim otopinama taninske kiseline (Sigma-Aldrich, St. Louis, USA) u rasponu koncentracija od 0,5 do 3 mg/mL ($y=0,9011x+0,0095$; $R^2=0,9993$). Ukupni udio fenola i udio zaostalih fenola (dobiveni nakon kompleksiranja tanina i kazeina) određeni su spektrofotometrijski na 760 nm prema metodi Singleton i sur. (1999). Sadržaj tanina u pripremljenim ekstraktima izračunat je jednadžbom (5) kao razlika između udjela ukupnih fenola i udjela zaostalih fenola. Rezultati su prikazani kao mg taninske kiseline po g odmašćenog uzorka (mgTA/g) i kao postotak tanina u ukupnom sadržaju fenola (%).

$$Tanini\left(\frac{mgTA}{g}\right) = udio\ ukupnih\ fenola - udio\ zaostalih\ fenola \qquad (5)$$

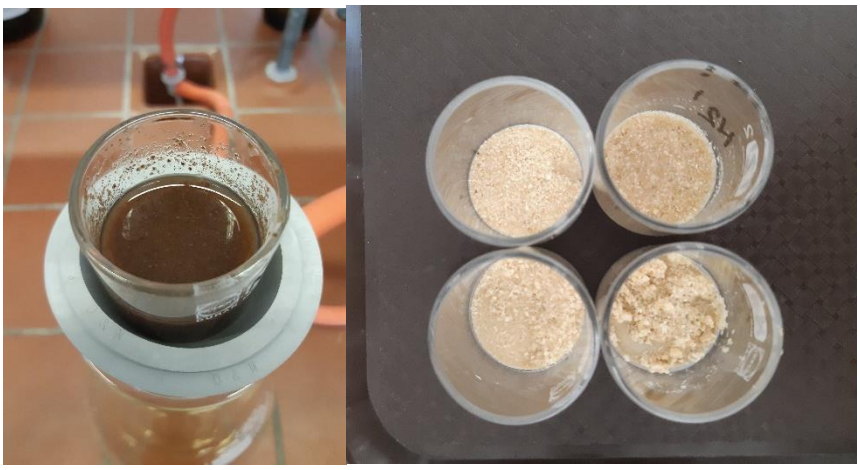
3.2.8. Određivanje udjela prehrambenih vlakana

Prehrambena vlakna su određena gravimetrijskom AOAC metodom 991.43 (AOAC, 1995). Uzorci su razgrađeni termostabilnom α-amilazom, proteazom i amiloglukozidazom (Megazyme set za analizu ukupnih prehrambenih vlakana, Megazyme Ltd., Bray, Irska). Udio netopivih prehrambenih vlakana (IDF, %) određen je gravimetrijski nakon filtracije (Slika 7), a topiva prehrambena vlakna (SDF, %) taloženjem iz dobivenog filtrata. Nakon korekcije za neprobavljene proteine (metoda po Kjeldahlu) i pepeo (spaljivanje pri 525 °C), udio ukupnih prehrambenih vlakana (TDF, %) izračunat je pomoću jednadžbi (6) i (7) kao zbroj IDF i SDF. Vrijednosti su preračunate na suhu tvar uzorka.

$$Udio\ prehrambenih\ vlakana = \frac{\frac{R_1+R_2}{2} - p - A - B}{\frac{m_1+m_2}{2}} \qquad (6)$$

$$B = \frac{BR_1+BR_2}{2} - BP - BA \qquad (7)$$

gdje je: R_1=masa ostatka uzorka 1 od m_1; R_2=masa ostatka uzorka 2 od m_2; m_1=masa uzorka 1; m_2=masa uzorka 2; A=masa pepela od R_1; P=količina proteina iz R_2; B=slijepa proba; BR=masa ostatka slijepe probe; BP=količina proteina iz slijepe probe iz BR_1; BA=masa pepela iz slijepe probe iz BR_2.



Slika 7 Gučevi tijekom i nakon filtracije

3.2.9. Određivanje meljivosti kakaove ljuske

Meljivost kakaove ljuske određena je prosijavanjem uzoraka samljevene kakaove ljuske na analitičkoj tresilici sa sitima (Retsch GmbH, AS200, Haan, Njemačka) i mjerenjem mase dobivenih frakcija. Ukupno 50 g uzorka prosijano je kroz šest sita (50, 71, 100, 125, 200 i 315 μm) tijekom 15 min. Nakon vaganja svake frakcije, rezultati su izraženi kao postotci mase kakaove ljuske koja je izvagana na svakom situ (%).

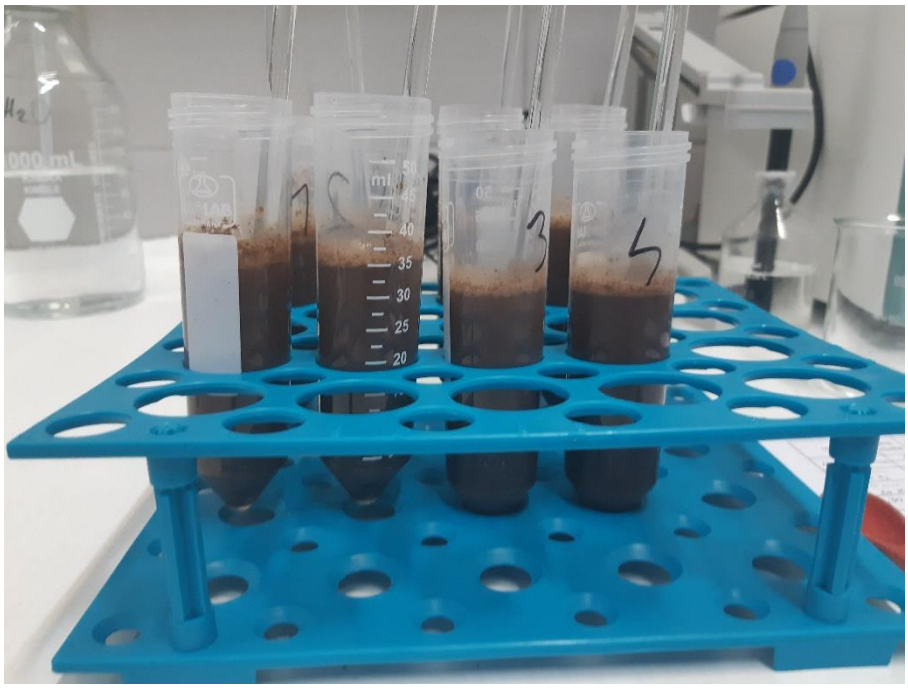
3.2.10. Kapacitet vezanja vode (WBC) i ulja (OBC)

Za određivanje WBC-a korištena je standardna metoda AACC 88-04 (AACC, 1983). U 2,5 g uzorka kakaove ljuske je dodano 30 mL vode (Slika 8). Ove suspenzije su ostavljene stajati na sobnoj temperaturi uz periodično miješanje. Nakon toga uzorci su centrifugirani pri 3000 o/min tijekom 15 minuta (Centra-MP4R, IEC, Mumbai, Indija). Supernatant je dekantiran, a preostali ostatak je izvagan. Analiza je provedena u dva ponavljanja. Rezultati su izračunati jednadžbom (8) i izraženi su kao grami apsorbirane H_2O po gramu kakaove ljuske (g/g).

$$WBC\ \left(\frac{g}{g}\right) = \frac{masa\ taloga}{masa\ suhe\ tvari\ početnog\ uzorka} \qquad (8)$$

Za određivanje OBC-a korišten je isti postupak kao i za određivanje WBC-a. Jedina razlika je bila što je za OBC umjesto vode korišteno hladno prešano ulje repice. Rezultati su izraženi kao grami apsorbiranog ulja po gramu kakaove ljuske (%), a izračunava se jednadžbom (9):

$$OBC\ \left(\frac{g}{g}\right) = \frac{masa\ taloga}{masa\ suhe\ tvari\ početnog\ uzorka} \qquad (9)$$



Slika 8 Određivanje kapaciteta vezanja vode i ulja

3.2.11. FTIR-ATR

FTIR-ATR spektri su snimljeni spektrometrom Cary 630 (Agilent, Santa Clara, CA, USA) u rasponu valnih duljina od 4000 do 650 cm^{-1}. Za svaki uzorak snimljena su 32 skeniranja koja su usrednjena uz spektralnu razlučivost od 16 cm^{-1}.

3.2.12. Mikrobiološka analiza kakaove ljuske

Priprema uzoraka za određivanje ispitivanih mikroorganizama je izvršena u aseptičnim uvjetima za određivanje *Salmonella* spp., ukupnog broja aerobnih mezofilnih bakterija, *Enterobacteriaceae*, kvasaca i plijesni. Prije svake odvage Stomacher vrećica za pripremu uzoraka je označena identičnom oznakom koja se nalazila na vrećici s uzorkom, koja je prije početka odvage (od 10 g ili 25 g) dezinficirana 70 %-tnim alkoholom. Za sve četiri metode je rađena posebna priprema uzorka, odnosno odvaga i razrjeđenje. Najprije je izvršena lagana ručna homogenizacija inicijalnog (primarnog) razrjeđenja, a potom su uzorci ostavljeni da odstoje tijekom 20 min na sobnoj temperaturi. Nakon toga pravila su se razrjeđenja i rađena nacjepljivanja.

Za određivanje *Salmonella* spp. korištena je metoda u skladu s normom Horizontalna metoda za detekciju *Salmonella* spp. BAS EN ISO 6579:2005, BAS EN ISO 6579/Cor2:2010 (ISO,

2010). U 25 g kakaove ljuske dodano je 225 mL puferirane peptonske vode (engl. *buffered peptone water* (BPW)) (Merck, Njemačka). Prekonoćna kultura je korištena za nacjepljivanje u tekuće hranjive podloge 1 mL u Muller-Kaufman tetrationatnom novocion bujonu (engl. *Muller-Kaufman tetrathionate novobiocin broth* (MKTT)) (Merck, Njemačka) i 0,1 mL u Rappaport-Vassiliadis soja peptonskom bujonu (engl. *Rappaport-Vassiliadis soya peptone broth* (RVS)) (Merck, Njemačka), a potom iz navedenih kultura nakon 24 h je izvršeno nacjepljivanje na ksiloza lizin dezoksikolatni agar (engl. *Xylose lysine deoxycholate agar* (XLD)) (Merck, Njemačka) i briljantni zeleni agar (engl. *Briliant green agar* (BGA)) (Merck, Njemačka).

Za određivanje ukupnog broja aerobnih mezofilnih bakterija u gramu uzorka korištena je metoda u skladu s normom Horizontalna metoda za brojanje mikroorganizama - Dio 1: Brojanje kolonija pri 30 °C tehnikom izlijevanja podloge BAS EN ISO 4833-1:2013 (ISO, 2013a). U 10 g uzorka dodano je 90 mL otopine BPW (Merck, Njemačka) i 0,5 % K_2SO_4 (Semikem) kako bi se smanjila antimikrobna aktivnost. Za navedenu metodu koristila se Stomacher vrećica s pregradnom mrežicom. Inicijalno (početno) razrjeđenje je razrijeđeno u koncentracijskom rasponu od 10^{-1} do 10^{-7}. Od svakog razrjeđenja odpipetiran je 1 mL alikvota u Petrijevu zdjelicu koja je potom zalivena s 12 do 15 mL agara standardnih metoda (engl. *Plate count agar* (PCA) (Merck, Njemačka). Inokulum s agarom pažljivo je promiješan rotiranjem zdjelice po podlozi, nakon skrutnjavanja sve ploče s okrenutim poklopcem na dolje inkubirane su kroz 72 h u aerobnim uvjetima na 30 °C. Nakon predviđenog vremena inkubacije izbrojane su porasle kolonije na agaru i utvrđen je broj bakterija u gramu uzorka.

Za određivanje *Enterobacteriaceae* korištena je metoda u skladu s normom Horizontalna metoda za detekciju i određivanje broja *Enterobacteriaceae* - Dio 2: Metoda brojanja kolonija BAS EN ISO 21528-2:2013 (ISO, 2013b). U 10 g samljevene kakaove ljuske dodano je 90 mL otopine BPW (Merck, Njemačka) i 0,5 % K_2SO_4 (Semikem) kako bi se smanjila antimikrobna aktivnost. Za navedenu metodu koristila se Stomacher vrećica s pregradnom mrežicom. Inicijalno (početno) razrjeđenje je razrijeđeno u koncentracijskom rasponu od 10^{-1} do 10^{-6}. Od svakog razrjeđenja odpipetiran je 1 mL alikvota u Petrijevu zdjelicu koja je potom zalivena najprije s 10 mL ljubičasto-crvenog žučnog agara (engl. *Violet Red Bile Glucose agara* (VRBG)) (Merck, Njemačka), a nakon što se prvi sloj skrutnuo s još 15 mL istog agara (Merck, Njemačka). Inokulum s agarom pažljivo je promiješan rotiranjem zdjelice po podlozi i nakon skrutnjavanja sve ploče s okrenutim poklopcem na dolje inkubirane su kroz 24 h u aerobnim uvjetima na 37 °C. Nakon predviđenog vremena inkubacije izbrojane su porasle kolonije na VRBG i izvršeno je daljnje precjepljivanje na hranjivi agar. Nacjepljivanjem na glukoza test i provjerom gotovim trakicama Bactident® Oxidase (Merck, Njemačka) za detekciju citokrom

oksidaze u mikroorganizmima utvrđene su *Enterobacteriaceae*, negativne na oksidazu, a pozitivne na glukozu.

Broj kvasaca i plijesni određen je u skladu s normom Horizontalna metoda za brojanje kvasaca i plijesni - Dio 2: Tehnika brojanja kolonija u proizvodima kod kojih je aktivitet vode manji ili jednak 0,95 BAS ISO 21527-2:2009 (ISO, 2009). U 10 g samljevene kakaove ljuske dodano je 180 mL 0,1 % peptonske vode (Liofilchem, Italija), što je kasnije uvršteno u proračun. Za navedenu metodu koristila se Stomacher vrećica bez pregradne mrežice. Inicijalno (početno) razrjeđenje je razrijeđeno u koncentracijskom rasponu od 10^{-1} do 10^{-3}. Alikvot od 0,1 mL niza razrjeđenja otopine nacijepljen je na DG 18 agar (Conda, Španjolska) u duplikatu i ravnomjerno razmazan L-štapićem po površini agara. Inokulirane ploče inkubirane su kroz 5 dana na 25 °C u aerobnim uvjetima nakon čega su izbrojane porasle kolonije kvasaca i plijesni te je određen njihov broj u gramu uzorka.

3.2.13. Određivanje utjecaja HVED-a i postupaka sušenja na svojstva kakaove ljuske

Za ovaj dio istraživanja odabrani su uvjeti tretmana kakaove ljuske koji su pokazali najbolji utjecaj na svojstva u prvom dijelu istraživanja, a tretman HVED-om ponovljen je kako slijedi.

3.2.13.1. Priprema uzoraka

Korištena kakaova ljuska dobivena je nakon prženja fermentiranih kakaovih zrna (West Africa mix, Huyser, Möller B.V., Edam, Nizozemska). Zrna su pržena na 135 °C tijekom 55 min nakon čega je ljuska odvojena od kotiledona. Kontrolni uzorak (uzorak netretirane kakaove ljuske (UCS)) pripremljen je mljevenjem ljuske sakupljene nakon odvajanja od kotiledona. Za pripremu svih uzoraka korištena je nemljevena kakaova ljuska kako bi se smanjila kontaktna površina kakaove ljuske i vode i time smanjio gubitak bioaktivnih komponenti. Kontrolni uzorci pripremljeni su miješanjem nemljevene kakaove ljuske u demineraliziranoj vodi (koncentracija 0,5 %) tijekom 10 minuta. Uzorci tretirani HVED-om pripremljeni su HVED obradom nemljevene kakaove ljuske u demineraliziranoj vodi. HVED generator se sastojao od 30 kV visokonaponskog impulsnog generatora (Barišić i sur., 2020a). Tretman je proveden na 70 Hz, 10 min u vodenoj suspenziji s koncentracijom od 0,5 % i na udaljenosti od 0,5 cm između igle i elektrode uzemljenja. Voda korištena za tretman imala je pH 5,86 ± 0,00 i vodljivost 12,05 ± 0,30 µS/cm. Prije tretmana suspenzije s kakaovom ljuskom imale su pH 5,14 ± 0,09 i električnu vodljivost 86,00 ± 4,00 µS/cm. Suspenzije nakon tretmana imale su pH 5,38 ± 0,10 i električnu vodljivost 247,50 ± 4,50 µS/cm. Tretirani uzorci podijeljeni su u dva poduzorka. Jedan dio kontrolnih uzoraka (WDCS uzorak) i HVED-tretiranih (HDCS uzorak) uzoraka sušeni su u

laboratorijskom sušioniku (Memmert, UFE 500) na 60 °C do konstantne mase, a drugi dio (uzorci WFCS i HFCS) je sušen u liofilizatoru (Christ, Alpha LSCplus) (Slika 9). Prije liofilizacije uzorci su zamrznuti na -80 °C. Tijekom glavnog sušenja tlak je bio 0,250 mbar, a tijekom završnog sušenja 0,050 mbar. Svi uzorci (25 g) mljeveni su u laboratorijskom mlinu (IKA, M20) tijekom 2 min. Kao takvi uzorci su pohranjeni (4 °C) do analize.

Udio vode, aktivitet vode, kapacitet vezanja vode i ulja, boja uzoraka, udio ukupnih tanina te udio metilksantina i fenolnih spojeva određeni su prema prethodno opisanim metodama. Osim toga, određeni su i specifični volumen, prividna i nasipna gustoća, udio ukupnih fenola, Klason lignin i termostabilnost, a metode su opisane u narednom tekstu.

Slika 9 Liofilizacija kakaove ljuske

3.2.13.2. Specifični volumen, prividna i nasipna gustoća

Specifični volumen, prividna i nasipna gustoća izmjereni su prema metodi koju su opisali de Escalada Pla i sur. (2012). Prividna gustoća određena je mjerenjem volumena koji zauzima 3 g kakaove ljuske u graduiranom cilindru od 10 mL. Cilindar je nježno udaran po površini stola sve dok nije došlo do smanjenja razine uzorka. Specifični volumen izračunat je kao inverzna

vrijednost prividne gustoće (ρa^{-1}). Nasipna gustoća (ρb) određena je sipanjem mljevene kakaove ljuske u graduirani cilindar do oznake za 10 mL. Cilindar s uzorcima izvagan je na analitičkoj vagi. Za svaki uzorak mjerenja su obavljena u tri ponavljanja te su izračunate srednje vrijednosti i standardna devijacija.

3.2.13.3. Udio ukupnih fenola (TPC)

Mljeveni uzorci su izvagani (1 g) i lipidi su uklonjeni ekstrahiranjem tri puta s po 10 mL n-heksana (masa uzoraka nakon odmašćivanja bila je 0,9381–0,9778 g). Uzorci su ostavljeni da se osuše preko noći i nakon toga je dodano 5 mL 70 %-tnog metanola za ekstrakciju u ultrazvučnoj kupelji (30 min, 80/320 W, 35 kHz). Nakon ekstrakcije uzorci su centrifugirani na 3000 o/min tijekom 10 minuta. Supernatant je prebačen u odmjernu tikvicu od 10 mL. Ekstrakcija je ponovljena još jednom, a tikvica sa supernatantom nadopunjena je 70 %-tnim metanolom (Belščak i sur., 2009). Za određivanje ukupnog sadržaja fenola (TPC) korištena je Folin–Ciocalteuova metoda (Singleton i sur., 1999). U odmjernu tikvicu (10 mL) dodano je 100 µL ekstrakta, 6 mL vode i 500 µL nerazrijeđenog Folin–Ciocalteu reagensa. Nakon 6 minuta dodano je 1,5 mL 20 %-tne Na_2CO_3 i tikvica je napunjena vodom. Uzorci su ostavljeni u mraku 2 sata nakon čega je izmjerena apsorbancija na 760 nm (spektrofotometar Shimadzu, UV-1800). Analize su provedene u tri ponavljanja za svaki uzorak. TPC je izražen kao mg galne kiseline (0,02-0,5 mg/mL galne kiseline korištene za kalibracijsku krivulju) po g odmašćenog uzorka.

3.2.13.4. Klason lignin

Sadržaj Klason lignina određen je metodom koju su opisali Kirk i Obst (1988). Uzorci kakaove ljuske su digestirani sa 72 %-tnom sumpornom kiselinom, nakon čega su smjese razrijeđene i sekundarna hidroliza je provedena u autoklavu na 120 °C tijekom 1 sata. Smjese su filtrirane, osušene i u ostacima je određen udio pepela. Rezultati su izraženi kao postotak lignina netopivog u 72 % H_2SO_4.

3.2.13.5. Diferencijalna motridbena kalorimetrija

Za određivanje termostabilnosti kakaove ljuske korištena je diferencijalna motridbena kalorimetrija (DSC 822^e, Mettler Toledo, Švicarska). DSC je kalibriran s indijem prije upotrebe. Uzorci kakaove ljuske izvagani su (4-5 mg) u aluminijske posudice od 40 µL. Kao referentni materijal korištena je prazna aluminijska posudica. Uzorci su zagrijavani od 25 do 420 °C uz konstantnu brzinu zagrijavanja od 20 °C/min. Svojstva faza temperature staklastog prijelaza

(T_g), kao što su početak (T_{go}), srednja točka (T_{gm}) i krajnja točka (T_{ge}) i promjene specifične topline (ΔH), analizirane su DSC termogramom pomoću softvera STAR^e Evaluation V6_V12 Conversation. Svaki uzorak je analiziran u dva ponavljanja i izračunate su srednje vrijednosti i standardne devijacije.

3.2.14. Statistička analiza

Statistička analiza provedena je primjenom softvera STATISTICA® 13.4.0.14 (1984–2018 TIBCO Software Inc).

Za utvrđivanje statistički značajne razlike uvjeta tretiranja na udio metilksantina i fenolnih komponenti provedena je faktorska analiza varijance (engl. *Factorial ANOVA*) (p<0,05).

Za utvrđivanje statistički značajne razlike uvjeta tretiranja na udio akrilamida, 5-hidroksimetilfurfurala, udio vode, aktivitet vode i boju također je provedena faktorska analiza varijance (engl. *Factorial ANOVA*) (p<0,05). Osim toga, za te parametre utvrđen je i Pearsonov koeficijent korelacije (p<0,05).

Za određivanje statistički značajne razlike učinaka tretmana na udio ukupnih tanina, prehrambenih vlakana, meljivosti kakaove ljuske i kapacitet vezanja vode i ulja korišteni su analiza varijance glavnih efekata (engl. *Main effects ANOVA*) i faktorska analiza varijance (engl. *Factorial ANOVA*). p-Vrijednost koja se smatrala značajnom bila je 0,05. Osim toga, određeni su i Pearsonovi koeficijenti korelacije (p<0,05).

Za određivanje utjecaja postupaka sušenja i HVED-tretmana na svojstva kakaove ljuske korištena je jednofaktorska analiza varijance uz korekciju s Welch F-testom. Rezultati su smatrani značajno različitim pri p-vrijednosti od 0,05.

4. REZULTATI I RASPRAVA

4.1. UTJECAJ TRETMANA VISOKONAPONSKIM ELEKTRIČNIM PRAŽNJENJEM NA FENOLNE KOMPONENTE I METILKSANTINE PRISUTNE U KAKAOVOJ LJUSCI

Udio fenolnih komponenti (galna kiselina, kafeinska kiselina, *p*-kumarinska kiselina, (+)-katehin, (-)-epikatehin i (-)-epikatehin galat) i metilksantina (teobromin i kafein) u netretiranoj kakaovoj ljusci, kao i sadržaj analiziranih komponenti nakon različitih tretmana prikazan je u Tablici 2. Kakaova ljuska je bogata fenolnim spojevima i metilksantinima koji migriraju iz kotiledona u ljusku tijekom procesa fermentacije i prženja. Također se tijekom daljnjih procesa prerade kakaovca može se primijetiti daljnja degradacija bioaktivnih komponenti (Hernández-Hernández i sur., 2017; Kim i Keeney, 1984; Martínez i sur., 2012). Visok sadržaj fenola, a samim time i jako antioksidativno djelovanje, uz visoki udio vlakana pokazuju da dodavanje kakaove ljuske kao funkcionalne komponente u proizvodnji čokolade i drugih prehrambenih proizvoda ima veliki potencijal. Glavne bioaktivne komponente u netretiranoj kakaovoj ljusci su bili metilksantini, teobromin prosječne vrijednosti 3,906 ± 0,070 mg/g i kafein prosječne vrijednosti 0,646 ± 0,055 mg/g. Rezultati dobiveni u ovom istraživanju u skladu su s literaturnim podatcima (Bonvehí i Jordà, 1998; Hartati, 2010).

Koncentracija teobromina u kakaovoj ljusci može biti čak i do 21 g/kg, ali također je poznato i da koncentracija teobromina ovisi o podrijetlu zrna, fermentaciji i uvjetima tijekom procesa prženja (Adamafio, 2013). (+)-Katehin je bio najzastupljenija fenolna komponenta u kakaovoj ljusci (prosječno 0,290 ± 0,005 mg/g), a zatim (-)-epikatehin i galna kiselina s prosječnim vrijednostima 0,165 ± 0,099 mg/g i 0,147 ± 0,041 mg/g (Tablica 2). Hernández-Hernández i sur. (2017) su utvrdili veće vrijednosti (+)-katehina i (-)-epikatehina u kakaovoj ljusci od rezultata dobivenih u ovom istraživanju. U oba istraživanja kakaova ljuska bila je fermentirana, ali u ovom istraživanju proveden je i proces prženja nakon fermentacije. Prženje može uzrokovati promjenu kemijskog sastava, povećanje udjela (+)-katehina i smanjenje udjela (-)-epikatehina zbog izomerizacije (Abbe i Amin, 2008), što djelomično može objasniti razliku u sadržaju fenolnih spojeva.

Tablica 2 Udio bioaktivnih komponenti u kakaovoj ljusci prije i nakon tretmana (Barišić i sur., 2020b)

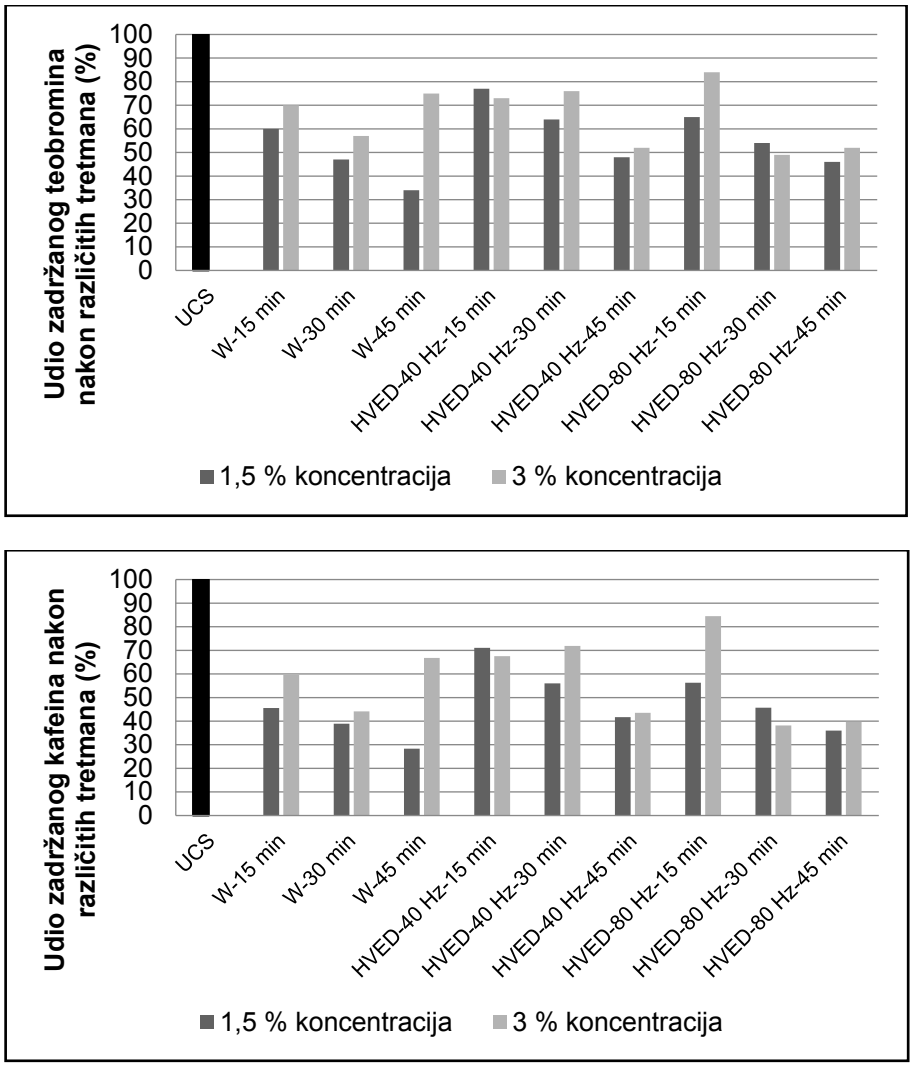
		TEO mg/g	**CAF mg/g**	**CAT mg/g**	**EPI mg/g**	**EPG mg/g**	**GA mg/g**	**CA mg/g**	***p*-CA mg/g**
Netretirana kakaova ljuska (UCS)		3,906± 0,070	0,646± 0,055	0,290± 0,005	0,165± 0,099	0,009± 0,000	0,147± 0,041	0,004± 0,001	0,017± 0,002
Tretman									
Koncentracija 1,5 %	W-15 min	2,335± 0,721	0,296± 0,066	0,026± 0,009	0,037± 0,004	0,004± 0,001	0,011± 0,002	n.d.	n.d.
	W-30 min	1,832± 0,195	0,253± 0,027	0,030± 0,005	0,033± 0,003	0,004± 0,001	0,009± 0,002	n.d.	n.d.
	W-45 min	1,334± 0,161	0,814± 0,023	0,017± 0,002	0,029± 0,002	0,003± 0,000	0,004± 0,001	n.d.	n.d.
	HVED-40 Hz-15 min	3,008± 0,109	0,462± 0,024	0,054± 0,003	0,074± 0,003	0,008± 0,000	0,021± 0,002	n.d.	0,008± 0,007
	HVED-40 Hz-30 min	2,481± 0,089	0,364± 0,019	0,058± 0,003	0,055± 0,002	0,006± 0,000	0,014± 0,001	n.d.	n.d.
	HVED-40 Hz-45 min	1,868± 0,323	0,271± 0,053	0,036± 0,005	0,041± 0,006	0,004± 0,000	0,008± 0,003	n.d.	n.d.
	HVED-80 Hz-15 min	2,547± 0,389	0,366± 0,068	0,048± 0,014	0,059± 0,012	0,006± 0,001	0,015± 0,005	n.d.	n.d.
	HVED-80 Hz-30 min	2,122± 0,224	0,297± 0,036	0,045± 0,005	0,049± 0,004	0,006± 0,001	0,010± 0,002	n.d.	n.d.
	HVED-80 Hz-45 min	1,813± 0,022	0,234± 0,007	0,030± 0,000	0,042± 0,001	0,004± 0,000	0,007± 0,000	n.d.	n.d.
Koncentracija 3 %	W-15 min	2,739± 0,185	0,388± 0,035	0,077± 0,006	0,059± 0,004	0,006± 0,001	0,027± 0,003	n.d.	0,014± 0,001
	W-30 min	2,209± 0,020	0,287± 0,005	0,053± 0,000	0,043± 0,000	0,005± 0,000	0,015± 0,000	n.d.	n.d.
	W-45 min	2,933± 0,051	0,434± 0,006	0,074± 0,001	0,054± 0,001	0,006± 0,000	0,021± 0,001	n.d.	0,013± 0,000
	HVED-40 Hz-15 min	2,832± 0,017	0,439± 0,009	0,064± 0,001	0,074± 0,001	0,007± 0,000	0,022± 0,000	n.d.	0,013± 0,000
	HVED-40 Hz-30 min	2,974± 0,031	0,467± 0,003	0,065± 0,001	0,074± 0,001	0,006± 0,000	0,020± 0,000	n.d.	0,013± 0,000
	HVED-40 Hz-45 min	2,032± 0,124	0,283± 0,024	0,037± 0,002	0,051± 0,001	0,004± 0,000	0,010± 0,002	n.d.	n.d.
	HVED-80 Hz-15 min	3,283± 0,020	0,549± 0,002	0,044± 0,001	0,085± 0,000	0,007± 0,000	0,027± 0,000	n.d.	0,014± 0,000
	HVED-80 Hz-30 min	1,928± 0,060	0,248± 0,011	0,037± 0,001	0,051± 0,001	0,005± 0,000	0,009± 0,001	n.d.	n.d.
	HVED-80 Hz-45 min	2,036± 0,081	0,258± 0,008	0,039± 0,002	0,050± 0,002	0,005± 0,000	0,011± 0,000	n.d.	n.d.

W-voda; TEO-teobromin, CAF-kafein, CAT-(+)-katehin, EPI-(-)-epikatehin, EPG-(-)-epikatehin galat, GA-galna kiselina, CA-kafeinska kiselina, *p*-CA-*p*-kumarinska kiselina; n.d.-nije detektiran

Ekstrakcija fenola i metilksantina iz kakaovih zrna i njegovih nusproizvoda značajno ovisi, prije svega, o primijenjenoj metodi ekstrakcije a kasnije i o parametrima ekstrakcije (Jokić i sur., 2018). Bolje iskorištenje tijekom ekstrakcije bioaktivnih komponenti iz kakaove ljuske postiže se primjenom inovativnih metoda ekstrakcije (npr. superkritični CO_2, ekstrakcija etanolom pod visokim tlakom, supkritična voda ili ekstrakcija potpomognuta pulsirajućim električnim poljem)

u usporedbi s konvencionalnim metodama ekstrakcije (Barbosa-Pereira i sur., 2018; Jokić i sur., 2018; Mazzutti i sur., 2018). HVED je korišten za ekstrakciju bioaktivnih komponenti iz mnogih prehrambenih proizvoda (Barba i sur., 2015b; Barba i sur., 2015c; Li i sur., 2019; Parniakov i sur., 2014a; Parniakov i sur., 2014b). Kako navode spomenuti autori, primjena HVED-a uzrokuje električni izboj u vodi te zajedno s različitim sekundarnim pojavama (kavitacija mjehurića, udarni valovi visoke amplitude, itd.) uzrokuje fragmentaciju čestica i oštećenje stanične stijenke. Uvjeti ekstrakcije, kao što su snaga visokog napona, ukupni unos energije, temperatura i vrijeme obrade, značajno utječu na iskorištenje. Uobičajena primjena HVED-a je za ekstrakciju, ali cilj ovog rada bio je ispitati primjenu HVED-a na zadržavanje bioaktivnih komponenti u kakaovoj ljusci. Pojavu većeg zadržavanja fenolnih komponenti u uzorcima koji su tretirani plazmom priopćilo je nekoliko autora (Muhammad i sur., 2018; Sarangapani i sur., 2017). Točni mehanizmi koji dovode do zadržavanja fenolnih komponenti nakon različitih tretmana električnim pražnjenjem još nisu razjašnjeni. Jedan od predloženih mehanizama je interakcija fenola s drugim sastojcima prisutnim u tretiranim materijalima (npr. vlaknima), ali potrebna su daljnja istraživanja kako bi se optimirali uvjeti obrade i osigurala minimalna degradacija bioaktivnih komponenti.

Kao što se može vidjeti u Tablici 2 i Slikama 10-12, značajno smanjenje udjela fenolnih komponenti i metilksantina postignuto je i u kontrolnim uzorcima i u uzorcima tretiranim HVED-om u usporebi s netretiranim uzorkom kakaove ljuske. Općenito, HVED tretman pri 40 Hz imao je manji utjecaj na udio bioaktivnih komponenti kada se usporedi s kontrolnim uzorcima i primjenom HVED-a pri 80 Hz, ali daljnja istraživanja trebala bi uključiti veći broj uzoraka kako bi se to potvrdilo. Najveći postotak zadržavanja među fenolnim komponentama dobiven je za (-)-epikatehin i (-)-epikatehin galat, dok je kafeinska kiselina određena samo u netretiranoj kakaovoj ljusci. Kao što se može vidjeti na Slici 11, 89 % i 78 % početne koncentracije (-)-epikatehin galata određeno je nakon HVED tretmana pri 40 Hz, tijekom 15 minuta i pri koncentraciji 3 %, odnosno 1,5 %.



Slika 10 Udio zadržanih metiksantina nakon različitih tretmana (UCS-netretirana kakaova ljuska; W-miješanje u vodi; HVED-visokonaponsko električno pražnjenje) (Barišić i sur., 2020b)

Tablica 3 Analiza varijance (ANOVA) (Barišić i sur., 2020b)

Varijabla	SS	DF	MS	F-vrijednost	*p*-vrijednost
Teobromin (TEO)					
Koncentracija (C)	2,08	1	2,08	36,3	0,0000
Miješanje (MT)	5,63	2	2,82	49,3	0,0000
Treatman (T)	0,90	2	0,45	7,9	0,0016
C*MT	0,44	2	0,22	3,8	0,0312
C*T	0,99	2	0,49	8,7	0,0009
MT*T	1,78	4	0,44	7,8	0,0001
C*MT*T	1,93	4	0,48	8,4	0,0001
Error	1,94	34	0,06		
Total	16,05	51			
Kafein (CAF)					
Koncentracija (C)	0,0619	1	0,0619	60,6	0,0000
Miješanje (MT)	0,1785	2	0,0892	87,3	0,0000
Treatman (T)	0,0517	2	0,0259	25,3	0,0000
C*MT	0,0103	2	0,0052	5,1	0,0120
C*T	0,0208	2	0,0104	10,2	0,0003
MT*T	0,0910	4	0,0227	22,3	0,0000
C*MT*T	0,0798	4	0,0199	19,5	0,0000
Error	0,0347	34	0,0010		
Total	0,5349	51			
(+)-Katehin (CAT)					
Koncentracija (C)	0,0034	1	0,0034	141,5	0,0000
Miješanje (MT)	0,0017	2	0,0008	34,4	0,0000
Treatman (T)	0,0012	2	0,0006	24,4	0,0000
C*MT	0,0005	2	0,0003	10,3	0,0003
C*T	0,0048	2	0,0024	98,5	0,0000
MT*T	0,0013	4	0,0003	13,0	0,0000
C*MT*T	0,0007	4	0,0002	7,2	0,0002
Error	0,0008	34	0,0000		
Total	0,0151	51			
(-)-Epikatehin (EPI)					
Koncentracija (C)	0,0023	1	0,0023	147,7	0,0000
Miješanje (MT)	0,0036	2	0,0018	113,7	0,0000
Treatman (T)	0,0033	2	0,0016	104,0	0,0000
C*MT	0,0001	2	0,0000	2,0	0,1532
C*T	0,0002	2	0,0001	6,0	0,0059
MT*T	0,0012	4	0,0003	19,3	0,0000
C*MT*T	0,0008	4	0,0002	12,8	0,0000
Error	0,0005	34	0,0000		
Total	0,0123	51			
(-)-Epikatehin galat (EPG)					
Koncentracija (C)	0,0000	1	0,0000	12,4	0,0012
Miješanje (MT)	0,0000	2	0,0000	81,6	0,0000
Treatman (T)	0,0000	2	0,0000	18,2	0,0000
C*MT	0,0000	2	0,0000	9,8	0,0004
C*T	0,0000	2	0,0000	25,5	0,0000
MT*T	0,0000	4	0,0000	15,2	0,0000
C*MT*T	0,0000	4	0,0000	4,4	0,0055
Error	0,0000	34	0,0000		
Total	0,0001	51			

Tablica 3 nastavak (Barišić i sur., 2020b)

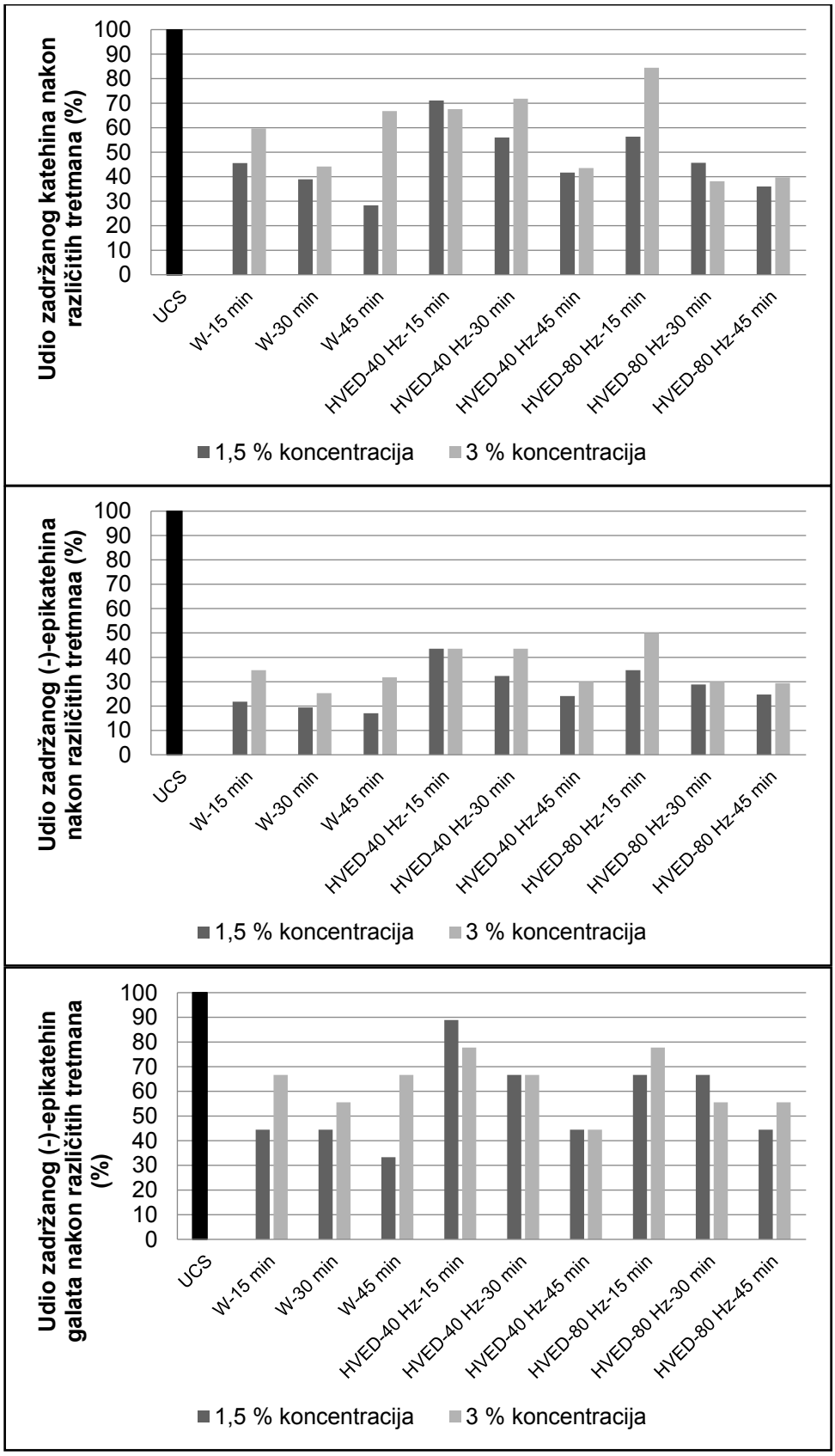
Varijabla	SS	DF	MS	F-vrijednost	*p*-vrijednost
Galna kiselina (GA)					
Koncentracija (C)	0,0006	1	0,0006	199,5	0,0000
Miješanje (MT)	0,0010	2	0,0005	164,5	0,0000
Treatman (T)	0,0001	2	0,0000	9,7	0,0005
C*MT	0,0001	2	0,0000	12,3	0,0001
C*T	0,0002	2	0,0001	36,5	0,0000
MT*T	0,0002	4	0,0000	13,6	0,0000
C*MT*T	0,0002	4	0,0000	13,3	0,0000
Error	0,0001	34	0,0000		
Total	0,0026	51			
***p*-Kumarinska kiselina (*p*-CA)**					
Koncentracija (C)	0,0005	1	0,0005	171,26	0,0000
Miješanje (MT)	0,0004	2	0,0002	64,81	0,0000
Treatman (T)	0,0001	2	0,0001	16,52	0,0000
C*MT	0,0001	2	0,0001	18,04	0,0000
C*T	0,0000	2	0,0000	6,83	0,0032
MT*T	0,0003	4	0,0001	23,08	0,0000
C*MT*T	0,0004	4	0,0001	29,40	0,0000
Error	0,0001	34	0,0000		
Total	0,0020	51			

SS: suma kvadrata; DF: stupnjevi slobode; MS: prosječna vrijednost kvadrata * $p<0,05$ statistička značajnost

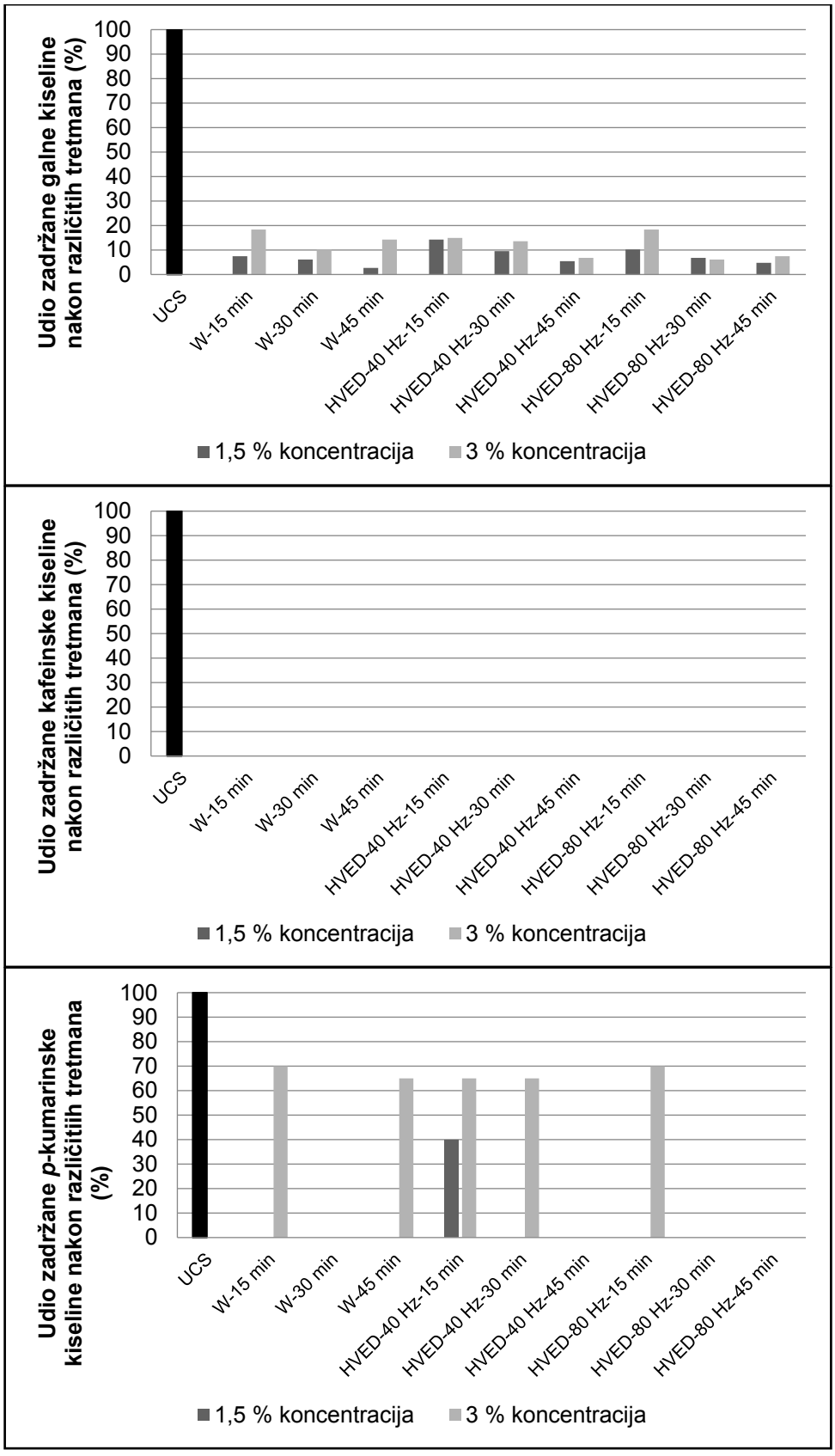
Glavne fenolne komponente u netretiranoj kakaovoj ljusci, (+)-katehin i galna kiselina, pokazale su najveće smanjenje udjela nakon tretmana (Slike 11 i 12). Nakon tretmana HVED-om udio galne kiseline bio je manji od 20 % u usporedbi s početnom koncentracijom, (+)-katehina manji od 30 % u usporedbi s početnom koncentracijom, a zabilježen je manji pad nakon tretmana pri koncentraciji 3 % nego pri 1,5 %. Koncentracija kakaove ljuske u otopini tijekom tretmana utjecala je i na zadržavanje *p*-kumarinske kiseline. Naime, *p*-kumarinska kiselina mogla se detektirati samo nakon tretmana pri koncentraciji 1,5 %, pri 40 Hz i tijekom 15 minuta, dok je za 3 %-tnu otopinu postotak zadržavanja *p*-kumarinske kiseline bio između 65 i 70 % nakon kraćih tretmana, bez obzira na vrstu tretmana (Slika 12). Teobromin i kafein bili su manje podložni utjecaju tretmana nego fenolne komponente (Slika 10). Najveći postotak zadržavanja (84 % za obje komponente) određen je u uzorcima tretiranim HVED-om pri 80 Hz tijekom 15 minuta.

Trend smanjenja udjela bioaktivnih komponenti pri duljem tretmanu uočen je samo kod uzoraka koji su tretirani pri koncentraciji 1,5 %. Razlike u dobivenim rezultatima, s obzirom na primijenjene uvjete tretiranja su statistički ispitane. Provedena je analiza varijance (ANOVA), a rezultati su prikazani u Tablici 3. Kao što se vidi u Tablici 3, koncentracija, vrijeme miješanja i primijenjeni tretman, kao i njihove kombinacije statistički su značajno utjecale na udio

bioaktivnih komponenti (*p*-vrijednost je bila niža od 0,05), osim za (-)-epikatehin, gdje kombinacija koncentracije i vremena miješanja (C × MT) nije bila statistički značajna (*p* = 0,1532). Statistički značajne razlike između uvjeta obrade i postotka zadržavanja analiziranih komponenti upućuju na mogući mehanizam interakcije fenola s ostalim komponentama hrane zbog čega bi bili manje skloni ekstrakciji u vodu uslijed veće molekularne mase, promjene električnog pražnjenja i veće veličine čestica.



Slika 11 Udio zadržanih katehina nakon različitih tretmana (UCS-netretirana kakaova ljuska; W-miješanje u vodi; HVED-visokonaponsko električno pražnjenje) (Barišić i sur., 2020b)



Slika 12 Udio zadržanih fenolnih kiselina nakon različitih tretmana (UCS-netretirana kakaova ljuska; W-miješanje u vodi; HVED-visokonaponsko električno pražnjenje) (Barišić i sur., 2020b)

4.2. UDIO AKRILAMIDA I 5-HIDROKSIMETILFURFURALA U KAKAOVOJ LJUSCI TRETIRANOJ VISOKONAPONSKIM ELEKTRIČNIM PRAŽNJENJEM

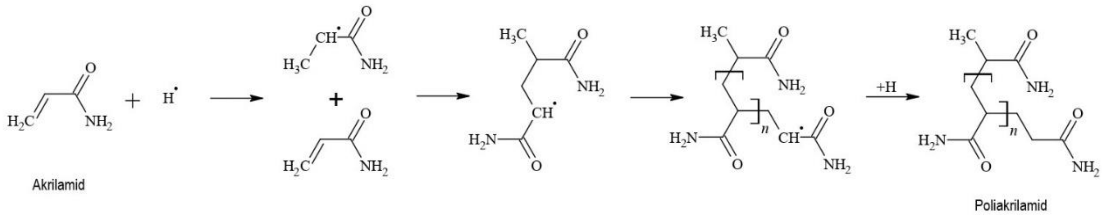
4.2.1. Akrilamid

Akrilamid nastaje tijekom Maillardovih reakcija iz slobodnih aminokiselina (uglavnom asparagina) i reducirajućih šećera. Kod hrane bogate lipidima (kakaovac sadrži oko 50 % kakaovog maslaca) također se navodi dodatni mehanizam putem akroleina i akrilne kiseline koji nastaju razgradnjom lipida na visokim temperaturama (Zyzelewicz i sur., 2017; Krishnakumar i Visvanathan, 2014). U netretiranoj kakaovoj ljusci utvrđen je udio akrilamida od 166 μg/kg (Tablica 4) što odgovara literaturnim podatcima za udio akrilamida u proizvodima od kakaovca (Krishnakumar i Visvanathan, 2014) i vrijednostima koje su poznate za kavu (200 μg/kg) i pekarske proizvode (112 μg/kg) (WHO i FAO, 2002). Udio akrilamida (koji nastaje tijekom prženja kakaovih zrna) bio je najveći u netretiranoj kakaovoj ljusci, što se može vidjeti u Tablici 4. Svi daljnji tretmani rezultirali su značajnim smanjenjem udjela akrilamida, u većini uzoraka udio je bio ispod granice kvantifikacije, a u nekima i ispod granice detekcije. Razlog za takvo smanjenje mogla bi biti njegova visoka topljivost u vodi (Farah i sur., 2012), pa tako većina zaostaje u vodi korištenoj tijekom tretmana ljuske. Utvrđen je statistički značajan utjecaj koncentracije, miješanja, tretmana, kombinacije koncentracije i tretmana te kombinacije sva tri parametra na udio akrilamida (Tablica 6). Glavna karakteristika HVED tretmana je da može narušiti staničnu stijenku i stoga se koristi kao metoda za ekstrakciju. Iz Tablice 4 može se zaključiti da, iako je sama voda (kontrolni uzorci) kao otapalo imala značajan utjecaj na smanjenje udjela akrilamida, HVED je bio učinkovitiji. To implicira da je akrilamid bilo puno lakše izdvojiti iz tretirane kakaove ljuske zbog narušavanja stanične stijenke i da bi HVED mogao biti dobra metoda za uklanjanje akrilamida. Međutim, za utvrđivanje ovog odnosa potrebna su daljnja istraživanja kako bi se provjerila prisutnost akrilamida u ekstraktu, te kako bi se utvrdio učinak na druge sastojke kakaove ljuske. Osim toga, Tablica 5 pokazuje da postoji obrnuti odnos između boje i akrilamida. Odnosno, kako se sadržaj akrilamida smanjivao, uzorci su postajali tamniji. To implicira da je dio akrilamida mogao biti izložen daljnjim reakcijama unutar kakaove ljuske. Akrilamid je mogao polimerizirati u poliakrilamid (Slika 13) pod utjecajem ultraljubičastog svjetla i oksidanasa (HVED). Postoje istraživanja koja istražuju mogućnost polimerizacije akrilamida ultraljubičastim svjetlom i električnim pražnjenjem (Capek, 2016; Novak i sur., 1995), ali ne i u preradi hrane. U hrani je zabilježena polimerizacija akrilamida pri visokim temperaturama (iznad 100 °C) i tijekom procesa kataliziranog bazom tijekom dugotrajnog skladištenja otopina (Adams i sur., 2010).

Tablica 4 Aktivitet vode, udio vode, 5-hidroksimetilfurfurala i akrilamida u netretiranoj i tretiranim kakaovim ljuskama (Barišić i sur., 2020c)

Uzorak	a_w	Udio vode (%)	5-HMF (mg/kg)	Akrilamid (µg/kg)
UCS	0,372	5,71 ± 0,31	133,34 ± 64,37	166,00 ± 8,92
1,5 %, 15 min	0,552	12,22 ± 0,00	12,02 ± 0,10	<LOQ
1,5 %, 30 min	0,570	12,62 ± 0,01	8,86 ± 0,04	25,50 ± 0,50
1,5 %, 45 min	0,579	13,52 ± 0,03	3,54 ± 0,05	28,00 ± 2,00
1,5 %, 15 min, 40 Hz	0,557	13,12 ± 0,02	8,34 ± 0,05	<LOQ
1,5 %, 30 min, 40 Hz	0,532	11,36 ± 0,11	10,22 ± 0,14	<LOQ
1,5 %, 45 min, 40 Hz	0,545	12,09 ± 0,01	9,29 ± 0,08	<LOQ
1,5 %, 15 min, 80 Hz	0,577	13,08 ± 0,02	11,47 ± 0,01	27,50 ± 0,50
1,5 %, 30 min, 80 Hz	0,547	12,28 ± 0,08	10,18 ± 0,16	<LOQ
1,5 %, 45 min, 80 Hz	0,567	12,57 ± 0,07	5,91 ± 0,13	<LOQ
3 %, 15 min	0,559	14,06 ± 0,07	36,94 ± 0,12	21,50 ± 0,50
3 %, 30 min	0,561	14,03 ± 0,04	24,99 ± 0,10	<LOQ
3 %, 45 min	0,572	15,03 ± 0,05	25,32 ± 0,29	<LOD
3 %, 15 min, 40 Hz	0,580	12,78 ± 0,03	25,56 ± 0,16	<LOQ
3 %, 30 min, 40 Hz	0,591	13,23 ± 0,02	24,50 ± 0,24	<LOQ
3 %, 45 min, 40 Hz	0,579	13,16 ± 0,08	24,59 ± 0,09	<LOQ
3 %, 15 min, 80 Hz	0,597	12,80 ± 0,01	32,75 ± 0,08	<LOQ
3 %, 30 min, 80 Hz	0,583	13,75 ± 0,00	25,61 ± 0,22	<LOD
3 %, 45 min, 80 Hz	0,780	13,84 ± 0,02	22,05 ± 0,22	<LOD

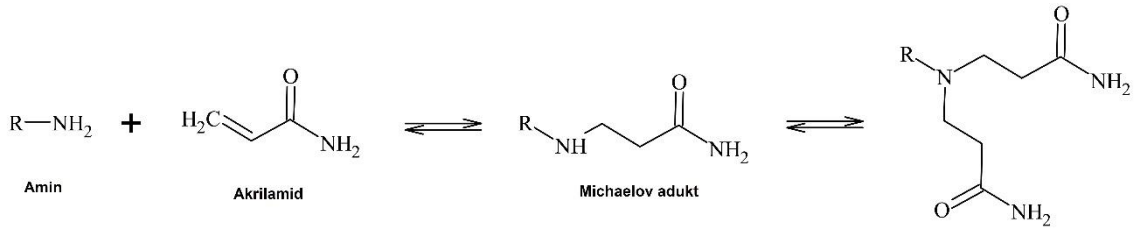
UCS: netretirana kakaova ljuska; a_w: aktivitet vode; 5-HMF: 5-hidroksimetilfurfural; standardna devijacija (n=2); LOQ: 20 µg/kg; LOD: 10 µg/kg



Slika 13 Polimerizacija akrilamida (Barišić i sur., 2020c)

Druga reakcija koja se mogla dogoditi tijekom HVED tretmana je Michaelova adicija, gdje je akrilamid mogao reagirati kao Michaelov akceptor, a slobodne aminokiseline kao Michaelovi donori (Slika 14). Predložena hipoteza može se djelomično objasniti sljedećim:

- Reakciju kataliziraju alkalni uvjeti (Adams i sur., 2010), a i Li i sur. (2019) su zabilježili povećanje pH vode nakon tretmana HVED-om.
- Slobodne aminokiseline reagiraju s akrilamidom u modelskim sustavima stvarajući Michaelov adukt (Adams i sur., 2010; Zamora i sur., 2010), a kakaova zrna sadrže dovoljne količine slobodnih aminokiselina (leucin, valin, alanin, izoleucin, fenilalanin) (Beckett i sur., 2017). Adeyeye i sur. (2014) izvijestili su da, između ostalog, kakaova ljuska sadrži glicin, metionin i lizin, za koje je dokazano da međusobno reagiraju s akrilamidom. Iako se aminokiseline smatraju glavnim prekursorima arome u kakaovcu, samo 25 % njih će reagirati i sudjelovati u razvoju arome (Beckett i sur., 2017). Dio njih sudjelovat će u Maillardovim reakcijama i dati Amadorijeve spojeve, no postoji mogućnost da će dio njih stupiti u interakciju s akrilamidom.
- Reakcija je reverzibilna iako je energija aktivacije veća za povratnu reakciju (Adams i sur., 2010) što bi moglo objasniti fenomen uočen u ovom istraživanju – za uzorke tretirane vodom u 1,5 % suspenziji, produljenje tretmana povećava količinu akrilamida otkrivenog u ljusci od <LOQ za tretman od 15 min, do 25,5 µg/kg i 28,0 µg/kg tijekom 30 min, odnosno 45 min.
- Tretman u ovom istraživanju proveden je u vodenim suspenzijama, a Adams i sur. (2010) navode da vodeni medij pogoduje smanjenju sadržaja akrilamida Michaelovom adicijom.



Slika 14 Michaelova adicija (Barišić i sur., 2020c)

U prženoj kavi je u novijim istraživanjima zabilježena prisutnost melanoidina (Oracz i Nebesny, 2019; Pastoriza i sur., 2012), a samim time postoji mogućnost da bi ovi konačni produkti Maillardovih reakcija mogli nastati i u kakaovim zrnima (Barišić i sur., 2019). Ako je to točno, melanoidini također mogu djelovati kao Michaelovi donori u reakciji s akrilamidom (Pastoriza i

sur., 2012) tijekom HVED tretmana. Takva reakcija akrilamida s melanoidinima bi zapravo bila razlog promjene boje i potamnjivanja uzoraka (Tablica 7).

Tablica 5 Pearsonovi koeficijenti korelacije (Barišić i sur., 2020c)

Varijabla	L	a*	b*	C	h°	∆E	5-HMF	AA	a_w	Udio vode
L	1,000									
a*	0,141	1,000								
b*	0,455	**0,897**	1,000							
C	0,421	**0,921**	**0,998**	1,000						
h°	**0,619**	**0,707**	**0,945**	**0,926**	1,000					
∆E	**-0,968**	-0,372	**-0,661**	**-0,632**	**-0,779**	1,000				
5-HMF	0,196	**0,670**	**0,708**	**0,711**	**0,616**	-0,360	1,000			
AA	**0,475**	**0,507**	**0,630**	**0,622**	**0,601**	**-0,575**	**0,881**	1,000		
a_w	**-0,558**	**-0,535**	**-0,665**	**-0,655**	**-0,670**	**0,638**	**-0,577**	**-0,668**	1,000	
Udio vode	**-0,736**	**-0,469**	**-0,661**	**-0,644**	**-0,692**	**0,805**	**-0,757**	**-0,843**	**0,723**	1,000

∆E: ukupna promjena boje; 5-HMF: 5-hidroksimetilfurfural; AA: akrilamid; a_w: aktivitet vode

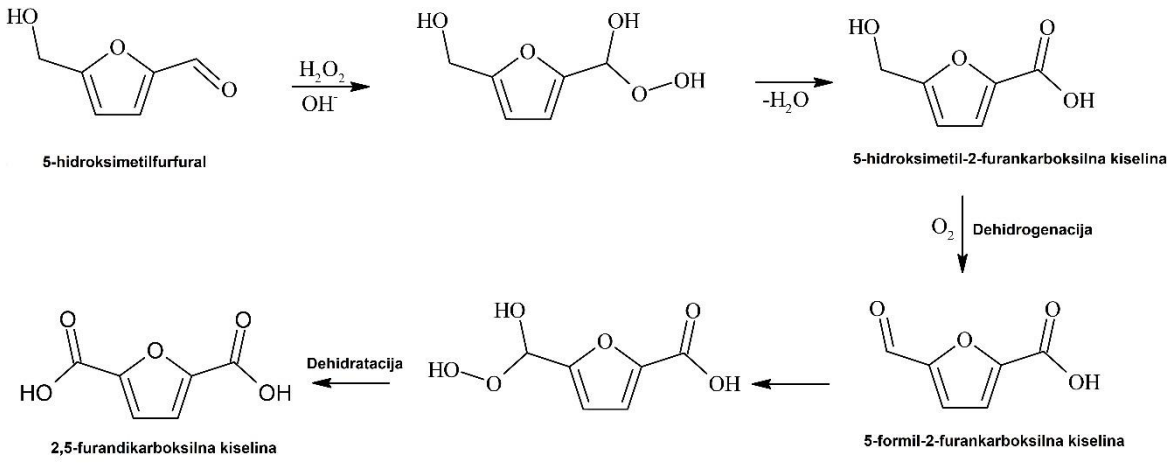
Hrana i nusproizvodi koji nastaju preradom hrane imaju složen sastav koji uključuje različite makro i mikro-molekule koje mogu međusobno reagirati i stvarati kompleksan sustav. Primjena električnog polja u takvom sustavu dovodi do povećanja energije u česticama što dovodi do stvaranja vibracija, pobuđivanja, vezanja, disocijacije i ionizacije. Različiti ioni mogu biti uključeni u reakcijske mehanizme kao što su ion-ion (neutralizacija), ion-molekula, Penning ionizacija, itd. Također, mogu se potaknuti i različite fotokatalizirane reakcije: emisija, apsorpcija i ionizacija (Misra i sur., 2016). Međutim, budući da se reakcija provodila u otapalu, neke reakcije su se mogle dogoditi i zbog međudjelovanja vode, komponenti hrane i HVED-a (Gavahian i sur., 2018). Zbog svega navedenog vrlo je teško utvrditi stvarno nastale mehanizme u tako složenim sustavima.

Pérez-Andrés i sur. (2018) u svom radu navode da hladna plazma može potaknuti oksidaciju lipida i proteina i promjene sekundarne strukture proteina, kao i uzrokovati inhibiciju enzima. Promjene u strukturi i konformaciji proteina utječu na njihovu reaktivnost, uključujući reaktivnost asparagina tijekom stvaranja akrilamida. Kalum i Hendriksen (2016) izvijestili su o smanjenju stvaranja akrilamida u prženom krumpiru nakon što su uzorci bili obrađeni pulsirajućim električnim poljem (Dourado i sur., 2019).

4.2.2. 5-Hidroksimetilfurfural

5-hidroksimetilfurfural (5-HMF) također nastaje tijekom prženja Maillardovom reakcijom. Tablica 4 pokazuje da je netretirana kakaova ljuska imala najveću koncentraciju 5-HMF. Svi primijenjeni tretmani i njihove kombinacije rezultirali su značajnim smanjenjem koncentracije 5-HMF-a u kakaovoj ljusci. Najznačajnije smanjenje bilo je u uzorcima koji su obrađeni samo u vodi, vjerojatno zato što 5-HMF prelazi u vodu zbog svoje visoke topljivosti u vodi (Gomes i sur., 2015). To je potkrijepljeno činjenicom da je značajnije smanjenje koncentracije 5-HMF uočeno u koncentracijama suspenzije od 1,5 %, jer bi u takvim uvjetima više 5-HMF-a moralo prijeći u vodu da bi se postigla ravnoteža. Nadalje, uzorak s najnižom koncentracijom 5-HMF-a (3,54 ± 0,05 mg/kg) tretiran je u vodi 45 minuta pri koncentraciji 1,5 %. U uzorcima tretiranim HVED-om, smanjenje koncentracije 5-HMF-a se dogodilo u manjoj mjeri.

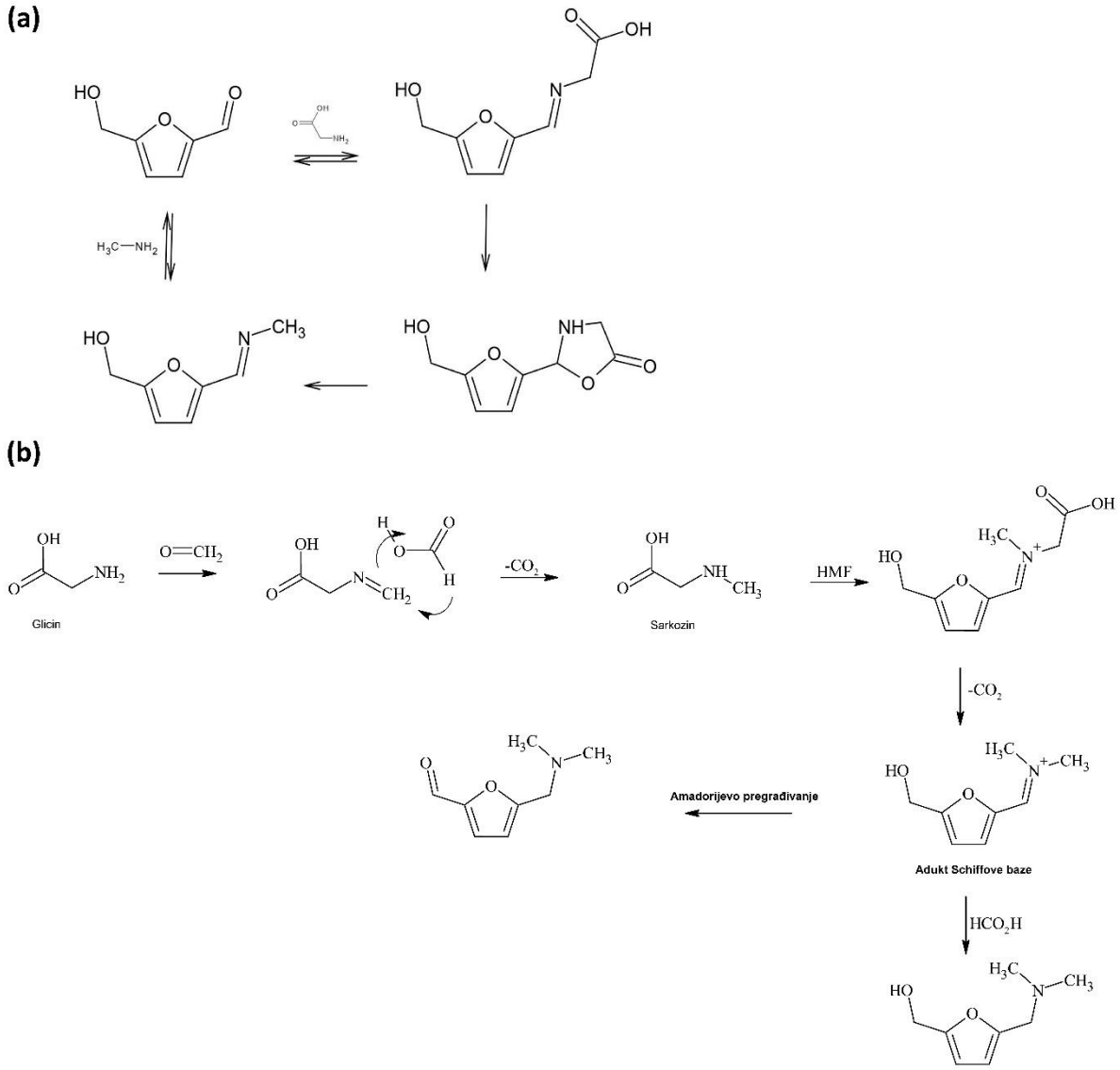
Iako je ovo istraživanje pokazalo smanjenje sadržaja 5-HMF-a u kakaovoj ljusci, Jokić i sur. (2019) u svom istraživanju nisu zabilježili prisutnost 5-HMF-a u ekstraktu kakaove ljuske dobivenom tretmanom HVED-om. Zajedno sa smanjenjem L* vrijednosti u ovom istraživanju (Tablica 7) koje pokazuje potamnjivanje uzoraka nakon tretmana, ovo može ukazivati na nastavak Maillardove reakcije i pretvorbu 5-HMF-a u daljnje Maillardove produkte izazvane HVED-om. Budući da je HVED oksidacijska metoda, može rezultirati pretvorbom 5-HMF-a u 2,5-furandikarboksilnu kiselinu (FDCA) u vodenom mediju (Slika 15). FDCA je vrlo stabilna komponenta koja je slabo topiva u većini uobičajenih otapala (Lewkowski, 2001). U posljednje vrijeme mnogo istraživanja se bavi pretvorbom 5-HMF-a u FDCA jer je FDCA obećavajuća zamjena za tereftalat u poliesterima (Koopman i sur., 2010).



Slika 15 Reakcija stvaranja FDCA (2,5-furandikarboksilne kiseline) iz 5-HMF-a (5-hidroksimetilfurfurala) (Barišić i sur., 2020c)

Osim toga, 5-HMF može sudjelovati i u reakcijama s aminokiselinama u uznapredovalim Maillardovim reakcijama (Slika 16a), a zabilježeno je i da može tvoriti Shiffovu bazu s glicinom (Slika 16b) (Nikolov i Yaylayan, 2011). Iako se one smatraju termički induciranim reakcijama, energija oslobođena HVED tretmanom također bi ih mogla potaknuti. Rezultati analize sadržaja 5-HMF-a pokazali su statistički značajan utjecaj koncentracije, miješanja, tretmana, kombinacije koncentracije i tretmana te kombinacije sva tri parametra (Tablica 6).

U budućim istraživanjima potrebno je istražiti mogućnost korištenja HVED-a u FDCA sintezi. Ova metoda ima potencijal postati novom "zelenom" tehnikom za uklanjanje visokih koncentracija 5-HMF iz hrane, ali i za proizvodnju FDCA koji se sve više koristi u industriji.



Slika 16 a) Reakcija 5-hidroksimetilfurfurala s aminokiselinama b) Formacija Schiffove baze 5-hidroksimetilfurfurala s glicinom (Barišić i sur., 2020c)

Tablica 6 Faktorska analiza varijance (Barišić i sur., 2020c)

		SS	DF	MS	F-vrijednost	*p*-vrijednost
L*	Intercept	177855,1	1	177855,1	174272844	<0,001*
	Koncentracija (C)	157,3	1	157,3	154098	<0,001*
	Miješanje (M)	11,0	2	5,5	5369	<0,001*
	Tretman (T)	14,9	2	7,4	7289	<0,001*
	C*M	4,0	2	2,0	1957	<0,001*
	C*T	14,9	2	7,5	7320	<0,001*
	M*T	3,9	4	1,0	950	<0,001*
	C*M*T	8,4	4	2,1	2057	<0,001*
	Error	0,1	72	0,0		
a*	Intercept	6896,751	1	6896,751	1960542	<0,001*
	Koncentracija (C)	0,166	1	0,166	47	<0,001*
	Miješanje (M)	0,282	2	0,141	40	<0,001*
	Tretman (T)	0,248	2	0,124	35	<0,001*
	C*M	0,018	2	0,009	3	<0,001*
	C*T	0,769	2	0,385	109	<0,001*
	M*T	0,110	4	0,027	8	<0,001*
	C*M*T	0,339	4	0,085	24	<0,001*
	Error	0,253	72	0,004		
b*	Intercept	22379,84	1	22379,84	11806480	<0,001*
	Koncentracija (C)	0,05	1	0,05	29	<0,001*
	Miješanje (M)	6,62	2	3,31	1747	<0,001*
	Tretman (T)	3,43	2	1,72	905	<0,001*
	C*M	0,45	2	0,23	120	<0,001*
	C*T	3,27	2	1,63	862	<0,001*
	M*T	0,56	4	0,14	74	<0,001*
	C*M*T	9,08	4	2,27	1198	<0,001*
	Error	0,14	72	0,00		
C	Intercept	29278,92	1	29278,92	40415688	<0,001*
	Koncentracija (C)	0,16	1	0,16	224	<0,001*
	Miješanje (M)	6,28	2	3,14	4336	<0,001*
	Tretman (T)	3,49	2	1,74	2406	<0,001*
	C*M	0,42	2	0,21	287	<0,001*
	C*T	3,91	2	1,95	2698	<0,001*
	M*T	0,63	4	0,16	218	<0,001*
	C*M*T	8,37	4	2,09	2888	<0,001*
	Error	0,05	72	0,00		
h°	Intercept	334297,3	1	334297,3	8314246	<0,001*
	Koncentracija (C)	0,5	1	0,5	13	<0,001*
	Miješanje (M)	6,3	2	3,1	78	<0,001*
	Tretman (T)	2,4	2	1,2	30	<0,001*
	C*M	0,5	2	0,3	6	<0,001*
	C*T	1,3	2	0,6	16	<0,001*
	M*T	0,5	4	0,1	3	0,025837*
	C*M*T	10,5	4	2,6	65	<0,001*
	Error	2,9	72	0,0		

Tablica 6 nastavak (Barišić i sur., 2020c)

		SS	DF	MS	F-vrijednost	*p*-vrijednost
∆**E**	Intercept	1576,425	1	1576,425	990799,2	<0,001*
	Koncentracija (C)	99,747	1	99,747	62692,3	<0,001*
	Miješanje (M)	17,060	2	8,530	5361,1	<0,001*
	Tretman (T)	22,596	2	11,298	7100,9	<0,001*
	C*M	4,121	2	2,061	1295,1	<0,001*
	C*T	7,049	2	3,525	2215,3	<0,001*
	M*T	1,169	4	0,292	183,7	<0,001*
	C*M*T	15,719	4	3,930	2469,8	<0,001*
	Error	0,115	72	0,002		
Udio vode (%)	Intercept	6164,344	1	6164,334	1242533	<0,001*
	Koncentracija (C)	10,802	1	10,802	2177	<0,001*
	Miješanje (M)	1,543	2	0,772	156	<0,001*
	Tretman (T)	5,482	2	2,741	553	<0,001*
	C*M	2,229	2	1,115	225	<0,001*
	C*T	1,110	2	0,555	112	<0,001*
	M*T	2,411	4	0,603	121	<0,001*
	C*M*T	2,217	4	0,554	112	<0,001*
	Error	0,089	18	0,005		
Akrilamid	Intercept	2584,028	1	2584,028	70,63402	<0,001*
	Koncentracija (C)	521,361	1	521,361	14,25133	0,001386*
	Miješanje (M)	737,556	2	368,778	10,08049	0,001156*
	Tretman (T)	977,056	2	488,528	13,35383	<0,001*
	C*M	14,222	2	7,111	0,19438	0,825050
	C*T	447,722	2	223,861	6,11921	0,009385*
	M*T	402,611	4	100,653	2,75133	0,060265
	C*M*T	983,944	4	245,986	6,72399	0,001711*
	Error	658,500	18	36,583		
5-HMF	Intercept	11528,92	1	11528,92	261974,1	<0,001*
	Koncentracija (C)	2933,97	1	2933,97	66669,2	<0,001*
	Miješanje (M)	224,91	2	112,45	2555,3	<0,001*
	Tretman (T)	14,16	2	7,08	160,9	<0,001*
	C*M	51,92	2	25,96	589,9	<0,001*
	C*T	43,76	2	21,88	497,2	<0,001*
	M*T	126,62	4	31,65	719,3	<0,001*
	C*M*T	12,55	4	3,14	71,3	<0,001*
	Error	0,79	18	0,04		

SS: suma kvadrata; DF: stupnjevi slobode; MS: prosječna vrijednost kvadrata *p<0,05 statistička značajnost; 5-HMF: 5-hidroksimetilfurfural

4.2.3. Boja

Promjena boje tretiranih uzoraka prikazana je u Tablici 7. Smanjenje L* vrijednosti ukazuje da je kod svih tretiranih uzoraka došlo do potamnjivanja. Takav učinak može se pripisati sušenju uzoraka tijekom kojeg je moglo doći do Maillardovih reakcija (Lario i sur., 2004). Yaylayan (2003) je zabilježio da Amadorijevo pregrađivanje aminokiselina s α-hidroksikarbonilnim spojevima može voditi Maillardovu reakciju uglavnom prema kromogenim putovima i stvaranju melanoidina. Drugi razlog potamnjivanja uzoraka može biti povećana vlaga i a_w u usporedbi s netretiranim uzorkom (Tablica 4). Naime, prema Romanu i sur. (2010) povećana vlaga u uzorcima uzrokuje bolje širenje svjetlosti u uzorku što rezultira manjom refleksijom. Veća ukupna promjena boje (ΔE) i veće smanjenje L* vrijednosti zabilježeno je u uzorcima tretiranim vodom u usporedbi s uzorcima tretiranim HVED-om, što je u korelaciji s a_w i sadržajem vode u uzorcima. Međutim, dio proteina mogao bi se ekstrahirati tijekom HVED tretmana, smanjujući opseg Maillardovih reakcija i potamnjivanje uzoraka (Parniakov i sur., 2014b; Sarkis i sur., 2015). Primjetan je i blagi pad vrijednosti a*, b*, C i h° u svim obrađenim uzorcima. Pozitivne vrijednosti a* i b* ukazuju da su svi uzorci u domeni crvene i žute boje. Statistička analiza pokazala je da postoji statistički značajan utjecaj svih parametara i njihovih kombinacija na vrijednosti L*, a* b*, C, h° i ΔE (Tablica 6).

Tablica 7 Boja samljevenih uzoraka kakaove ljuske (Barišić i sur., 2020c)

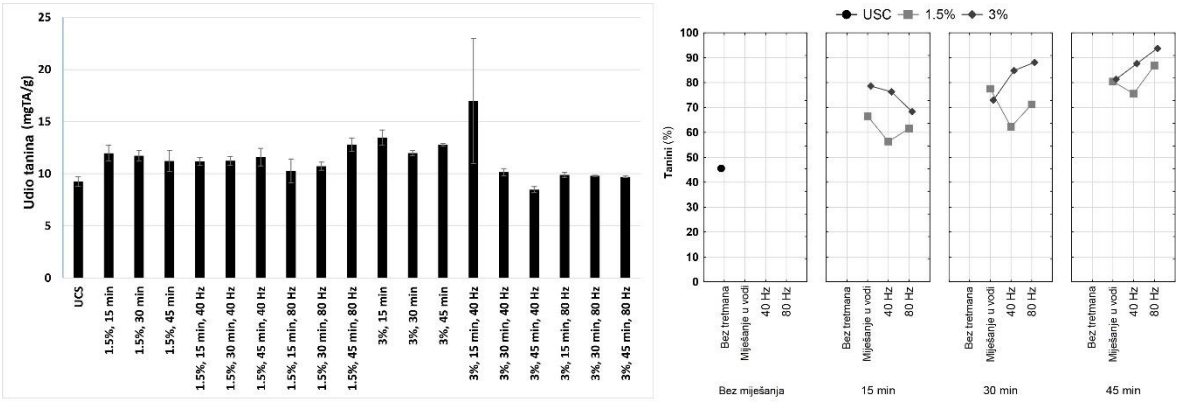
Uzorak	L*	a*	b*	C	h°	ΔE
UCS	47,856 ± 3,08	9,23 ± 0,44	17,98 ± 0,08	20,21 ± 0,13	62,84 ± 1,20	
1,5 %, 15 min	46,51 ± 0,02	8,51 ± 0,08	15,48 ± 0,07	17,66 ± 0,03	61,19 ± 0,32	2,93 ± 0,05
1,5 %, 30 min	45,76 ± 0,02	8,59 ± 0,12	15,44 ± 0,06	17,67 ± 0,02	60,77 ± 0,25	3,36 ± 0,03
1,5 %, 45 min	45,27 ± 0,01	8,45 ± 0,03	15,02 ± 0,04	17,24 ± 0,03	60,64 ± 0,14	4,01 ± 0,03
1,5 %, 15 min, 40 Hz	46,06 ± 0,03	9,00 ± 0,06	16,97 ± 0,02	19,22 ± 0,01	62,07 ± 0,20	2,08 ± 0,03
1,5 %, 30 min, 40 Hz	45,71 ± 0,02	8,70 ± 0,04	15,45 ± 0,03	17,73 ± 0,03	60,64 ± 0,15	3,36 ± 0,03
1,5 %, 45 min, 40 Hz	45,79 ± 0,04	8,77 ± 0,04	15,58 ± 0,04	17,88 ± 0,02	60,62 ± 0,15	3,20 ± 0,05
1,5 %, 15 min, 80 Hz	45,26 ± 0,03	8,80 ± 0,05	15,65 ± 0,03	17,96 ± 0,02	60,64 ± 0,17	3,51 ± 0,03
1,5 %, 30 min, 80 Hz	46,15 ± 0,02	8,74 ± 0,04	15,94 ± 0,02	18,18 ± 0,01	61,27 ± 0,13	2,71 ± 0,02
1,5 %, 45 min, 80 Hz	45,47 ± 0,02	8,83 ± 0,02	16,16 ± 0,03	18,42 ± 0,01	61,35 ± 0,08	3,03 ± 0,02
3 %, 15 min	43,12 ± 0,02	8,89 ± 0,02	16,21 ± 0,03	18,49 ± 0,03	61,25 ± 0,04	5,07 ± 0,03
3 %, 30 min	41,73 ± 0,06	8,79 ± 0,04	15,60 ± 0,05	17,91 ± 0,02	60,62 ± 0,18	6,59 ± 0,07
3 %, 45 min	41,53 ± 0,02	8,91 ± 0,04	15,65 ± 0,03	18,01 ± 0,04	60,35 ± 0,10	6,75 ± 0,03
3 %, 15 min, 40 Hz	44,13 ± 0,03	8,88 ± 0,05	16,14 ± 0,03	18,42 ± 0,02	61,19 ± 0,18	4,17 ± 0,04
3 %, 30 min, 40 Hz	44,45 ± 0,03	8,85 ± 0,03	16,21 ± 0,03	18,47 ± 0,02	61,36 ± 0,10	3,86 ± 0,04
3 %, 45 min, 40 Hz	43,73 ± 0,03	8,72 ± 0,03	15,85 ± 0,05	18,09 ± 0,03	61,18 ± 0,15	4,67 ± 0,04
3 %, 15 min, 80 Hz	44,42 ± 0,02	8,91 ± 0,05	16,44 ± 0,05	18,70 ± 0,03	61,53 ± 0,18	3,78 ± 0,03
3 %, 30 min, 80 Hz	42,40 ± 0,02	8,63 ± 0,05	15,15 ± 0,03	17,44 ±0,01	60,32 ± 0,18	6,18 ± 0,03
3 %, 45 min, 80 Hz	42,67 ± 0,01	8,59 ± 0,07	14,90 ± 0,04	17,20 ± 0,01	60,04 ± 0,28	6,07 ± 0,02

UCS: netretirana kakaova ljuska; ΔE: ukupna promjena boje; ± standardna devijacija (n=5)

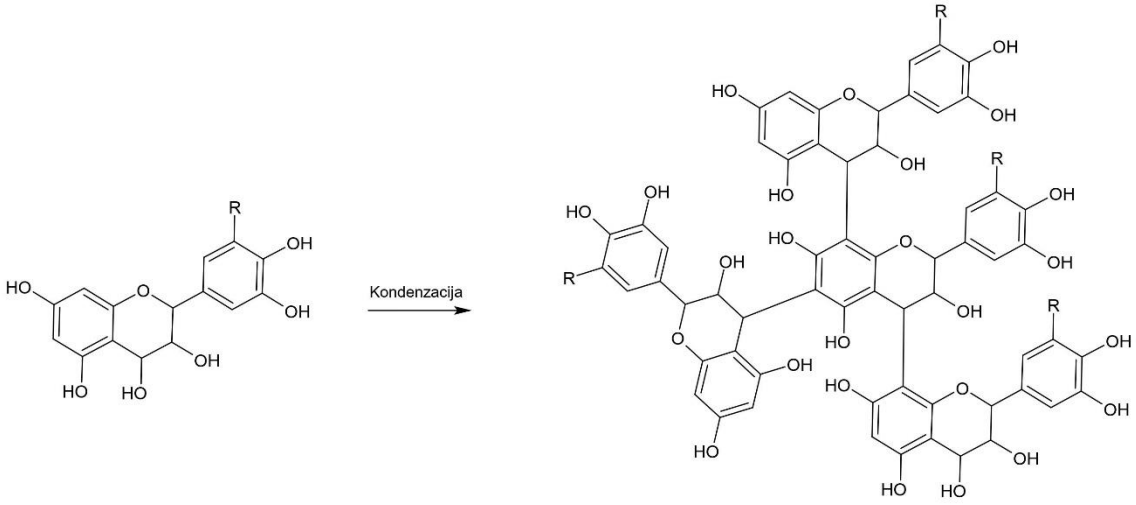
4.3. INDUCIRA LI TRETMAN VISOKONAPONSKIM ELEKTRIČNIM PRAŽNJENJEM PROMJENE U SVOJSTVIMA TANINA I VLAKANA KAKAOVE LJUSKE

4.3.1. Udio tanina

Sadržaj tanina u netretiranoj kakaovoj ljusci i tretiranim uzorcima prikazan je na Slici 17, gdje su prikazani rezultati za udio tanina (mg TA/g odmašćenog uzorka) i postotke tanina u ukupnim fenolima (%). Vidi se da je netretirana ljuska imala najmanji udio tanina, a sadržaj tanina se povećavao sa svim tretmanima. U uzorcima tretiranim HVED-om udio tanina u ukupnim fenolima kretao se od 45,03 do 65,09 %. U prethodno prikazanim rezultatima (Tablica 2; Barišić i sur. 2020b) utvrđeno je smanjenje sadržaja svih glavnih fenolnih spojeva u kakaovoj ljusci tretiranoj HVED-om (katehina, epikatehina, epikatehin galata, galne kiseline, kafeinske kiseline i *p*-kumarinske kiseline). Ove komponente se mogu ekstrahirati vodom, a smanjenje udjela ovih spojeva u tretiranoj ljusci može biti posljedica ekstrakcije (kako su utvrdili Jokić i sur. 2019), međutim, polifenoli su također skloni reakcijama kondenzacije u prikladnim uvjetima (Slika 18). Budući da cilj ovog istraživanja nije bio utvrditi učinak HVED tretmana na ekstrakciju bioaktivnih spojeva, kakaova ljuska prije tretmana nije mljevena, za razliku od istraživanja Jokić i sur. (2019). Iskorištenje ekstrakcije također ovisi o intenzitetu električnog polja, kontaktnoj površini između materijala i otapala, omjeru tekućine i materijala itd. S obzirom na navedeno, uvjeti tijekom HVED tretmana primijenjeni u ovom istraživanju nisu povoljni za ekstrakciju bioaktivnih komponenti (Li i sur., 2019). Zbog toga je ekstrakcija polifenolnih spojeva bila otežana. HVED stvara različite reaktivne spojeve koji su mogli izazvati polimerizaciju. Dakle, HVED je izvor radikala koji mogu lako oksidirati tanine, što dovodi do povećanja njihove rigidnosti. Suprotno dobivenim rezultatima, Delsart i sur. (2015) i Lukić i sur. (2019) su zabilježili smanjenje ukupnog sadržaja tanina u crnom vinu tretiranom HVED-om i hladnom plazmom, pripisujući ga oksidaciji tanina tijekom tretmana.



Slika 17 Udio tanina (izražen na masu odmašćenog uzorka) u kakaovoj ljusci prije i nakon tretmana HVED-om i postotak tanina u ukupnim fenolima (UCS, netretirana kakaova ljuska) (Barišić i sur., 2020d)



Slika 18 Kondenzacija polifenola (Barišić i sur., 2020d)

Međutim, treba imati na umu da se tretman hladnom plazmom i HVED-om razlikuje po tome što hladna plazma uključuje uvođenje plina u tekućinu, te da postoje velike razlike u kemijskom sastavu, uglavnom polifenolnom profilu, tretiranih uzoraka. Osim toga, budući da je vrijeme tretmana u ovom istraživanju bilo znatno duže, oksidirani tanini i drugi fenoli mogli su biti uključeni u međusobne reakcije, uglavnom zato što su oksidirani fenoli hidrofobni. Također, zabilježeno je da se hidrofobne reakcije mogu pojaviti među polifenolima i izazvati njihovu agregaciju (Guyot i sur., 2008).

Rezultati udjela tanina, izraženih kako u uzorku tako i u ukupnim fenolima, pokazuju da su tanini vrlo otporni na HVED tretman. Iznimku predstavlja uzorak tretiran pri koncentraciji 3,0 % i pri 40 Hz, gdje je došlo do značajnog smanjenja sadržaja tanina s povećanjem vremena

tretmana. Kod ostalih tretmana je uočeno blago povećanje njihovog sadržaja (Slika 17), vjerojatno zbog oksidacije i agregacije tanina, ali i zbog gubitka dijela topivih tvari tijekom tretmana, što je dovelo do promjene omjera komponenti u uzorcima.

Tablica 8 Faktorska analiza varijance (Barišić i sur., 2020d)

		SS	DF	MS	F-vrijednost	*p*-vrijednost
OBC (g/g)	Intercept	392,4578	1	392,4578	533,103,9	<0,001 *
	Koncentracija (C)	0,2885	1	0,2885	391,8	<0,001 *
	Miješanje (M)	0,1274	2	0,0637	86,5	<0,001 *
	Tretman (T)	0,1614	2	0,0807	109,6	<0,001 *
	C*M	0,0200	2	0,0100	13,6	<0,001 *
	C*T	0,0474	2	0,0237	32,2	<0,001 *
	M*T	0,0516	4	0,0129	17,5	<0,001 *
	C*M*T	0,0821	4	0,0205	27,9	<0,001 *
	Error	0,0133	18	0,0007		
WBC (g/g)	Intercept	2384,278	1	2384,278	313,752,4	<0,001 *
	Koncentracija (C)	15,149	1	15,149	1993,6	<0,001 *
	Miješanje (M)	3,762	2	1,881	247,6	<0,001 *
	Tretman (T)	0,040	2	0,020	2,6	0,101638
	C*M	0,130	2	0,065	8,5	0,002470 *
	C*T	0,177	2	0,088	11,6	<0,001 *
	M*T	0,266	4	0,066	8,8	<0,001 *
	C*M*T	0,516	4	0,129	17,0	<0,001 *
	Error	0,137	18	0,008		
Tanini (mg TA/g odmašćenog uzorka)	Intercept	4714,410	1	4714,410	1021,198	<0,001 *
	Koncentracija (C)	0,030	1	0,030	0,006	0,936726
	Miješanje (M)	13,343	2	6,671	1,445	0,261805
	Tretman (T)	17,066	2	8,533	1,848	0,186168
	C*M	24,240	2	12,120	2,625	0,099895
	C*T	10,946	2	5,473	1,186	0,328333
	M*T	29,441	4	7,360	1,594	0,218875
	C*M*T	24,668	4	6,167	1,336	0,294923
	Error	83,098	18	4,617		
Tanini (% ukupnih polifenola)	Intercept	114,239,9	1	114,239,9	3614,463	<0,001 *
	Koncentracija (C)	202,5	1	202,5	6,406	0,020914 *
	Miješanje (M)	453,1	2	226,5	7,167	0,005134 *
	Tretman (T)	84,5	2	42,3	1,337	0,287391
	C*M	75,8	2	37,9	1,200	0,324306
	C*T	49,7	2	24,9	0,786	0,470508
	M*T	146,0	4	36,5	1,155	0,363192
	C*M*T	102,3	4	25,6	0,809	0,535434
	Error	568,9	18	31,6		

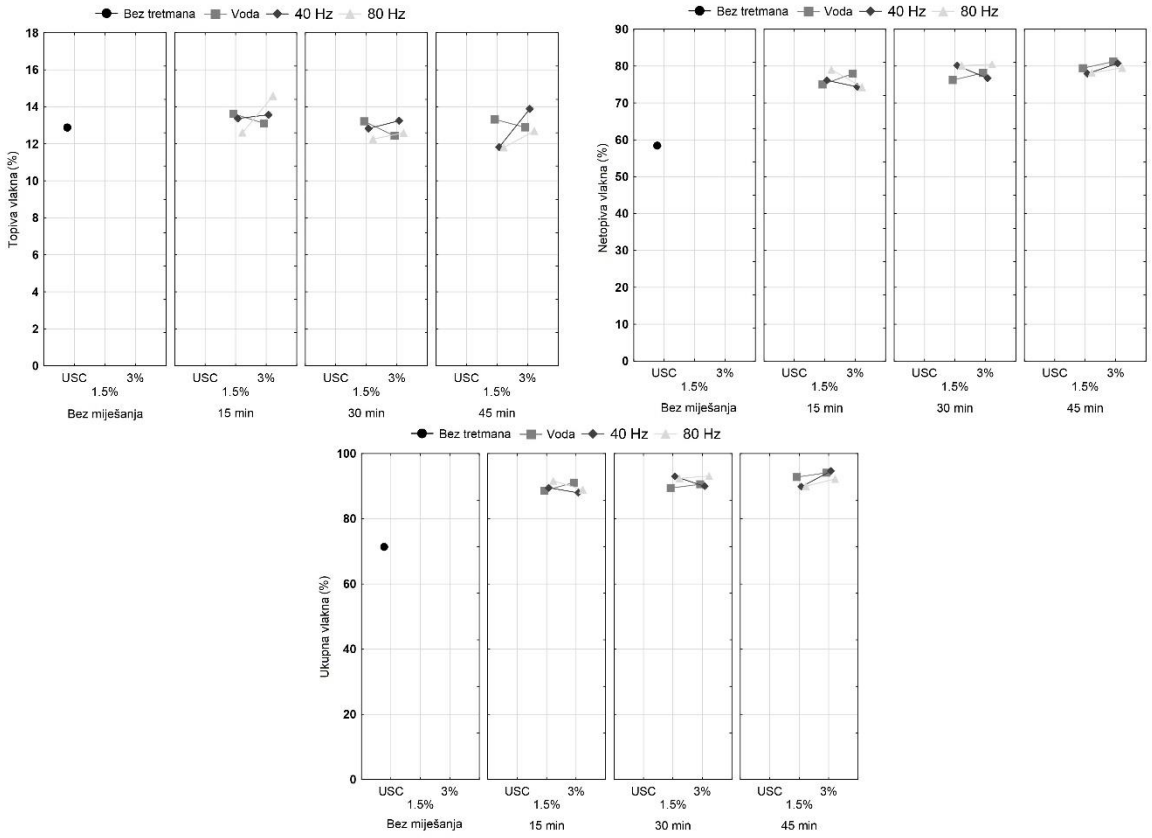
OBC: kapacitet vezanja ulja; WBC: kapacitet vezanja vode; SS: suma kvadrata; DF: stupnjevi slobode; MS: prosječna vrijednost kvadrata * p<0,05 statistička značajnost

Statistička analiza potvrdila je da su tanini vrlo otporni na HVED tretman, budući da nije utvrđena statistička značajnost. Nadalje, faktorska analiza varijance pokazala je da je utjecaj koncentracije i vremena miješanja na postotak tanina u ukupnim fenolima statistički značajan

(Tablica 8). Koeficijent korelacije pokazuje da je udio tanina (%) u korelaciji s najmanjim i najvećim česticama, netopivim i ukupnim vlaknima. To može ukazivati na to da tanini utječu na udio vlakana u kakaovoj ljusci, budući da se udio netopivih i ukupnih vlakana povećava s povećanjem udjela tanina.

4.3.2. Prehrambena vlakna

Udjeli topivih, netopivih i ukupnih vlakana u uzorcima kakaove ljuske prikazani su na Slici 19. Vidi se da je sadržaj netopivih i ukupnih vlakana u tretiranim uzorcima veći nego u netretiranoj kakaovoj ljusci. Utjecaj HVED-a na topiva prehrambena vlakna nije bio statistički značajan (Tablica 9). Povećanje udjela netopivih vlakana nakon tretmana bilo je statistički značajno za vrijeme miješanja pri čemu je vidljiv pozitivan trend. Veći utjecaj na povećanje sadržaja netopivih vlakana bio je u HVED tretiranim uzorcima pri koncentraciji od 1,5 % nego pri 3,0 % zbog većeg unosa energije pri nižoj koncentraciji suspenzije.



Slika 19 Udio topivih, netopivih i ukupnih vlakana u kakaovoj ljusci prije i nakon tretmana HVED-om (USC: netretirana kakaova ljuska) (Barišić i sur., 2020d)

Povećanje sadržaja vlakana u tretiranoj kakaovoj ljusci može se objasniti činjenicom da su se tijekom različitih tretmana vlakna vjerojatno vezala s ostalim komponentama kakaove ljuske. Međutim, neka istraživanja su pokazala da rezultati dobiveni gravimetrijskim određivanjem vlakana mogu biti povećani zbog prisutnosti netopivih proteina i kondenziranih tanina

(Lecumberri i sur., 2007; Bravo i sur., 1994; Saura-Calixto, 1987). Metoda korištena u ovom istraživanju ima korak za isključivanje neprobavljenih proteina iz rezultata za sadržaj vlakana, međutim, kondenzirani tanini mogu utjecati na uočeno povećanje. Kondenzirani tanini su, uz otporne proteine i produkte Maillardovih reakcija, dio takozvanog Klason lignina (Lecumberri i sur., 2007), koji se ne smatra uvijek vlaknom. Kako su prikazali Perez i sur. (2015), pržena ljuska sadrži velike količine slobodnih aminokiselina i šećera, a prethodni rezultati u ovom istraživanju (Tablica 2; Barišić i sur., 2020b) pokazali su značajne količine katehina. HVED stvara slobodne radikale i nabijene čestice i visoko reaktivne spojeve (H^+, OH^-, H_2O_2), koji su mogli izazvati uznapredovale Maillardove reakcije i reakcije kondenzacije katehina u kondenzirane tanine (Slika 18). Akrilamid i 5-HMF najvjerojatnije reagiraju sa slobodnim radikalima stvorenim tijekom HVED tretmana i stvaraju nove spojeve koji bi mogli biti dio Klason lignina (Poglavlje 4.2; Barišić i sur., 2020c). To bi moglo pridonijeti povećanju udjela netopivih prehrambenih vlakana posebno zato što su kondenzirani tanini i produkti uznapredovalih Maillardovih reakcija netopivi u vodi. Osim toga, uočeno je da uzorci tretirani HVED-om imaju više neprobavljenih proteina od netretiranih uzoraka (rezultati nisu prikazani). Smanjena probavljivost proteina može biti posljedica stvaranja kompleksa s taninima, posebno zato što HVED tretman uzrokuje promjenu pH i površinskog naboja, što bi mogli biti povoljni uvjeti za kompleksiranje. Već je zabilježena smanjena *in vitro* i *in vivo* probavljivost proteina zbog stvaranja kompleksa s taninima za sirak i nekoliko vrsta bagrema. Osim toga, zabilježeno je i protein-protein kompleksiranje izazvano taninima i inhibicija enzima taninima (Ozdal i sur., 2013). Iako su napravljene korekcije za proteine, ostale komponente koje su bile vezane za njih nisu ovdje uključene.

Tablica 9 Analiza varijance glavnih efekata (Barišić i sur., 2020d)

	Grupirajuća varijabla	SS	DF	MS	F-vrijednost	*p*-vrijednost
0–50 µm	Intercept	169,1845	1	169,1845	59,27547	0,000006 *
	Koncentracija	20,7446	1	20,7446	7,26806	0,019455 *
	Miješanje	2,4918	2	1,2459	0,43652	0,656146
	Tretman	2,8862	2	1,4431	0,50561	0,615433
	Error	34,2505	12	2,8542		
51–71 µm	Intercept	2025,733	1	2025,733	451,2232	<0,001 *
	Koncentracija	54,266	1	54,266	12,0875	0,004574 *
	Miješanje	12,171	2	6,085	1,3555	0,294609
	Tretman	1,952	2	0,976	0,2174	0,807671
	Error	53,873	12	4,489		
72–100 µm	Intercept	1371,127	1	1371,127	705,1271	<0,001 *
	Koncentracija	18,601	1	18,601	9,5659	0,009312 *
	Miješanje	0,303	2	0,152	0,0780	0,925440
	Tretman	1,729	2	0,864	0,4446	0,651247
	Error	23,334	12	1,945		
101–125 µm	Intercept	525,4466	1	525,4466	970,2258	<0,001 *
	Koncentracija	0,9016	1	0,9016	1,6648	0,221262
	Miješanje	0,5083	2	0,2542	0,4693	0,636443
	Tretman	0,8918	2	0,4459	0,8234	0,462285
	Error	6,4989	12	0,5416		
126–200 µm	Intercept	3189,472	1	3189,472	1644,420	<0,001 *
	Koncentracija	1,189	1	1,189	0,613	0,448736
	Miješanje	2,527	2	1,264	0,651	0,538775
	Tretman	2,636	2	1,318	0,679	0,525389
	Error	23,275	12	1,940		
201–315 µm	Intercept	5128,784	1	5128,784	3745,356	<0,001 *
	Koncentracija	0,147	1	0,147	0,107	0,748864
	Miješanje	3,778	2	1,889	1,379	0,288933
	Tretman	4,432	2	2,216	1,618	0,238628
	Error	16,432	12	1,369		
>315 µm	Intercept	31,757,57	1	31,757,57	1505,873	<0,001 *
	Koncentracija	26,88	1	26,88	1,275	0,280959
	Miješanje	45,68	2	22,84	1,083	0,369462
	Tretman	52,88	2	26,44	1,254	0,320301
	Error	253,07	12	21,09		
Netopiva vlakna	Intercept	109,774,9	1	109,774,9	30,704,36	<0,001
	Koncentracija	0,1	1	0,1	0,02	0,883234
	Miješanje	36,8	2	18,4	5,15	0,024326 *
	Tretman	2,7	2	1,4	0,38	0,691002
	Error	42,9	12	3,6		
Topiva vlakna	Intercept	3038,191	1	3038,191	7378,913	<0,001*
	Koncentracija	0,962	1	0,962	2,336	0,152303
	Miješanje	2,116	2	1,058	2,570	0,117785
	Tretman	0,501	2	0,250	0,608	0,560488
	Error	4,941	12	0,412		
Ukupna vlakna	Intercept	149,338,0	1	149,338,0	43,452,47	<0,001*
	Koncentracija	1,6	1	1,6	0,47	0,508137
	Miješanje	21,7	2	10,9	3,16	0,079032
	Tretman	1,0	2	0,5	0,14	0,867489
	Error	41,2	12	3,4		

SS: suma kvadrata; DF: stupnjevi slobode; MS: prosječna vrijednost kvadrata *p<0,05 statistička značajnost

Već postoje neka istraživanja utjecaja električnog pražnjenja na vlakna. Yuan i sur. (2004) su zaključili da plazma poboljšava čvrstoću vlakana i hrapavost površine, što dovodi do većeg međufaznog kontakta. Osim toga, tijekom tretmana došlo je do oksidacije vlakana. Sinha i Panigrahi (2009) primijetili su povećanu hidrofobnost vlakana od jute nakon tretmana plazmom, vjerojatno zbog oksidacije ili smanjenja fenolnih i sekundarnih alkoholnih skupina. Do poboljšane otpornosti vlakana na savijanje došlo je zbog boljeg prianjanja između vlakana i matriksa. Bozaci i sur. (2009), te Karahan i Özdogan (2008) došli su do zaključka da vlakna nakon tretmana plazmom imaju povećanu hidrofilnost, grublju površinu i veći udio oštećenih vlakana. Potrebna su dodatna istraživanja kako bi se otkrilo mogu li predloženi mehanizmi biti primjenjivi na utjecaj HVED-a na vlakna u kakaovoj ljusci.

4.3.3. Meljivost kakaove ljuske

Najveća promjena u udjelu čestica kakaove ljuske nakon tretmana HVED-om bila je u rasponima veličine čestica 0–50 μm i >315 μm (Tablica 10). Netretirana kakaova ljuska imala je najveći postotak čestica između 0 i 50 μm i najmanji postotak čestica većih od 315 μm u usporedbi s tretiranim i kontrolnim uzorcima kakaove ljuske. Svaki tretman, bilo samo u vodi ili s HVED-om, doveo je do povećanja udjela čestica veličine veće od 315 μm i smanjenja udjela čestica veličine manje od 50 μm, što je i dokazano koeficijentom korelacije (Tablica 11). Postoji negativna korelacija između udjela najmanjih i najvećih čestica. Analiza varijance glavnih efekata pokazala je da postoji statistički značajna razlika između različitih koncentracija uzoraka tijekom tretmana za veličine čestica od 0–50 μm, 51–71 μm i 72–100 μm (Tablica 9). U svim tretiranim uzorcima uočeno je smanjenje postotka manjih čestica i povećanje udjela većih čestica. Minimalna promjena dogodila se u uzorku 1,5 %, 30 min, 40 Hz. Statistička analiza pokazala je da postoji korelacija između veličine čestica i udjela prehrambenih vlakana, što implicira da poteškoće u mljevenju HVED tretirane kakaove ljuske mogu biti uzrokovane povećanim udjelom vlakana.

Tablica 10 Meljivost kakaove ljuske prije (UCS) i nakon tretmana HVED-om (Barišić i sur., 2020d)

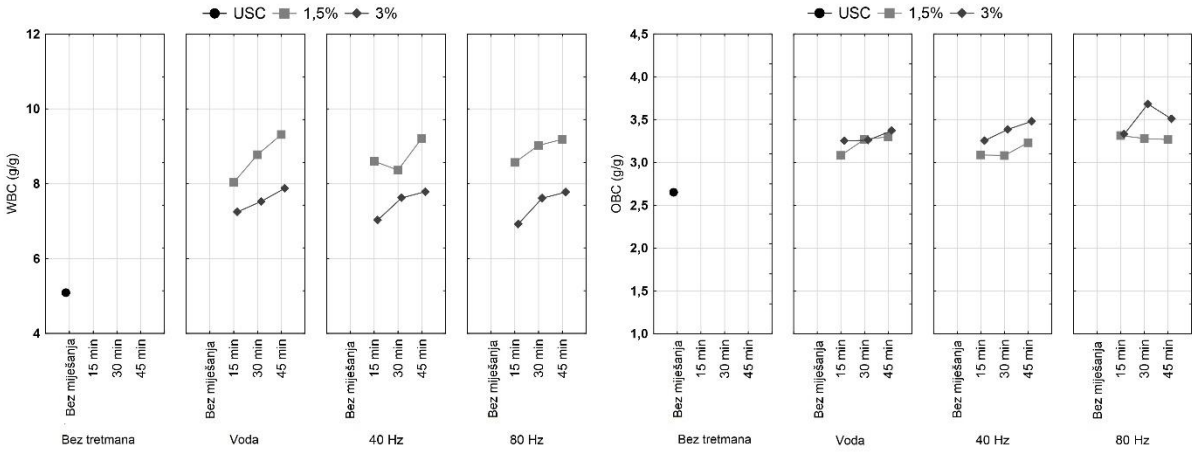
Uzorak	0–50 µm (%)	51–71 µm (%)	72–100 µm (%)	101–125 µm (%)	126–200 µm (%)	201–315 µm (%)	>315 µm (%)
UCS	15,19	21,89	11,83	7,94	18,24	14,10	10,81
1,5 %, 15 min	3,63	14,70	8,27	5,29	14,66	17,87	35,58
1,5 %, 30 min	3,12	12,64	7,83	5,07	13,12	16,76	41,47
1,5 %, 45 min	1,71	10,64	7,33	4,74	12,11	15,44	48,03
1,5 %, 15 min, 40 Hz	5,64	13,78	7,42	5,52	13,62	17,42	36,61
1,5 %, 30 min, 40 Hz	8,39	13,47	7,33	5,16	13,35	17,51	34,80
1,5 %, 45 min, 40 Hz	3,67	13,67	7,42	5,15	12,65	16,94	40,50
1,5 %, 15 min, 80 Hz	5,48	10,55	6,28	4,66	11,59	15,75	45,69
1,5 %, 30 min, 80 Hz	2,64	11,71	8,59	5,50	13,33	16,75	41,48
1,5 %, 45 min, 80 Hz	2,98	9,95	8,93	5,54	13,07	16,67	42,87
3,0 %, 15 min	3,52	9,98	7,22	4,64	11,72	15,36	47,56
3,0 %, 30 min	2,28	9,19	9,33	5,77	13,10	16,40	43,93
3,0 %, 45 min	2,16	9,26	10,98	6,40	15,05	17,31	38,84
3,0 %, 15 min, 40 Hz	1,36	8,89	11,45	5,79	15,12	18,76	38,64
3,0 %, 30 min, 40 Hz	0,64	4,04	11,23	7,40	15,23	18,69	42,77
3,0 %, 45 min, 40 Hz	2,08	8,84	10,14	5,24	12,78	16,18	44,75
3,0 %, 15 min, 80 Hz	1,50	11,73	10,83	6,07	15,48	19,18	35,20
3,0 %, 30 min, 80 Hz	1,32	6,51	9,05	4,92	12,47	15,72	50,01
3,0 %, 45 min, 80 Hz	3,07	11,41	7,46	4,41	11,17	15,14	47,33

Tablica 11 Pearsonovi koeficijenti korelacije (Barišić i sur., 2020d)

Varijabla	Tanini (%)	Tanini (mg TA/g)	Ukupna vlakna (%)	Topiva vlakna (%)	Netopiva vlakna (%)	OBC (g/g)	WBC (g/g)	>315 µm	201–315 µm	126–200 µm	101–125 µm	72–100 µm	51–71 µm	0–50 µm
Tanini (%)	1,000													
Tanini (mg TA/g)	0,067	1,000												
Ukupna vlakna (UV) (%)	0,627	0,127	1,000											
Topiva vlakna (TV) (%)	0,167	0,065	0,435	1,000										
Netopiva vlakna (NV) (%)	0,635	0,123	0,994	0,334	1,000									
OBC (g/g)	0,844	−0,100	0,780	0,334	0,776	1,000								
WBC (g/g)	−0,014	−0,118	0,464	0,306	0,450	0,233	1,000							
>315 µm	0,762	0,097	0,875	0,268	0,883	−0,862	0,349	1,000						
201–315 µm	0,018	0,357	0,318	0,582	0,264	0,114	0,096	0,018	1,000					
126–200 µm	−0,511	0,035	−0,722	−0,023	−0,751	−0,600	−0,391	−0,851	0,303	1,000				
101–125 µm	−0,365	−0,089	−0,666	−0,179	−0,674	−0,479	−0,422	−0,717	0,210	0,908	1,000			
72–100 µm	0,004	0,058	−0,425	0,098	−0,455	−0,103	−0,473	−0,467	0,312	0,803	0,819	1,000		
51–71 µm	−0,747	−0,154	−0,723	−0,351	−0,714	−0,838	−0,106	−0,795	−0,329	0,402	0,199	−0,083	1,000	
0–50 µm	−0,768	−0,244	−0,791	−0,519	−0,765	−0,827	−0,252	−0,809	−0,456	0,430	0,364	0,006	0,839	1,000

4.3.4. Kapacitet vezanja vode i ulja

Kapacitet vezanja vode (WBC) i kapacitet vezanja ulja (OBC) su važni parametri za preradu hrane i svaka promjena tih svojstava utječe na proces proizvodnje. WBC i OBC uzoraka kakaove ljuske prikazani su na Slici 20. Vidljivo je da je uzorak netretirane kakaove ljuske imao najniži WBC i OBC. Uzorci tretirani u koncentraciji od 1,5 % imali su veći WBC u usporedbi s uzorcima tretiranim u koncentraciji od 3,0 %. OBC je pokazao suprotan trend, gdje su uzorci tretirani pri koncentraciji 3,0 % imali veći kapacitet vezanja ulja od uzoraka tretiranih pri 1,5 %. Najveći porast može se primijetiti u uzorcima tretiranim 45 minuta u vodi i HVED-om. Statistička analiza pokazala je da postoji statistički značajna razlika između testiranih koncentracija i vremena miješanja, ali tretman (sa ili bez HVED-a) nije pokazao statističku značajnost. Sve kombinacije ovih učinaka pokazale su se značajnim (Tablica 8).



Slika 20 Kapacitet vezanja vode i ulja kakaove ljuske prije (USC) i nakon tretmana HVED-om (Barišić i sur., 2020d)

Prema istraživanju Sangnark i Noomhorm (2004), sposobnost vezanja vode i ulja je u korelaciji s veličinom čestica. Ovo istraživanje također je pokazalo korelaciju OBC-a s veličinom čestica (Tablica 11). Dodatno, poroznost, ukupna gustoća naboja i hidrofobna svojstva vlakana, koje se sve mogu promijeniti HVED tretmanom, mogu uvelike utjecati na WBC i OBC (Sangnark i Noomhorm, 2004; Ulbrich i Flöter, 2014). To se također može potkrijepiti i podatkom da postoji korelacija OBC-a s ukupnim sadržajem vlakana, netopivih vlakana i tanina u ovom istraživanju.

4.3.5. FTIR-ATR

Promjene u kemijskom sastavu nakon HVED tretmana potkrijepljene su FTIR-ATR analizom. Svi tretmani imali su sličan trend pa su samo reprezentativni spektri prikazani na Slici 21.

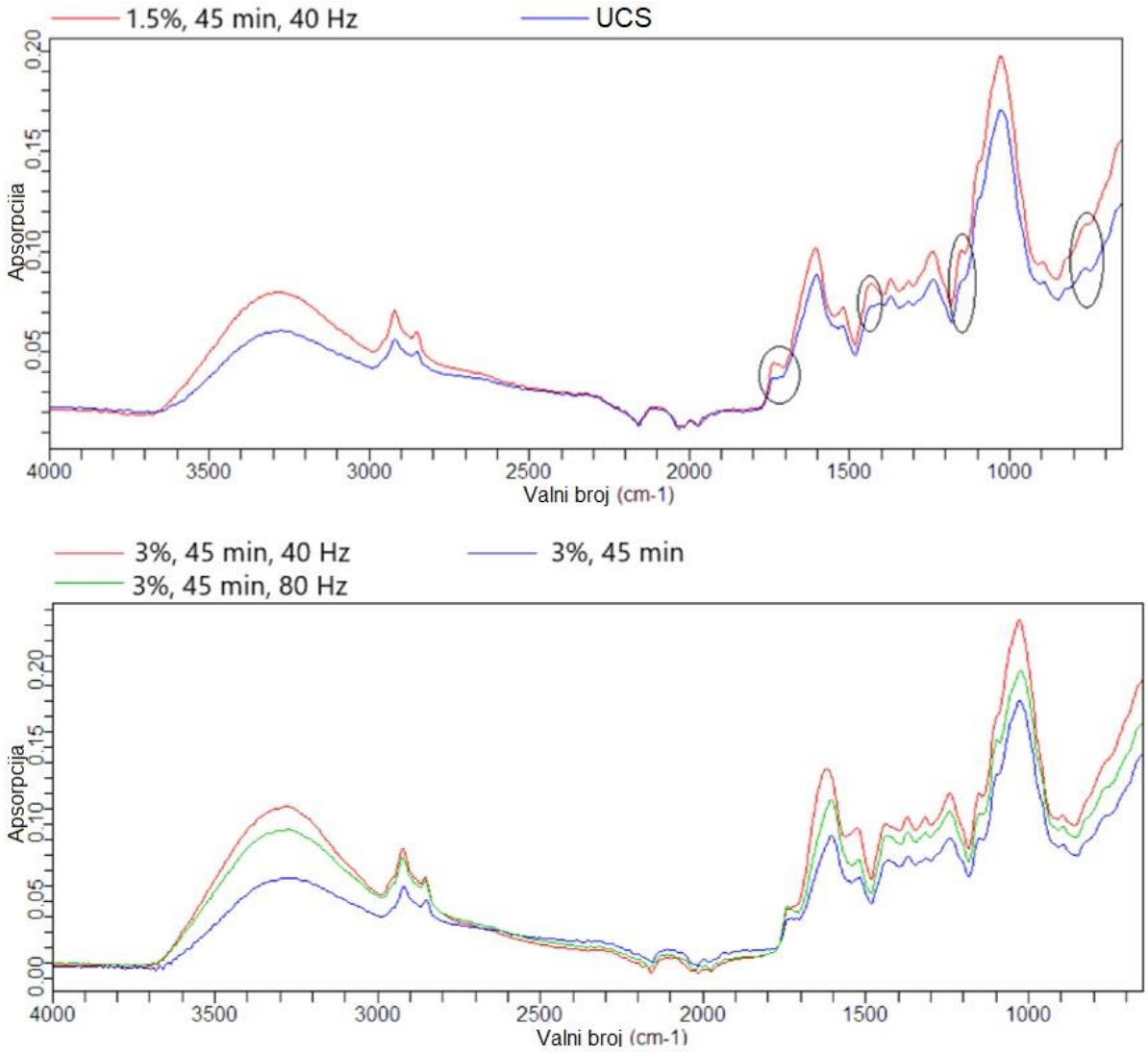
U netretiranoj kakaovoj ljusci C=O istezanje koje se proteže na 1737 cm^{-1} prikazano je samo s ramenom, a vrh je na 1602,8 cm^{-1}. Nakon tretmana pojavljuje se mali vrh na 1737 cm^{-1}. Karahan i Özdogan (2008) su ovaj vrh pripisali esterskim skupinama pektina. To implicira da se povećani sadržaj topivih vlakana može povezati s pojavom ovog vrha nakon tretmana. Međutim, prema Günzler i Gremlich (2003) i Grillo i sur. (2019b), ovo je također C=O istezanje u nekonjugiranim esterima, karboksilnim kiselinama, aldehidima i ketonima.

C-H asimetrične deformacijske vibracije u netretiranoj kakaovoj ljusci prikazane su kroz rame na 1410 cm^{-1}, dok se nakon tretmana vrh pojavljuje na 1431,3 cm^{-1}.

U netretiranoj kakaovoj ljusci vrh se nalazi na 1028,7 cm^{-1} s dva ramena na 1096 cm^{-1} i 1148 cm^{-1}. Tretmani nisu promijenili rame na 1096 cm^{-1}, za razliku od drugog koji se pomaknuo na 1155 cm^{-1} (C-H deformacija) i tu se pojavljuje mali vrh. Ovo je također blizu vrha (1152 cm^{-1}) koji je karakterističan za asimetrične vibracije C-O-C u ugljikohidratima i glukozidima prema Grillo i sur. (2019b).

Netretirana kakaova ljuska imala je mali vrh na 760 cm^{-1} (vibracije deformacije prstena). Nakon tretmana to se transferira na rame vrha.

Ove promjene u spektrima rezultat su kombiniranog učinka promjena u sastavu vlakana (omjer netopivo:topivo) i promjena u fenolnim komponentama. Bozaci i sur. (2009) također su primijetili pomak veza nakon obrade vlakana od jute hladnom plazmom. To su pripisali reakciji vlakana s reaktivnim spojevima iz plazme.



Slika 21 Reprezentativni FTIR-ATR spektar kakaove ljuske prije (UCS) i nakon tretmana HVED-om (Barišić i sur., 2020d)

4.4. MIKROBIOLOŠKA KVALITETA KAKAOVE LJUSKE

U Tablici 12 prikazane su vrijednosti udjela suhe tvari i aktiviteta vode u analiziranim uzorcima netretirane kakaove ljuske i kakaove ljuske tretirane HVED-om i osušene pri 40 °C. Temperatura sušenja odabrana je u skladu s preporukama za termički tretman sirovina bogatih polifenolima, kako bi se smanjila toplinska degradacija ovih bioaktivnih komponenti. Iz dobivenih rezultata vidljivo je da je miješanjem u vodi, bez obzira na to je li primijenjen tretman HVED-om ili ne, porastao aktivitet vode u uzorcima, ali je on i dalje značajno niži od 0,95.

Tablica 12 Udio suhe tvari i aktivitet vode (a_w) u prženoj kakaovoj ljusci prije (UCS) i nakon tretmana suspenzije neusitnjene ljuske koncentracije 1,5 % i 3,0 % (Doko i sur., 2019)

Tretman	1,5 %		3,0 %	
	Suha tvar (%)	a_w	Suha tvar (%)	a_w
UCS	94,29	0,372	94,29	0,372
15 min miješanje	87,78	0,552	85,93	0,559
30 min miješanje	87,38	0,570	85,96	0,561
45 min miješanje	86,45	0,579	84,97	0,572
15 min, 40 Hz	86,87	0,557	87,22	0,580
30 min, 40 Hz	88,63	0,532	86,76	0,591
45 min, 40 Hz	87,90	0,545	86,83	0,579
15 min, 80 Hz	86,92	0,577	87,19	0,597
30 min, 80 Hz	87,72	0,547	86,24	0,583
45 min, 80 Hz	87,43	0,567	86,15	0,780

U Tablicama 13 i 14 prikazani su rezultati mikrobiološke analize kakaove ljuske. Napravljena je „dvostruka slijepa provjera“ – za usporedbu su, osim netretirane i ljuske tretirane HVED-om pri dvije frekvencije, pripravljeni i uzorci koji su samo miješani u vodi tijekom odgovarajućeg vremena kako bi se razlučilo ima li na mikroorganizme utjecaja HVED ili samo voda.

Ni u jednom uzorku nije detektirana prisutnost *Salmonella* spp. Svi uzorci imali su veliki broj aerobnih mezofilnih bakterija (od $2{,}8 \times 10^5$ cfu/g u ljusci tretiranoj u 1,5 % suspenziji, 45 min i pri 40 Hz do $2{,}8 \times 10^7$ cfu/g u ljusci tretiranoj u 3 %-tnoj suspenziji tijekom 30 min na 80 Hz) i enterobakterija (od $1{,}9 \times 10^3$ cfu/g u ljusci tretiranoj u 3 %-tnoj suspenziji na 80 Hz tijekom 15 min do $3{,}5 \times 10^6$ cfu/g kod ljuske miješane u vodi u 1,5 %-tnoj suspenziji tijekom 30 min). Pri tome su svi uzorci tretirani u 1,5 %-tnoj suspenziji imali veći broj enterobakterija od netretirane kakaove ljuske, a u 3 %-tnoj suspenziji samo je kod jednog uzorka došlo do smanjenja njihovog broja. Nakon svih tretmana došlo je do porasta broja plijesni i kvasaca u odnosu na netretiranu ljusku, pri čemu je on nešto manji kod uzoraka na kojima je primijenjen HVED u 1,5 %-tnoj suspenziji, dok je u 3 %-tnoj suspenziji kod uzoraka tretiranih HVED-om taj broj čak veći nego kod uzoraka koji su samo miješani u vodi.

Tablica 13 Mikrobiološka kvaliteta pržene kakaove ljuske prije (UCS) i nakon tretmana 1,5 %-tne suspenzije neusitnjene ljuske (Doko i sur., 2019)

Uzorak	Metode i kriteriji prihvatljivosti			
	***Salmonella* spp. (nn.* u 25 g)**	***Enterobacteriaceae* (m=10 cfu/g, M=10^2 cfu/g)**	**Aerobne mezofilne bakterije (m=10^4 cfu/g, M=5x10^4 cfu/g)**	**Kvasci i plijesni (m=10^2 cfu/g, 10^3 cfu/g)**
UCS	n.n. u 25 g	2,7x10^3 cfu/g	1,4x10^6 cfu/g	<10 cfu/g
1,5 %, 15 min miješanje	n.n. u 25 g	1,6x10^5 cfu/g	3,2x10^6 cfu/g	1,8x10^3 cfu/g
1,5 %, 30 min miješanje	n.n. u 25 g	3,5x10^6 cfu/g	2,4x10^7 cfu/g	2,4x10^4 cfu/g
1,5 %, 45 min miješanje	n.n. u 25 g	1,2x10^6 cfu/g	2,3x10^7 cfu/g	2,6x10^4 cfu/g
1,5 %, 15 min, 40 Hz	n.n. u 25 g	1,5x10^6 cfu/g	1,6x10^7 cfu/g	1,5x10^4 cfu/g
1,5 %, 30 min, 40 Hz	n.n. u 25 g	1,5x10^5 cfu/g	2,2x10^6 cfu/g	3,6x10^3 cfu/g
1,5 %, 45 min, 40 Hz	n.n. u 25 g	1,4x10^4 cfu/g	2,8x10^5 cfu/g	1,8x10^3 cfu/g
1,5 %, 15 min, 80 Hz	n.n. u 25 g	5,1x10^4 cfu/g	6,8x10^6 cfu/g	1,8x10^3 cfu/g
1,5 %, 30 min, 80 Hz	n.n. u 25 g	5,5x10^4 cfu/g	1,7x10^7 cfu/g	9,0x10^3 cfu/g
1,5 %, 45 min, 80 Hz	n.n. u 25 g	7,6x10^5 cfu/g	2,6x10^7 cfu/g	1,9x10^4 cfu/g

*odsutnost

Ranija istraživanja, međutim, pokazala su da se HVED može koristiti u cilju smanjenja mikrobiološke kontaminacije. Tako su Ahmed i sur. (2017) objavili da su primjenom plazme u vodi inaktivirali *Escherichia coli*. Pri tome su utvrdili da i reaktivne čestice koje se generiraju i/ili dodaju tijekom tretmana (O_2, H_2O_2) imaju utjecaja na sterilizirajući efekt plazme, koji se zadržao i 72 h nakon tretmana. Ragni i sur. (2016) ispitali su utjecaj materijala od kojeg su izrađene elektrode za plinsku plazmu koja se generira dielektričnim pražnjenjem na *Escherichia coli* i *Listeria monocytogenes*. Utvrdili su da napon, jakost struje i aktivna snaga te reaktivne čestice (u ovom slučaju nitrati i nitriti) nemaju značajnog utjecaja na dekontaminaciju vode, ali su srebro i mesing imali značajan utjecaj. Butscher i sur. (2016) utvrdili su i da priroda tretiranog materijala značajno utječe na uspješnost procesa, pri čemu je smanjenje broja endospora *Geobacillus stearothermophilus* na polipropilenskim glatkim

površinama bilo značajno veće u odnosu na zrna pšenice, koja imaju neravnu površinu i duboku brazdicu.

Delsart i sur. (2015) primijenili su HVED u inaktivaciji mikroorganizama u vinu te su postigli značajno smanjenje broja plijesni i bakterija, ali tretman nije bio uspješan kao pulsirajuće električno polje, a došlo je i do sniženja udjela polifenola.

Tablica 14 Mikrobiološka kvaliteta pržene kakaove ljuske prije (UCS) i nakon tretmana 3,0 %-tne suspenzije neusitnjene ljuske (Doko i sur., 2019)

Uzorak	Metode i kriteriji prihvatljivosti			
	***Salmonella* spp. (nn.* u 25 g)**	***Enterobacteriaceae* (m=10 cfu/g, M=10^2 cfu/g)**	**Aerobne mezofilne bakterije (m=10^4 cfu/g, M=5x10^4 cfu/g)**	**Kvasci i plijesni (m=10^2 cfu/g, 10^3 cfu/g)**
UCS	n.n. u 25 g	2,7x10^3 cfu/g	1,4x10^6 cfu/g	<10 cfu/g
3,0 %, 15 min miješanje	n.n. u 25 g	1,0x10^4 cfu/g	7,4x10^5 cfu/g	2,7x10^3 cfu/g
3,0 %, 30 min miješanje	n.n. u 25 g	6,7x10^5 cfu/g	3,4x10^6 cfu/g	9,1x10^2 cfu/g
3,0 %, 45 min miješanje	n.n. u 25 g	1,8x10^5 cfu/g	3,0x10^6 cfu/g	9,1x10^2 cfu/g
3,0 %, 15 min, 40 Hz	n.n. u 25 g	7,9x10^3 cfu/g	1,7x10^6 cfu/g	1,3x10^5 cfu/g
3,0 %, 30 min, 40 Hz	n.n. u 25 g	9,1x10^3 cfu/g	6,0x10^5 cfu/g	1,1x10^4 cfu/g
3,0 %, 45 min, 40 Hz	n.n. u 25 g	2,9x10^4 cfu/g	8,0x10^5 cfu/g	9,1x10^3 cfu/g
3,0 %, 15 min, 80 Hz	n.n. u 25 g	1,9x10^3 cfu/g	1,0x10^6 cfu/g	3,2x10^4 cfu/g
3,0 %, 30 min, 80 Hz	n.n. u 25 g	1,3x10^5 cfu/g	2,8x10^7 cfu/g	9,5x10^4 cfu/g
3,0 %, 45 min, 80 Hz	n.n. u 25 g	3,1x10^4 cfu/g	1,2x10^6 cfu/g	3,2x10^4 cfu/g

*odsutnost

Do porasta broja mikroorganizama u ovom istraživanju najvjerojatnije nije došlo uslijed tretmana HVED-om, nego tijekom sušenja. Naime, temperatura sušenja bila je 40 °C, što nije značajno više od optimalne temperature za razvoj mikroorganizama te je tijekom sušenja moglo doći do oporavka preživjelih stanica i njihovog razmnožavanja. Do sličnih rezultata došlo se u radu Mandure (2016), gdje je utvrđeno da je nakon 18 sati na 30 °C došlo do rekuperacije

stanica *Escherichia coli*, iako je tretman plazmom pri 60, 90 i 120 Hz uzrokovao oksidacijski stres i odumiranje bakterija.

Kako bi se provjerila ova hipoteza, odabran je uzorak ljuske tretiran u 3 %-tnoj suspenziji 15 min na 40 Hz. Tretman je ponovljen u istim uvjetima, ali je odmah po završetku tretmana dekantiran višak vode, a tretirana ljuska zamrznuta i liofilizirana u laboratorijskom liofilizatoru (Alpha LSC Plus, Christ, Njemačka). Liofiliziranoj ljusci također je određena mikrobiološka kvaliteta te je utvrđeno da sadrži < 10 cfu/g enterobakterija, 2,4 x 10^6 cfu/g aerobnih mezofilnih bakterija i 10^2 kvasaca i plijesni, što ukazuje na uspješnost HVED-a u redukciji broja enterobakterija.

4.5. VALORIZACIJA KAKAOVE LJUSKE: UTJECAJ VISOKONAPONSKOG ELEKTRIČNOG PRAŽNJENJA I POSTUPAKA SUŠENJA NA SVOJSTVA KAKAOVE LJUSKE

S obzirom na prethodno dobivene rezultate, u sljedećem dijelu istraživanja ispitan je utjecaj postupka sušenja na svojstva kakaove ljuske. Za tretiranje kakaove ljuske HVED-om odabrano je vrijeme i frekvencija te su uzorci osušeni na 60 °C i liofilizacijom. Nakon toga su ispitana svojstava kakaove ljuske: udio vode, aktivitet vode, boja, kapacitet vezanja vode i ulja, specifični volumen, prividna i nasipna gustoća, spektrofotometrijski je određen udio ukupnih tanina i fenola, utvrđen je udio fenolnih komponenti i metilksantina HPLC-PDA metodom i određena su termofizikalna svojstava (DSC) kakaove ljuske.

4.5.1. Udio vode i aktivitet vode

U Tablici 15 može se vidjeti da je netretirana kakaova ljuska (UCS) imala najveći udio vode. HVED tretirani uzorci sušeni u ventilacijskom sušioniku (HDCS) imali su niži udio vode od uzoraka tretiranih samo miješanjem u vodi (WDCS) i sušenih u ventilacijskom sušioniku. To pokazuje da je HVED mogao poremetiti strukturu kakaove ljuske zbog elektroporacije i time uzrokovati lakše uklanjanje vode nego u uzorku koji nije tretiran HVED-om. Brahim i sur. (2017) zaključili su da su visokoenergetski udarni valovi generirani tijekom HVED tretmana izazvali fragmentaciju celuloze. Liofilizirani uzorci imali su najniži udio vode, ali nije bilo značajne razlike između kakaove ljuske miješane samo u vodi (WFCS) i HVED tretirane kakaove ljuske (HFCS) (Tablica 16) jer je liofilizacija puno učinkovitija za uklanjanje vezane i slobodne vode (Trelea i sur., 2015). Borchani i sur. (2011) su također zaključili da liofilizacija daje uzorke s nižim sadržajem vode u usporedbi sa sušenjem u sušioniku.

Aktivitet vode u svim uzorcima pokazao je isti trend kao i udio vode. Iako je netretirana kakaova ljuska imala najveći a_w, svi ostali uzorci imali su dovoljno niske vrijednosti da se spriječi rast mikroorganizama. Aktivitet vode pokazuje da liofilizirani uzorci nisu sadržavali vodu koja bi bila dostupna za kemijske i biokemijske reakcije. Iako su WFCS i HFCS imali isti aktivitet vode, HFCS je imao nešto veći udio vode. To ukazuje da je HVED uzrokovao vezanje vode u matriksu kakaove ljuske. HVED je utjecao na lakše oslobađanje slobodne vode tijekom sušenja, ali je također uzrokovao da se dio vode više veže unutar matriksa kakaove ljuske što se vidi iz sadržaja vode u uzorcima WFCS i HFCS.

Tablica 15 Udio vode, aktivitet vode (a_w), kapacitet vezanja vode (WBC) i ulja (OBC) kakaove ljuske (Barišić i sur., 2022)

Uzorak	Udio vode (%)	a_w	WBC (g/g)	OBC (g/g)
UCS	9,651 ± 0,048	0,471 ± 0,002	5,288 ± 0,038	1,598 ± 0,013
WDCS	6,687 ± 0,027	0,153 ± 0,001	6,237 ± 0,056	1,578 ± 0,000
HDCS	4,936 ± 0,023	0,057 ± 0,001	6,575 ± 0,086	1,523 ± 0,072
WFCS	3,100 ± 0,005	0	6,047 ± 0,062	2,054 ± 0,043
HFCS	3,780 ± 0,013	0	6,136 ± 0,012	2,031 ± 0,085

*UCS-netretirana kakaova ljuska; WDCS-kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS- kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS- kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS- kakaova ljuska tretirana HVED-om i sušena liofilizacijom

4.5.2. Kapaciteti vezanja vode i ulja

Kapaciteti vezanja vode i ulja su fizikalna svojstva koja daju informacije o volumenu pora i ponašanju materijala u matriksu hrane (de Escalada Pla i sur., 2012). Liofilizirani uzorci i oni sušeni u sušioniku imali su više vrijednosti WBC-a u usporedbi s netretiranom kakaovom ljuskom, ali je povećanje nešto izraženije u uzorcima osušenim u sušioniku. Guillon i Champ (2000) utvrdili su da procesi prerade mogu modificirati fizikalna svojstva matriksa vlakana što direktno utječe na hidratacijska svojstva. HVED stvara reaktivne spojeve koji bi mogli reagirati s matriksom kakaove ljuske i učiniti ga osjetljivijim na upijanje vode. U istraživanju Maceda i sur. (2020), gdje je hladna plazma korištena za modificiranje vlakana, rezultati su pokazali da je u modificiranim vlaknima povećan kapacitet vezanja ulja. Ovaj fenomen može biti posljedica površinske erozije vlakana i preuređivanja hidrofobnih skupina na površini uslijed čega se povećava afinitet za nepolarne tekućine. Rezultati ovog istraživanja pokazuju da je HVED tretman uzrokovao povećan afinitet za polarne tekućine poput vode, što ukazuje da plazma stvorena u vodi vjerojatno inducira povećanje broja hidrofilnih skupina na površini vlakana.

Kapacitet vezanja ulja (OBC) ovisi o sastavu materijala, svojstvima površine i hidrofobnoj prirodi čestica (Borchani i sur., 2011). Tablica 15 pokazuje da su liofilizirani uzorci imali najveći OBC. De Escalada Pla i sur. (2012) su također zaključili da se OBC povećava kada se primjenjuje liofilizacija u usporedbi s prirodnim sušenjem i sušenjem na zraku. Naveli su da materijal može apsorbirati više ulja kada ima veći specifični volumen. Rezultati dobiveni u ovom istraživanju također su pokazali istu korelaciju. Ova dva svojstva vrlo su važna za određivanje ponašanja određenog materijala u prehrambenim sustavima.

Tablica 16 Jednofaktorska analiza varijance uz korekciju s Welch F-testom (Barišić i sur., 2022)

	Grupirajuća varijabla	Welch *p*		Grupirajuća varijabla	Welch *p*
Suha tvar	Sušenje	**0,012761**	**Nasipna gustoća**	Sušenje	**0,000010**
	Tretman	0,650726		Tretman	0,377573
a_w	Sušenje	-	**Teobromin**	Sušenje	0,672998
	Tretman	0,366180		Tretman	**0,015449**
WBC	Sušenje	**0,053271**	**Kafein**	Sušenje	0,892681
	Tretman	0,214023		Tretman	**0,018278**
OBC	Sušenje	**0,000091**	**(+)-Katehin**	Sušenje	**0,024334**
	Tretman	0,858107		Tretman	0,162426
L*	Sušenje	**0,016322**	**(-)-Epikatehin**	Sušenje	**0,003978**
	Tretman	0,355509		Tretman	0,923919
a*	Sušenje	**0,004862**	**(-)-Epikatehin galat**	Sušenje	**0,063741**
	Tretman	0,612508		Tretman	0,393741
b*	Sušenje	**0,055428**	**Galna kiselina**	Sušenje	-
	Tretman	**0,000424**		Tretman	0,889654
C	Sušenje	0,141808	**Kafeinska kiselina**	Sušenje	0,371941
	Tretman	**0,000005**		Tretman	**0,006793**
h°	Sušenje	**0,013098**	***p*-Kumarinska kiselina**	Sušenje	0,813538
	Tretman	**0,048762**		Tretman	**0,000550**
ΔE	Sušenje	0,083833	**Udio ukupnih polifenola**	Sušenje	**0,003429**
	Tretman	**0,000173**		Tretman	0,445195
Specifični volumen	Sušenje	**<0,000001**	**Klason lignin**	Sušenje	0,470626
	Tretman	0,901643		Tretman	0,426702
Prividna gustoća	Sušenje	**<0,000001**	**Tanini (%)**	Sušenje	**0,005559**
	Tretman	0,975265		Tretman	**0,007030**

a_w: aktivitet vode; WBC: kapacitet vezanja vode; OBC: kapacitet vezanja ulja; ΔE: ukupna promjena boje

4.5.3. Boja

Sirovine koje se koriste za proizvodnju prehrambenih proizvoda utječu na organoleptičke parametre, a boja je jedna od njih koja je potrošačima vrlo važna. Parametri boje uzoraka kakaove ljuske prikazani su u Tablici 17. Uzorci tretirani HVED-om imali su niže L* vrijednosti nakon sušenja u sušioniku (60 °C) i veće L* vrijednosti nakon liofilizacije (-40 do 20 °C), što pokazuje da sušenje u sušioniku uzrokuje tamnjenje uzorka, a liofilizacija posvjetljivanje uzoraka tretiranih HVED-om. Svi uzorci su bili u domeni crvene i žute boje što se vidi iz pozitivnih a* i b* vrijednosti. Poznato je da visoke temperature mogu uzrokovati neenzimsko posmeđivanje što dovodi do tamnjenja uzoraka (Borchani i sur., 2011). Potamnjivanje uzoraka nakon zagrijavanja može se djelomično objasniti neenzimskom oligomerizacijom katehina koja rezultira stvaranjem žutih do smeđih kompleksa (Gadkari i Balaraman, 2015). U ovom istraživanju kakaova ljuska je termički obrađena (pržena) prije ovdje primijenjenih tretmana, pa se kao razlog može isključiti enzimsko posmeđivanje. Međutim, neenzimske reakcije posmeđivanja započete tijekom prženja pospješuju se naknadnom termičkom obradom u

ovom istraživanju (sušenjem). Budući da se liofilizacija provodi na mnogo nižim temperaturama, očekuje se da će rezultirati višim L* vrijednostima (svjetlijom bojom).

Tablica 17 Parametri boje uzoraka kakaove ljuske (Barišić i sur., 2022)

Uzorak	L*	a*	b*	C	h°	ΔE
UCS	46,72±0,00	9,59±0,03	18,72±0,02	21,03±0,02	62,86±0,09	
WDCS	47,03±0,01	8,48±0,01	17,15±0,03	19,13±0,03	63,68±0,04	1,94±0,02
HDCS	44,18±0,01	8,77±0,03	16,47±0,03	18,66±0,03	61,96±0,11	3,49±0,02
WFCS	46,28±0,09	8,53±0,07	17,17±0,04	19,18±0,01	63,58±0,22	1,93±0,02
HFCS	47,91±0,02	8,33±0,05	16,96±0,03	18,90±0,03	63,85±0,16	2,47±0,03

*UCS-netretirana kakaova ljuska; WDCS-kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS- kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS- kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS- kakaova ljuska tretirana HVED-om i sušena liofilizacijom

Uzorak HDCS imao je najnižu L* vrijednost što implicira da je HVED tretman pridonio stvaranju prekursora za stvaranje boje, koji su reagirali tijekom sušenja u sušioniku i rezultirali tamnijim uzorkom. U prethodnom dijelu istraživanja (Poglavlje 4.2; Barišić i sur., 2020c) gdje je određen sadržaj 5-hidroksimetilfurfurala i akrilamida u HVED tretiranoj kakaovoj ljusci navedeno je da tretman može pogodovati daljnjoj reakciji ovih komponenti. Sušenje je imalo statistički značajan učinak na L* i a* parametre, dok je HVED tretman imao statistički značajan učinak na C i ΔE. Također, Tablica 17 pokazuje da su na b* i h° utjecali i sušenje i HVED tretman. Nakon svih tretmana uzorci su imali manje izraženu crvenu i žutu boju, što je prikazano nižim vrijednostima a* i b* parametara. Također, HVED je imao veći učinak na smanjenje intenziteta žute boje od kontrolnih uzoraka miješanih u vodi. Uzorci WDCS, WFCS i HFCS imali su ΔE vrijednosti u rasponu koji ukazuje da promjenu boje može lako primijetiti obučeni analitičar, a ΔE vrijednost za uzorak HDCS je pokazala da je promjena boje vidljiva i netreniranom oku (Jukić i sur., 2007).

4.5.4. Specifični volumen, prividna i nasipna gustoća

Fizikalna svojstva (specifični volumen, prividna i nasipna gustoća) kakaove ljuske prikazana su u Tablici 18. Ovi parametri važni su za transport, skladištenje i pakiranje praškastih materijala i hrane (Chaloeichitratham i sur., 2018). Najveće promjene su vidljive za uzorke koji su sušeni liofilizacijom kao rezultat sublimacije vode tijekom tog procesa, što dovodi do visoke poroznosti uzoraka (Chaloeichitratham i sur., 2018). Nasipna gustoća bila je nešto niža za uzorke tretirane HVED-om. Ovaj parametar ovisi o sadržaju vode i volumenu pora na koje utječe struktura matriksa i u kojoj je mjeri on narušen (de Escalada Pla i sur., 2012). Uzorci

tretirani HVED-om imali su manji udio vode, a HVED je mogao utjecati na volumen pora kakaove ljuske, što je već dokazano na anorganskim spojevima (De Coste i sur., 2015), ali i organskim stanicama (Moreau i sur., 2008). Statistička analiza pokazala je da postupak sušenja ima značajan utjecaj na ove parametre, dok HVED nema. Stoga se može zaključiti da na te parametre najviše utječe količina vode u uzorcima i način na koji voda izlazi iz materijala, odnosno posljedice koje nastaju i utječu na strukturu materijala.

Tablica 18 Specifični volumen, prividna i nasipna gustoća uzoraka kakaove ljuske (Barišić i sur., 2022)

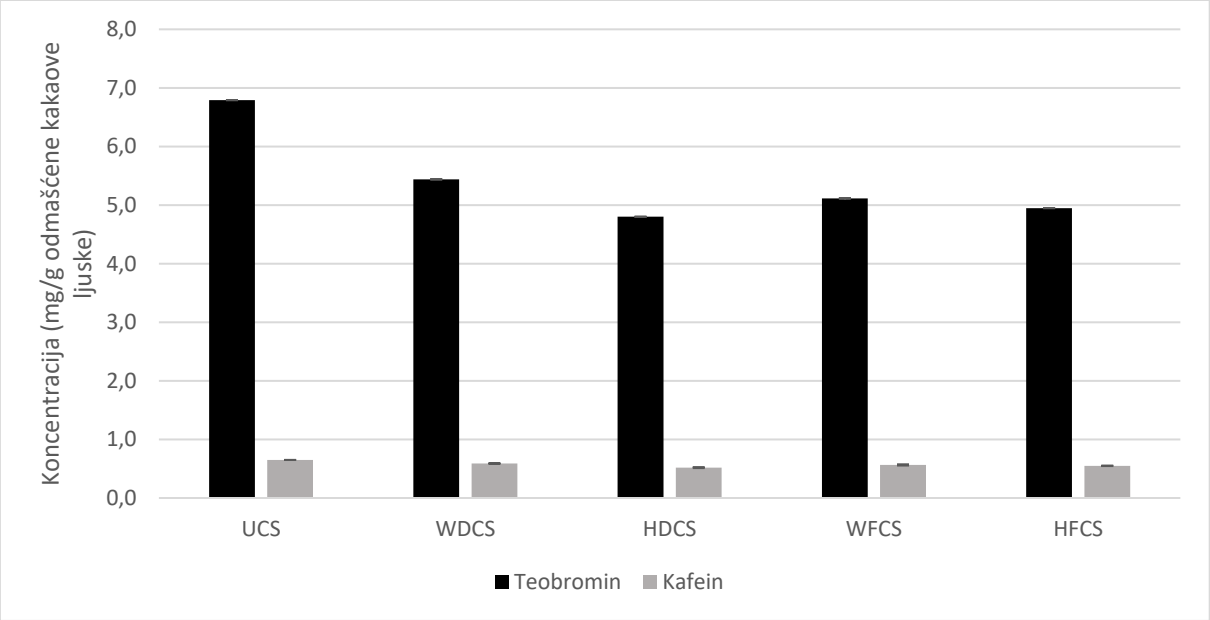
Uzorak	Specifični volumen (cm^3/g)	Prividna gustoća (g/cm^3)	Nasipna gustoća (g/cm^3)
UCS	2,300±0,054	0,435±0,010	0,481±0,014
WDCS	2,022±0,031	0,495±0,008	0,438±0,009
HDCS	2,067±0,047	0,484±0,011	0,411±0,011
WFCS	2,922±0,068	0,342±0,008	0,346±0,013
HFCS	2,811±0,016	0,356±0,002	0,319±0,012

*UCS-netretirana kakaova ljuska; WDCS-kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS- kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS- kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS- kakaova ljuska tretirana HVED-om i sušena liofilizacijom

4.5.5. Metilksantini, fenolne komponente, ukupni udio fenola i tanina

UCS je imala najveći sadržaj teobromina i kafeina (Slika 22). HVED tretman je imao statistički značajan utjecaj na sadržaj ovih metilksantina, dok postupak sušenja nije. To pokazuje da su metilksantini stabilni na temperaturi 60 °C, dok HVED uzrokuje smanjenje sadržaja metilksantina. U slučaju liofilizacije nije bilo velike razlike u sadržaju metilksantina između kakaove ljuske koja je tretirana HVED-om i miješana samo u vodi. To može biti zbog HVED-inducirane ekstrakcije ovih spojeva u vodi, oksidacije izazvane radikalima stvorenim tijekom HVED tretmana ili kombinacije oba fenomena gdje je viša temperatura (60 °C) uzrokovala daljnju oksidaciju. Moguća reakcija oksidacije kafeina izazvana Fentonom i/ili Fentonovim reagensom, kao što su prikazali de Oliveira i sur. (2015), potkrijepljeno je smanjenjem sadržaja ^{56}Fe i ^{57}Fe u kakaovoj ljusci nakon tretmana HVED-om i činjenicom da H_2O_2 nastaje u vodi tijekom HVED tretmana, a Dalmázio i sur. (2005) predložili su mehanizme razgradnje kafeina kroz oksidaciju, reakciju s $OH^{\cdot}$ radikalima i molekulama vode, što je sve prisutno u vodi tijekom tretmana HVED-om. Također, Stadler i sur. (1996) su pokazali da katehini, koji su prisutni u kakaovoj ljusci, potiču oksidaciju kafeina. Jokić i sur. (2019) zaključili su da je HVED potaknuo ekstrakciju teobromina i kafeina, ali prinos tih spojeva u vodenim ekstraktima ovisi o uvjetima

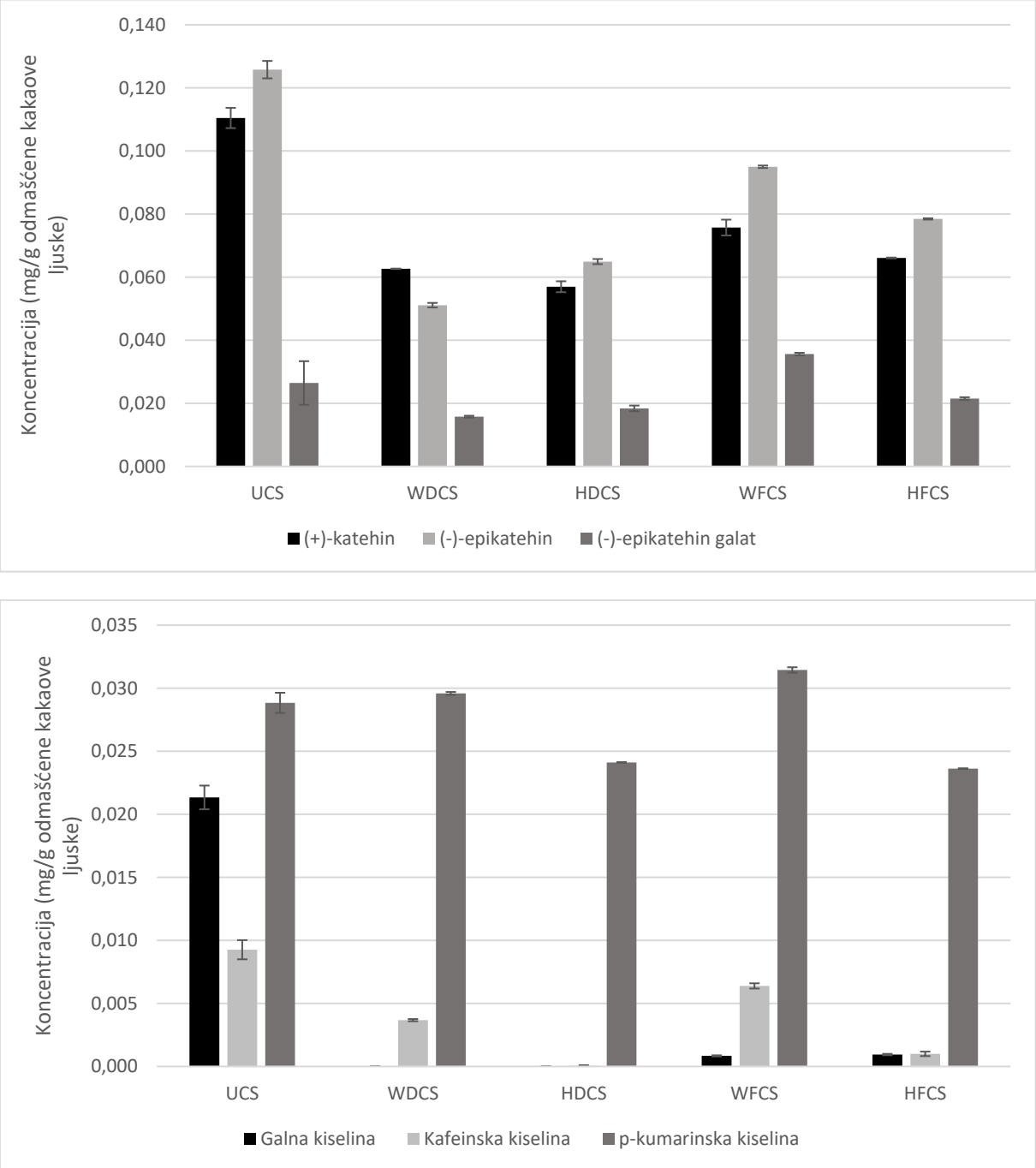
obrade (omjer otapalo:sirovina, frekvencija i vrijeme HVED tretmana). Zbog složenosti mogućih reakcija potrebno je daljnje istraživanje drugim instrumentalnim tehnikama kako bi se potvrdilo koji od predloženih mehanizama prevladava.



Slika 22 Udio metilksantina u uzorcima kakaove ljuske (UCS - netretirana kakaova ljuska; WDCS-kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS - kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS - kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS - kakaova ljuska tretirana HVED-om i sušena liofilizacijom) (Barišić i sur., 2022)

UCS je imala najveći udio (+)-katehina, (-)-epikatehina i (-)-epikatehin galata (Slika 23). Također, liofilizirani uzorci imali su veći udio ovih spojeva od uzoraka sušenih u sušioniku. Sušenje je pokazalo statistički značajan učinak na sadržaj ovih spojeva, dok tretman nije. Jedan od razloga za ovu situaciju je činjenica da su katehini vrlo osjetljivi na visoke temperature, oksidaciju, svjetlost i alkalnu sredinu. Tijekom toplinske obrade dolazi do mnogo različitih reakcija između katehina i drugih komponenti prisutnih u kakaovoj ljusci (npr. proteina, enzima i kafeina), ali i između samih katehina (Gadkari i Balamaran, 2015). Fan i sur. (2016) proveli su istraživanje toplinske stabilnosti različitih katehina i pokazali da je epikatehin najmanje postojan pri visokim temperaturama (60 i 90 °C) među ispitanim katehinima. Najniža stabilnost epikatehina pripisana je reakciji epimerizacije. (-)-Epikatehin je i u ovom istraživanju pokazao najmanju stabilnost tijekom zagrijavanja (Slika 23). Uzorak HDCS imao je veći sadržaj (-)-epikatehina i (-)-epikatehin galata nego WDCS uzorak. Liofilizirani uzorci imali su suprotan trend. Iz prethodnih rezultata je vidljivo (Tablica 2; Barišić i sur., 2020b) da su HVED tretirani i sušeni uzorci imali veći sadržaj fenolnih komponenti od uzoraka tretiranih u vodi i

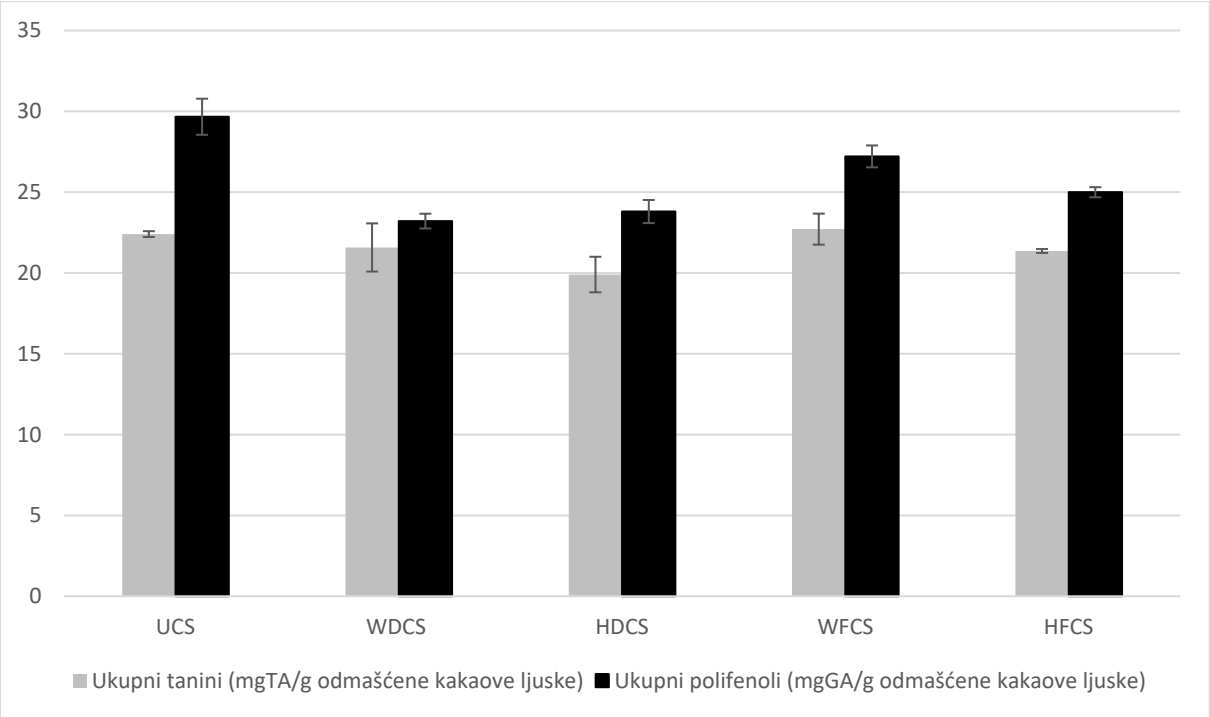
sušenih u sušioniku, ali je primijenjena temperatura sušenja bila niža (40 °C). (-)-Epikatehin i (-)-epikatehin galat također su imali najveći postotak zadržavanja. Kao što je prethodno navedeno, HVED tretman mogao bi potaknuti interakcije fenolnih spojeva s vlaknima i zaštititi fenole tijekom sušenja u sušioniku.



Slika 23 Udio polifenola u uzorcima kakaove ljuske (UCS - netretirana kakaova ljuska; WDCS-kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS - kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS - kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS- kakaova ljuska tretirana HVED-om i sušena liofilizacijom) (Barišić i sur., 2022)

Galna kiselina pokazala je najmanju stabilnost nakon tretmana i različitih postupaka sušenja. Nije detektirana nakon sušenja u sušioniku, a vrlo niske koncentracije su utvrđene nakon liofilizacije (Slika 23). Rezultati razgradnje fenolnih kiselina mogu se djelomično objasniti razlikama u toplinskoj stabilnosti fenolnih kiselina. Naime, Setyaningsih i sur. (2016) uočili su da se galna kiselina razgrađuje na 60 °C dok su *p*-kumarinska kiselina i kafeinska kiselina ostale stabilne na toj temperaturi. Statistička analiza (Tablica 16) pokazala je da je tretman imao statistički značajan učinak na kafeinsku i *p*-kumarinsku kiselinu. Slika 23 prikazuje da je uzorak WFCS imao najveći sadržaj *p*-kumarinske kiseline. Postoji mogućnost da je ovu komponentu bilo lakše izdvojiti nakon liofilizacije zbog pora stvorenih tijekom sublimacije.

UCS je imao najveći, a WDCS najmanji sadržaj ukupnih fenola (TPC) između svih uzoraka kakaove ljuske (Slika 24). Statistička analiza pokazala je da tretman ima značajan učinak na TPC (Tablica 16). Liofilizirani uzorci imali su veći sadržaj ukupnih fenola od uzoraka sušenih u sušioniku. To je u skladu sa istraživanjem Borchani i sur. (2011) gdje su liofilizirani uzorci imali najveći TPC u usporedbi s uzorcima sušenim u sušioniku i na suncu. Poznato je da nusproizvodi prehrambene industrije imaju određeni postotak fenolnih komponenti, što je važno za ponovnu uporabu takvih materijala i pozitivnog učinka fenolnih spojeva na zdravlje ljudi i prevenciju bolesti (Borchani i sur., 2011). Orphanides i sur. (2013) i Roslan i sur. (2020) također su izvijestili o nižoj razgradnji fenolnih komponenti i TPC-a u liofiliziranim uzorcima u usporedbi s konvencionalno zagrijavanim uzorcima.



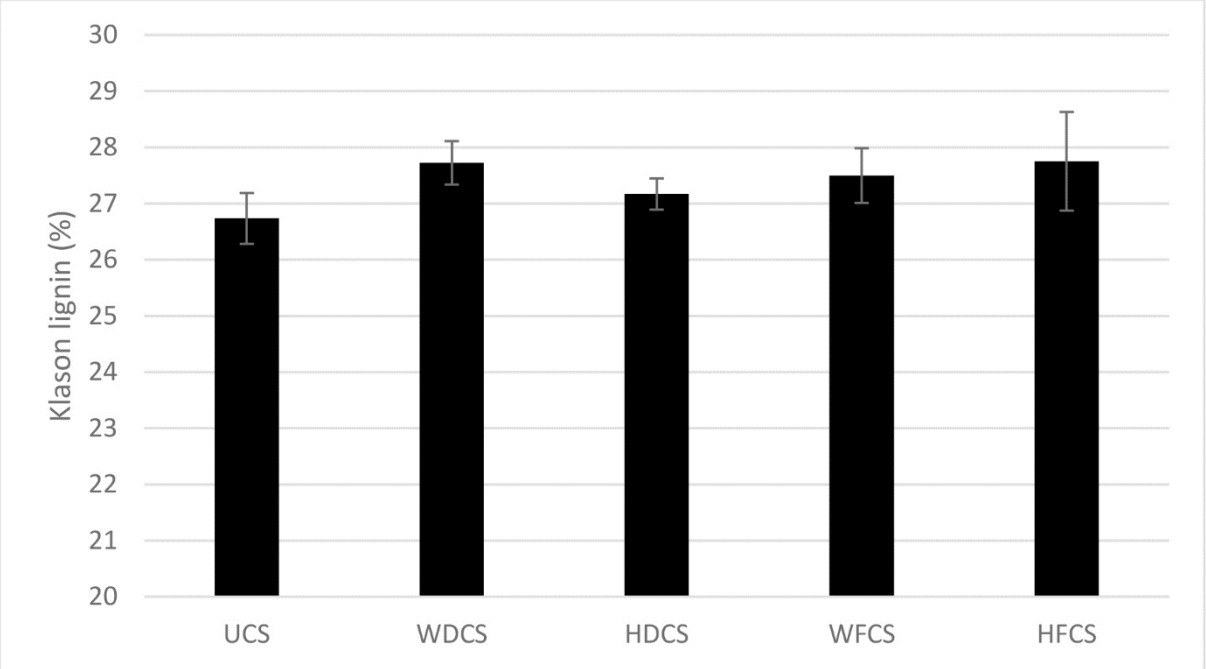
Slika 24 Udio ukupnih polifenola i tanina u uzrocima kakaove ljuske (UCS-netretirana kakaova ljuska; WDCS-kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS- kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS-kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS- kakaova ljuska tretirana HVED-om i sušena liofilizacijom) (Barišić i sur., 2022)

Sadržaj tanina u uzorcima kakaove ljuske pokazao je sličan trend kao i ukupni sadržaj fenola, iako su tanini bili otporniji na HVED tretman i postupak sušenja (Slika 24). Što se tiče rezultata fenolnih komponenti, može se zaključiti da su veće molekule otpornije na HVED tretman i uvjete sušenja. U prilog tome govore i rezultati za galnu kiselinu, koja nije utvrđena nakon sušenja u sušioniku na 60 °C.

4.5.6. Klason lignin

U prethodnim rezultatima, analiza sadržaja prehrambenih vlakana pokazala je da je HVED tretman utjecao na povećanje sadržaja netopivih prehrambenih vlakana (Slika 19; Barišić i sur. 2020d). Budući da je već objavljeno da je sadržaj netopivih prehrambenih vlakana u kakaovoj ljusci precijenjen zbog visokog sadržaja Klason lignina (Redgwell i sur., 2003), u ovom dijelu istraživanja određena je ta frakcija kako bi se utvrdilo je li Klason lignin frakcija uzrokovala povećanje udjela netopivih prehrambenih vlakana nakon HVED-a. Klason lignin predstavlja lignin koji nije topiv u 72 % sumpornoj kiselini. Dobro je poznato da frakcija netopiva u kiselini ne mora biti samo lignin. U istraživanju Li i sur. (2007) rezultati su pokazali da je približno 50

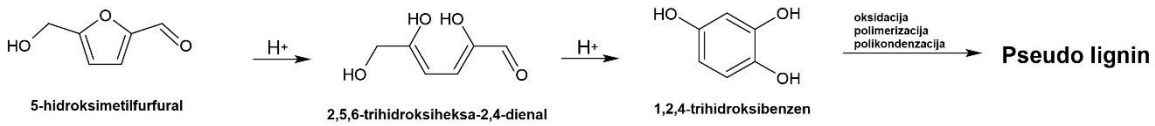
% Klason lignina u hidrotermalno obrađenim uzorcima lignin. Ovaj fenomen se također vidi u materijalima tretiranim kiselinom i naziva se pseudo-lignin. Rezultati određivanja Klason lignina dobiveni u ovom istraživanju pokazuju da su svi tretirani uzorci kakaove ljuske imali veći sadržaj Klason lignina od netretirane kakaove ljuske. HVED tretman i postupci sušenja su mogli imati utjecaj na takav trend (Slika 25). Struktura slična ligninu može se stvoriti reakcijom supstitucije na aromatskim prstenovima lignina (Shinde i sur., 2018).



Slika 25 Udio Klason lignina u uzorcima kakaove ljuske (UCS - netretirana kakaova ljuska; WDCS - kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS - kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS - kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS - kakaova ljuska tretirana HVED-om i sušena liofilizacijom) (Barišić i sur., 2022)

U prethodnim rezultatima o utjecaju HVED-a na 5-hidroksimetilfurfural (5-HMF) predložen je mehanizam daljnje reakcije ovog spoja i stvaranja novih organskih spojeva (Slika 16; Barišić i sur., 2020c). Jedna od predloženih formacija pseudo-lignina je pretvorba 5-HMF-a u druge aromatske komponente. Ovi ključni međuprodukti mogu se polimerizirati u trodimenzionalni polimer ili se mogu vezati na postojeći lignin koji tada djeluje kao Klason lignin. Dosadašnji rezultati za kakaovu ljusku tretiranu HVED-om pokazali su formiranje ramena na FTIR-ATR spektru što je karakteristično za aromatske C-H vibracije lignina (Slika 21; Barišić i sur., 2020d). Budući da je lignin aromatski biopolimer, ova tvorba spomenutog ramena mogla bi biti jedan od pokazatelja povećanog sadržaja Klason lignina. Jedan od predloženih mehanizama za stvaranje pseudo-lignina prikazan je na Slici 26. Predložena reakcija može se provesti u

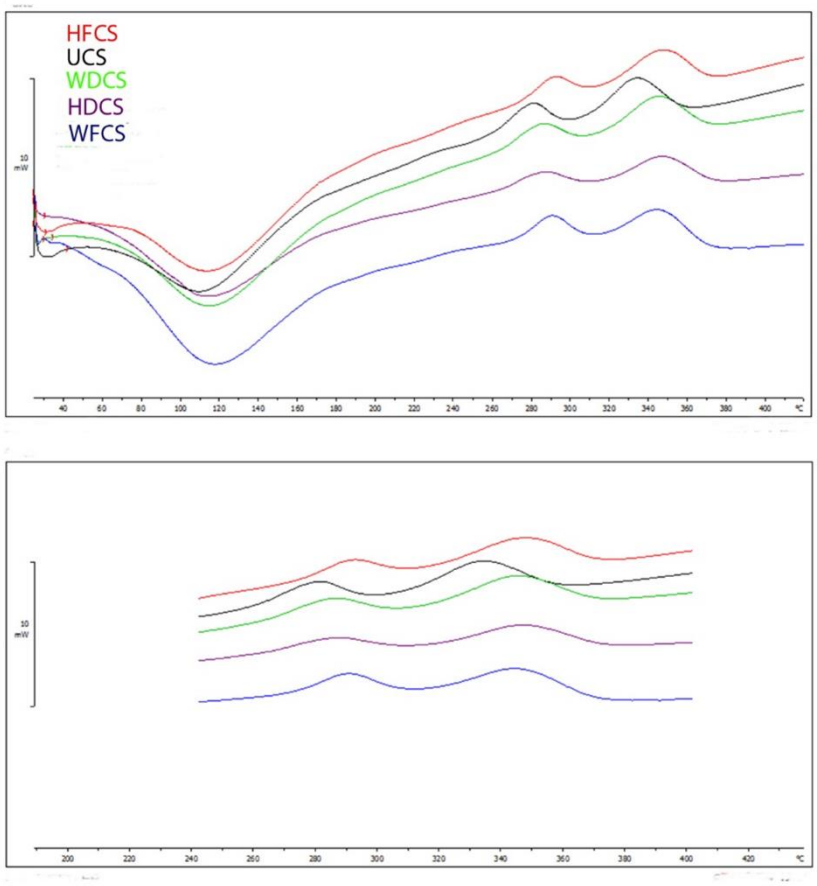
vodenom mediju s prisutnim H^+, što je vrlo moguće u ovom istraživanju, zbog električnih pražnjenja koja se stvaraju izravno u vodi. Inače, tijekom obrade lignoceluloznih materijala opisano je da ova reakcija počinje od ugljikohidrata iz kojih nastaje 5-HMF (Shinde i sur., 2018), no u ovom slučaju, budući da je 5-HMF već prisutan u kakaovoj ljusci, reakcija bi se dogodila s manje koraka i u kraćem vremenu.



Slika 26 Predloženi mehanizam za stvaranje pseudo lignina (Barišić i sur., 2022)

4.5.7. Diferencijalna motridbena kalorimetrija (DSC)

Diferencijalna motridbena kalorimetrija pokazala je endotermni vrh od 110 – 120 °C i dva egzotermna vrha u području 280 – 347 °C za sve uzorke kakaove ljuske. Egzotermni vrhovi koji se javljaju nakon 280 °C karakteristični su za hemicelulozu, celulozu i lignin (Bargougui i sur., 2018). Slika 27 pokazuje da su se vrhovi pomaknuli na više temperature u svim tretiranim uzorcima, što pokazuje da su oni termostabilniji. Najveća termostabilnost na prvom vrhu uočena je za liofilizirani HVED-tretirani uzorak (HFCS), gdje se vrh pojavio na 292,39 °C, dok je u netretiranoj kakaovoj ljusci taj vrh bio na 280,86 °C. Drugi vrh također je bio pomaknut u HVED tretiranim uzorcima (HFCS i HDCS) i iznosio je 347 °C, dok je u netretiranoj kakaovoj ljusci isti vrh bio na 335,27 °C. U istraživanju koje su proveli Bargougui i sur. (2018), gdje je modificirana kakaova ljuska, ova dva vrha također su pomaknuta na više temperature. Zaključili su da je to zbog morfoloških promjena u materijalu. Prethodni rezultati HVED-tretirane kakaove ljuske pokazali su da se na FTIR-ATR spektrima pojavio veći vrh, karakterističan za deformaciju C-H, vibracije CH_2 i CH_3 skupina ugljikohidrata (Slika 21; Barišić i sur., 2020d). Ovi rezultati, u kombinaciji s DSC analizom kakaove ljuske tretirane HVED-om, pokazuju da HVED uzrokuje određenu modifikaciju ugljikohidrata prisutnih u kakaovoj ljusci. Također, jedan od razloga povećane termostabilnosti kakaove ljuske nakon tretmana mogao bi biti i povećani sadržaj lignina. U istraživanju koje su proveli Fu i sur. (2020) lignocelulozne nanofibrile s većim udjelom lignina pokazale su više temperature razgradnje.



Slika 27 Krivulje diferencijalne motridbene kalorimetrije (gornja slika-kompletne krivulje; donja slika-uvećani egzotermni vrhovi karakteristični za hemicelulozu, celulozu i lignin) uzoraka kakaove ljuske (UCS - netretirana kakaova ljuska; WDCS - kakaova ljuska miješana u vodi i sušena u ventilacijskom sušioniku; HDCS - kakaova ljuska tretirana HVED-om i sušena u ventilacijskom sušioniku; WFCS - kakaova ljuska miješana u vodi i sušena liofilizacijom; HFCS - kakaova ljuska tretirana HVED-om i sušena liofilizacijom) (Barišić i sur., 2022)

Sumarni pregled utjecaja HVED-a na svojstva kakaove ljuske

Utvrđivanjem udjela bioaktivnih komponenti u kakaovoj ljusci zaključeno je da ljuska sadrži najviše metilksantina (teobromin i kafein). Najzastupljenija fenolna komponenta bio je (+)-katehin, a nakon njega (-)-epikatehin i galna kiselina. (-)-Epikatehin i (-)-epikatehin galat su među fenolnim spojevima, nakon tretmana HVED-om, ostali najstabilniji u kakaovoj ljusci, odnosno došlo je do najmanjeg gubitka ovih komponenti. Također se može vidjeti i da je tretiranje pri 1,5 %-tnoj koncentraciji, tijekom 15 minuta i 40 Hz kod većine komponenti (metilksantina i fenolnih spojeva) uzrokovalo najveće zadržavanje u usporedbi s ostalim tretmanima i kontrolnim uzorcima (miješanje u vodi). Duljim tretmanima i pri većoj frekvenciji došlo je do većeg gubitka ispitivanih komponenti, ali i teobromin, kafein, (+)-katehin, (-)-epikatehin, (-)-epikatehin galat i galna kiselina bili su nakon bilo kojeg tretmana HVED-om više očuvani nego u kontrolnim uzorcima gdje se vršilo samo miješanje u vodi. Kao što je već napomenuto, polifenoli prisutni u kakaovoj ljusci imaju slabiju bioraspoloživost što je vjerojatno rezultat interakcije ovih spojeva s vlaknima u kakaovoj ljusci (Jakobek i Matić, 2019). Postoji mogućnost da je HVED tretman utjecao na dodatne interakcije fenolnih komponenti i vlakana zbog čega su fenolne komponente više očuvane u tim uzorcima.

Udio tanina u tretiranim uzorcima pokazao je da su ove komponente vrlo otporne na djelovanje HVED-a, odnosno da nije došlo do smanjenja njihovog udjela. Pri tretmanima od 30 i 45 min pri koncentraciji 3 % povećanjem frekvencije došlo je i do povećanja udjela tanina u ukupnim fenolima. Jednim dijelom objašnjenje je i u promjeni omjera prisutnih komponenti u kakaovoj ljusci, ali ovo također pokazuje da je HVED tretman mogao utjecati na oksidaciju i agregaciju tanina zbog djelovanja radikala koji nastaju tijekom tretmana.

Netopiva, topiva i ukupna vlakna određena su u svim uzorcima kakaove ljuske. Rezultati su pokazali da se nakon tretmana HVED-om povećao udio netopivih i ukupnih vlakana. Budući da je za određivanje udjela vlakana korištena gravimetrijska metoda, moguće je da je udio vlakana prividno povećan jer ova metoda može uračunati neke komponente, kao što su produkti Maillardovih reakcija i Klason lignin, u rezultate za udio vlakana (Lecumberri i sur., 2007). Također, već je navedeno da je HVED utjecao na povećanje udjela tanina koji bi mogli utjecati na povećanje vlakana.

Budući da je cilj ove doktorske disertacije pronaći način za korištenje kakaove ljuske u proizvodnji prehrambenih proizvoda, kapacitet vezanja vode i ulja su vrlo bitni parametri koji trebaju biti ispitani. Tretman HVED-om utjecao je na povećanje kapaciteta vezanja vode i ulja u uzorcima kakaove ljuske što može biti iz razloga što električni izboji mogu promijeniti poroznost i hidrofobna svojstva vlakana (Sangnark i Noomhorm, 2004; Ulbrich i Flöter, 2014).

Meljivost kakaove ljuske je vrlo bitna karakteristika jer direktno utječe na isplativost korištenja ovog nusproizvoda u prehrambenoj industriji. Rezultati su pokazali da se nakon tretmana HVED-om meljivost smanjuje, odnosno smanjuje se udio čestica manjih od 50 μm i povećava udio čestica većih od 315 μm. U literaturnim podatcima već postoje rezultati koji govore da električna pražnjenja povećavaju čvrstoću i uzrokuju oksidaciju vlakana (Yuan i sur., 2004; Sinha i Panigrahi, 2009). Nadalje, iznad je spomenuto i povećanje udjela netopivih i ukupnih vlakana koji bi mogli biti direktno povezani s ovim parametrom, odnosno sa smanjenjem meljivosti.

Iz svih rezultata vidljivo je da je došlo do značajne modifikacije i promjene u kemijskom sastavu kakaove ljuske nakon tretmana HVED-om. FTIR-ATR analiza pokazala je da je došlo do promjena na esterskim grupama u pektinu, ali postoje indikacije da je došlo i do promjene na ugljikohidratima. Svi rezultati ukazuju na to da su reaktivni spojevi generirani tijekom HVED tretmana uzrokovali promjene u sastavu kakaove ljuske.

Analiziranjem udjela akrilamida u uzorcima kakaove ljuske došlo se do zaključka da je najveći udio ove nepoželjne komponente imao uzorak koji nije bio tretiran. Svi daljnji tretmani, kontrolni (miješanje u vodi) i HVED, imali su značajan utjecaj na smanjenje udjela akrilamida. Jedan od razloga je što akrilamid ima visoku topljivost u vodi (Farah i sur., 2012), ali isto tako kontrolni uzorci 1,5 %-tna suspenzija 30 min, 1,5 %-tna suspenzija 45 min i 3 %-tna suspenzija 15 min imali su, za razliku od većine uzoraka tretiranih HVED-om, udio akrilamida veći od limita detekcije i kvantifikacije. To ukazuje da je HVED uz vodu imao dodatan utjecaj na smanjenje udjela akrilamida.

Kao i kod akrilamida, udio 5-hidroksimetilfurfurala (5-HMF) je bio najveći u kakaovoj ljusci koja nije bila tretirana iako je u ovom slučaju veći utjecaj na smanjenje udjela ove komponente pokazao kontrolni tretman, a ne HVED. Budući da je nakon HVED-a došlo do potamnjivanja uzoraka, to bi moglo značiti da je 5-HMF sudjelovao u daljnjim Maillardovim reakcijama i da je došlo do stvaranja novih produkata koji nisu topivi u vodi.

Mikrobiološka analiza kakaove ljuske pokazala je da ni u jednom uzorku nije detektirana *Salmonella* spp. Budući da se nakon tretmana HVED-om i sušenjem na 40 °C udio enterobakterija povećao, ispitan je utjecaj sušenja kakaove ljuske na to povećanje. Rezultati su pokazali da je primjenom liofilizacije, koja se provodi pri niskim temperaturama i funkcionira na principu sublimacije vode iz uzorka, udio enterobakterija bio unutar kriterija prihvatljivosti nakon tretmana HVED-om. Iz ovog je vidljivo da sušenje na 40 °C pogoduje razvoju stanica mikroorganizama koje su inaktivirane primjenom HVED-a.

Nakon provedenih istraživanja vezanih za utjecaj HVED-a na svojstva kakaove ljuske, drugi dio istraživanja se fokusirao na utjecaj načina sušenja na zabilježene promjene. Primijenjeni

postupci sušenja su liofilizacija i sušenje u sušioniku pri 60 °C, a tretman HVED-om je proveden na 70 Hz kroz 10 min u koncentraciji 0,5 %.

Liofilizacija je utjecala na manji udio vode i niži aktivitet vode u usporedbi s klasičnim sušenjem. Nakon sušenja u sušioniku rezultati su pokazali da je udio vode u HVED tretiranom uzorku bio niži nego u kontrolnom uzorku (miješanje u vodi). Iz toga se može zaključiti da je elektroporacija uzrokovala lakši izlazak vode iz tretiranog materijala.

Kapaciteti vezanja vode i ulja pokazali su isti trend kao i u prvom dijelu istraživanja: svi tretirani uzorci imali su veći kapacitet vezanja od netretirane kakaove ljuske. Također, istraživanje je pokazalo da postupci sušenja imaju utjecaj na ove parametre budući da je kakaova ljuska sušena u sušioniku imala više vrijednosti kapaciteta vezanja vode, ali niži kapacitet vezanja ulja u usporedbi s liofliziranom kakaovom ljuskom.

Nasipna gustoća i specifični volumen analiziranih uzoraka kakaove ljuske pokazali su da je najveća promjena zabilježena kod uzoraka sušenih liofilizacijom. Također, uzorci tretirani HVED-om imali su nižu nasipnu gustoću u usporedbi s netretiranim što je znak utjecaja tretmana na strukturu i volumen pora kakaove ljuske.

Rezultati utjecaja postupaka sušenja na boju pokazali su da su uzorci sušeni u sušioniku pri 60 °C bili tamniji od uzoraka sušenih liofilizacijom. Sušenje pri višim temperaturama u kombinaciji s HVED-om pokazalo je najveći utjecaj na potamnjivanje uzoraka što je u skladu sa spomenutim reakcijama koje su se mogle dogoditi s 5-HMF-om.

Udio metilksantina i fenolnih komponenti bio je viši u uzorcima koji su sušeni liofilizacijom nego u onima sušenim u sušioniku. Također, potvrđeno je da prilikom sušenja u sušioniku uzorci tretirani HVED-om imaju veći udio (-)-epikatehina i (-)-epikatehin galata od kontrolnih uzoraka (miješanje u vodi). Ukupni udio polifenola bio je najveći u netretiranoj kakaovoj ljusci, a najmanji u kontrolnom uzorku sušenom u sušioniku. Udio tanina je također bio veći u netretiranoj ljusci, iako je pokazao veću otpornost prema tretmanima u usporedbi s ukupnim polifenolima.

Analizom udjela Klason lignina utvrđeno je da se udio ove frakcije povećao u svim tretiranim ljuskama u usporedbi s netretiranom. Literaturni podatci pokazuju da u rezultate ove analize mogu ulaziti i neke komponente koje se ponašaju kao lignin, a nazivaju se pseudo lignin (Shinde i sur., 2018). U prethodnim rezultatima je vidljivo da je došlo do promjena na 5-HMF, a moguće je da je sudjelovao u daljnjim reakcijama tijekom HVED tretmana. Jedan od mogućih puteva formiranja pseudo lignina je zapravo pretvorba 5-HMF-a u druge spojeve (Shinde i sur., 2018). FTIR-ATR analiza u prvom dijelu istraživanja je također pokazala da dolazi do promjena na strukturi lignina budući da je došlo do formacije ramena karakterističnog za aromatske C-H vibracije lignina.

Diferencijalna motridbena kalorimetrija je prilikom analiziranja kakaove ljuske prikazala formiranje egzotermnih vrhova koji su karakteristični za hemicelulozu, celulozu i lignin. Također, utvrđena je razlika u pikovima između tretiranih i netretiranog uzorka. Svi tretirani uzorci pokazali su veću stabilnost, a najveću je imao uzorak koji je bio tretiran HVED-om i liofiliziran. Ovi rezultati u skladu su i s FTIR-ATR spektrima koji su pokazali da se nakon HVED tretmana pojavio veći vrh karakterističan za CH_2- i CH_3-skupine ugljikohidrata.

5. ZAKLJUČCI

Na temelju provedenih istraživanja i dobivenih rezultata mogu se izvesti sljedeći zaključci:

1. Udio teobromina i kafeina u kakaovoj ljusci bio je najviši u usporedbi s ostalim bioaktivnim komponentama. Tretiranje HVED-om uzrokovalo je sniženje količine bioaktivnih spojeva, ali u usporedbi s kontrolnim uzorcima (miješani samo u vodi) HVED tretirani uzorci imali su viši udio ovih spojeva. Za očuvanje komponenti najboljim se pokazao tretman: koncentracija kakaove ljuske u vodi 1,5 %, trajanje tretmana 15 minuta i frekvencija 40 Hz.
2. Udio tanina u kakaovoj ljusci se povećavao produženjem vremena HVED tretmana i pri koncentraciji kakaove ljuske u vodi od 3 %. Osim toga, udio tanina u ukupnim fenolima se također povećao tretiranjem kakaove ljuske. Nadalje, udio vlakana u kakaovoj ljusci se nakon tretmana povećao, pogotovo udio netopivih i ukupnih vlakana, što može ukazati na fizikalne i kemijske promjene tijekom HVED tretmana.
3. Kapacitet vezanja ulja i vode povećao se nakon svih tretmana, a meljivost kakaove ljuske smanjila se HVED tretmanom. Rezultati su pokazali da se pri istim uvjetima mljevenja netretirane i tretiranih kakaovih ljuski udio manjih čestica smanjuje, a udio većih povećava u HVED tretiranim uzorcima. Samim time veličina čestica je mogla izravno utjecati na kapacitet vezanja vode i ulja.
4. Spektri dobiveni FTIR-ATR analizom pokazali su da se tijekom tretmana kakaove ljuske dogodio niz modifikacija koje su utjecale na sastav ljuske. Došlo je do promjena na esterskim grupama pektina, ali i promjena na ugljikohidratima.
5. Tretiranjem kakaove ljuske, bilo miješanjem u vodi ili HVED tretmanom, došlo je do smanjenja udjela akrilamida i 5-HMF-a. Udio akrilamida se više smanjio HVED tretmanom, dok se udio 5-HMF-a više smanjio samo miješanjem u vodi.
6. Mikrobiološka analiza kakaove ljuske pokazala je da HVED tretman i sušenje na 40 °C nisu povoljno utjecali na broj mikroorganizama. Također, utvrđeno je da je razvoj mikroorganizama bio potaknut sušenjem na 40 °C budući da je u uzorku koji je bio HVED tretiran i liofiliziran došlo do smanjenja udjela enterobakterija.
7. Dva postupka sušenja (u sušioniku pri 60 °C i liofilizacija) i HVED tretman pri 70 Hz, 10 min i pri koncentraciji 0,5 % pokazali su sljedeći utjecaj:

- Uzorci sušeni liofilizacijom imali su niži udio vode i aktivitet vode od uzoraka sušenih u sušioniku, a uzroci tretirani HVED-om i sušeni u sušioniku imali su niže vrijednosti ovih parametara u usporedbi s uzorkom koji je sušen u sušioniku i nije tretiran HVED-om.

- Najveći utjecaj na nasipnu gustoću i specifični volumen zabilježen je u liofiliziranim uzorcima.
- Liofilizirani uzorci imali su veći udio bioaktivnih komponenti u usporedbi s uzorcima sušenim u sušioniku. Između uzoraka sušenih pri 60 °C onaj koji je tretiran HVED-om imao je veći udio (-)-epikatehina i (-)-epikatehin galata.
- Svi tretmani uzrokovali su povećanje udjela Klason lignina i termostabilnosti kakaove ljuske.

8. Odabirom uvjeta tretiranja visokonaponskim električnim pražnjenjem i sušenja kakaove ljuske može se ciljano utjecati na funkcionalna svojstva, sigurnost i prehrambenu vrijednost kakaove ljuske, u skladu sa željenom namjenom.

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7. PRILOZI

Dijelovi ove doktorske disertacije objavljeni su u radovima prikazanim u prilogu.

PRILOG 1

sustainability

MDPI

Review

Difficulties with Use of Cocoa Bean Shell in Food Production and High Voltage Electrical Discharge as a Possible Solution

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Received: 7 April 2020; Accepted: 9 May 2020; Published: 13 May 2020

Abstract: The cocoa and chocolate industries have huge problems with the utilization of waste generated during the production process. Waste material generated during production include cocoa pod husk, pulp, and cocoa bean shell. Cocoa shell is a by-product that has great potential because of its composition. It consists of dietary fibers, proteins, polyphenols, methylxanthines, etc. However, despite its favorable composition, cocoa shell often cannot be used directly in food production because it may contain components that are harmful for human health. Cocoa shell can carry mycotoxins, different microorganisms, polycyclic aromatic hydrocarbons, and heavy metals. High voltage electrical discharge presents a novel non-thermal method that has great potential for the decontamination of waste materials and can also be used for extraction of valuable compounds from cocoa shell.

Keywords: cocoa shell; HVED; waste material; sustainable production

1. Introduction

The cocoa industry faces fluctuation of cocoa bean price and social and political instabilities in producing countries. The environmental aspects of the cocoa industry present some relevant issues, and one of them is management of waste material generated during production [1,2].

The market for functional food is constantly expanding, so it is not surprising that agro-industrial wastes are seen as new ingredients for this type of products [3]. Economic, social, and environmental sustainability is the goal of every food production, including that of the chocolate industry. Many institutions want to ensure waste management to achieve this goal [4].

Cocoa shell is a by-product of the cocoa industry that has a high nutritional value. It can be used in the food industry, as well as in pharmaceutical, cosmetic, and agricultural industries. A more detailed review of its use in different industries is given in the paper by Panak Balentić et al. [5].

The production of cocoa beans can be divided into three stages:

- Growing, harvesting, and pre-processing;
- Primary cocoa processing and production of semi-finished products; and
- Chocolate industry—manufacturing of finished products [6].

High voltage electrical discharge (HVED) has become very interesting to many scientists because it can degrade organic compounds and inactivate bacteria, viruses, and yeasts [7]. This treatment leads to a number of chemical and physical processes: production of ultraviolet light, shock waves, production of reactive species, etc. [8]. All these changes are responsible for HVED's capability to be a disinfection and extraction technology.

This review shows the benefits and shortcomings of using cocoa shell in food production. A possible solution for problems that occur with the use of cocoa shell in food production is stated.

2. Cocoa Shell

The cocoa industry generates large amounts of waste that consist of pod husk, pulp, and bean shell. Namely, cocoa beans, which are the main ingredient in chocolate production, are removed from the cocoa pod, after which they are fermented and dried. Cocoa bean shell is removed from seeds before or after roasting of the beans [9].

After the separation of the shell from the seed, it is usually discarded or sold as agricultural mulch. Since the shell presents 12%-20% of the bean, it is obvious that this is the largest waste generated after processing the beans [9–11]. According to International Cocoa Organization [12], the world generation of cocoa waste can be estimated to 700 thousand tons per year.

Cocoa shell has an interesting composition. It is rich in dietary fibers, proteins [13], polyphenols [14,15], methylxanthines [16], etc.

Dietary fibers are generally divided into soluble and insoluble fibers. The soluble/insoluble ratio is very important in human nutrition, and cocoa bean shell has a ratio close to desirable, giving it potential for direct implementation in food [17]. Dietary fibers of cocoa shell are mainly composed of pectin and cellulose [13]. In addition, cocoa shell is rich in flavanols (catechin and epicatechin), which have an antioxidant activity, and methylxanthines (theobromine and caffeine), which have an effect on the human nervous system [18,19]. Okiyama et al. [20] investigated the lipid profile of cocoa shell and concluded that it is similar to that of cocoa butter, which could lead to its application as a partial substitute for cocoa butter.

2.1. Use of Cocoa Shell in Food Production

Cocoa shell composition has driven many scientists into implementing cocoa shell directly in food products and investigating the properties and sensorial acceptance of the obtained products. In addition, there are research that investigated the application of different components of cocoa shell as a food ingredient. This subsection gives an overview of papers that have addressed this topic.

Martinez-Cervera et al. [21] used soluble dietary fiber extracted from cocoa shell in the production of chocolate muffins. Fibers were used as fat replacers and the results showed decreased hardening during storage, good texture, higher moisture, and pleasant color of enriched muffins. Soluble dietary fibers from cocoa shell were also used in production of wheat bread, showing a softening effect [22]. It was concluded that these fibers can be used up to 6% and do not have a negative effect on sensory acceptability and storage of bread. Enrichment of products like muffins and bread, which are consumed often, with dietary fibers from the cocoa shell can have beneficial effects on glucose absorption, as was shown in an in vitro study by Nsor-Atindana et al. [23].

Mazzutti et al. [19] used cocoa shell to obtain lipid-enriched extract and phenolic-rich extract. These products showed great potential for incorporation in food products. Another study conducted with extracts of cocoa shell aimed to protect polyphenols that are present in this kind of extract. Papillo et al. [24] used spray-drying with maltodextrins to achieve this. Results showed that polyphenols in these extracts were protected during baking and storage.

Alkalized cocoa shell has also found its way into food production. Bernaert and Rysscher [25] used it for production of a cocoa beverage with a unique taste and rich in dietary fibers. In another study, they concluded that cocoa shell powder could be used in different food products as a replacement for cocoa powder [26]. Alkalized cocoa shell was used also in the production of cookies, and the obtained

product showed higher resistance to breaking compared to wheat cookies [27]. Another study was conducted to investigate functional beverages with cocoa shell [28], where beverages with the highest content of bioactive compounds were the least appreciated by consumers. This was probably because of polyphenols and methylxanthines that give an astringent taste to these products.

Some direct implementations of cocoa shell in food products without previous processing include the production of pork sausages [29] and extruded snack products [30]. Pork sausages with levels of cocoa shell of 1% or lower had improved color, viscosity, moisture content, and emulsion stability. An interesting discovery was also that the addition of cocoa bean shell could inhibit lipid oxidation in these kinds of products. Jozinović et al. [30] added cocoa shell in extruded snack products in amounts of 5%, 10%, and 15%. This enrichment increased resistant starch and polyphenol content. Although physical properties were slightly poorer than in conventional products, they were still acceptable.

Cocoa shell will also be interesting for incorporation in chocolates because it would not need to be transported from chocolate factories. It would be directly used in chocolate production, which would decrease the cost of its use. A great deal of research has been done with focus on enrichment of chocolates with fiber sources where they replaced sugar or fat [31]. This gives promising hope that the use of cocoa shell in chocolate production could come to life.

2.2. Problems with Use of Cocoa Shell in Food Production

Since it is obvious that cocoa bean shell has great potential and it is rich in many bioactive components that can benefit human health, why is it not used in food production yet? One of the reasons is that cocoa shell may contain undesirable components that need to be removed before its incorporation in food products. Some of these components are mycotoxins, heavy metals, polycyclic aromatic hydrocarbons (PAHs), and microorganisms.

2.2.1. Mycotoxins

Cocoa beans are fermented, dried, and stored most commonly in unhygienic conditions. That is obvious from the fact that they are often contaminated with *Aspergillus*, *Eurotium*, and *Absidia* species [10]. Copetti et al. [32] reported that ochratoxin A, which is produced by fungi of *Aspergillus* and *Penicillium* genera, are concentrated in cocoa shell. This toxin is present in a wide variety of foods like coffee beans, dried fruit, and cereals [33]. Only a small part of this toxin is present in cocoa nibs. Aflatoxins B1, B2, G1, and G2 have been found in cocoa shell. It was concluded that they appeared more frequently in cocoa shell than other parts of bean, and that they appeared in 11% of the samples [34]. These components are very stable and cannot be completely destroyed during processes conducted during the production of chocolate [35].

2.2.2. Heavy Metals

Cocoa bean may be contaminated with heavy metals because of environmental and external influences [36]. A major concern is the presence of nickel (Ni), cadmium (Cd), chromium (Cr), and lead (Pb) [37]. Most research mentioned below were conducted to examine heavy metal contents in chocolate and cocoa products [38,39]. Increased contamination was mostly because of the use of fertilizers, pesticides, insecticides, etc. If these activities are not controlled and managed according to Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP), they can lead to increased content of heavy metals [40]. Additionally, fermentation, drying, crushing, and contact with metal devices during processing can affect the content of heavy metals [41].

Cocoa shell in most cases has higher content of these compounds because of its high absorption capacity. This characteristic is used in a few researches to examine cocoa shell as a new adsorbent for the removal of heavy metals from polluted water [42,43].

2.2.3. Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are known as genotoxic carcinogens and in cocoa beans can be produced during drying and roasting [44,45]. They are formed in roasted foods rich in carbohydrates through two processes: pyrolysis and pyrosynthesis [46,47]. The increased content of PAHs in cocoa beans is most often the result of inappropriate drying. The highest risk of contamination is present in artificial drying when producers use firewood or fossil fuel [48]. In addition, Ciecierska [45] concluded that even low temperatures during roasting of beans favor the development of PAHs. Since, most often, cocoa beans are roasted with cocoa shell, there is a high possibility that the cocoa shell is also contaminated with PAHs. Agus et al. [49] reported that roasted cocoa shell had lower amounts of PAHs than dried cocoa shell. This could be due to migration of these compounds in cocoa nibs during roasting.

2.2.4. Microorganisms

During drying in cocoa farms, birds and insects frequently come into contact with cocoa seeds. They are transmitters of *Escherichia coli* and *Salmonella* [6]. Although cocoa beans are subjected to roasting, the research of Izurieta and Komitopoulou [50] showed that *Salmonella* strains present on cocoa shell were heat resistant. To minimize risk of contamination of cocoa beans, good hygiene and manufacturing practices should be implemented in cocoa farms. Leaving cocoa beans unprotected should be avoided so that contact with transmitters of contamination can be reduced [51].

3. High Voltage Electrical Discharge (HVED)

High voltage electrical discharge treatment is a low-energy and non-thermal technology that has great potential for use in the utilization of by-products in the food industry.

3.1. Work Principle of HVED Device

HVED technology is based on generation of electric discharges directly in water. This contact generates physio-chemical changes and chemical processes in water [52]. Applying high voltage and intensity pulses of short duration between two electrodes submerged in liquid leads to ionization. During this process, three phases occur: electric pulse generation, current discharge, and electric arc formation [53]. The process is accelerated with bubbles present in the solution or generated during localized heating. Electron avalanche from high-voltage- to ground electrode will occur if the potential difference between electrodes is sufficient. During this process, due to large amounts of energy used, a number of oxidizing species is generated [4].

HVED systems can be divided into batch, continuous, and circulating systems. The basic mechanism of these three systems is the same, but local electric field concentration modes are different [54].

For use of HVED technology in extraction or any other treatment, there are two important parameters: total HVED treatment duration (t_{HVED}) (Equation (1)) and HVED energy input (w_{HVED}) (Equation (2)) [54]:

$$t_{HVED}(s) = n \times t_i \tag{1}$$

where n is number of discharges and t_i is discharge duration (s).

$$w_{HVED}\left(\frac{kJ}{kg}\right) = \frac{E_p \times n}{m} \tag{2}$$

where E_p is energy of one pulse (kJ), n is number of discharges, and m is mass of suspension (kg).

During HVED treatment, photonic dissociation of water occurs, which leads to emission of UV-light and $OH^{\cdot}$ radicals. The UV-light can inactivate cells by damaging DNA and the created shock waves can fragmentize tissue of products that are treated. The electric strength of the field is directly proportional to the poration of the cell membrane, and this phenomenon is called electroporation [54,55].

3.2. Description of HVED Device

Since there are many custom-made devices that are used in research and some of them are well described by Takaki et al. [56], only the high voltage electrical discharge device (Figure 1) that was custom made for Faculty of Food Technology Osijek by Ingeniare CPTS1 is described in this subsection as an example. The pulse generator of high voltage contains a 30 kV impulse generator with a variable pulse frequency of 20 Hz to 100 Hz. The impulse generator can be replaced so that the voltage can be adjusted according to the needs of the experiment. The generator scheme (Figure 2) shows that it consists of a high-voltage DC generator, an energy tank (capacitor), a high-voltage switch, a chamber, and an automatic control unit. The automatic control unit provides capability to control time of treatment, pulse frequency, and mixing speed.

Figure 1. High voltage electrical discharge device.

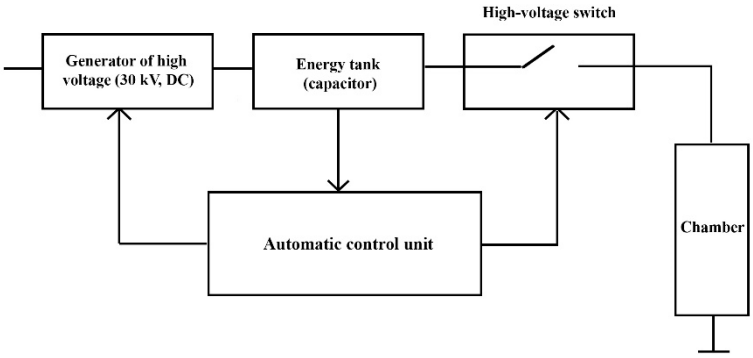

Figure 2. Scheme of generator.

High voltage electrodes are located in the treatment chamber and are attached to the electrode carrier with the ability to adjust the distance between the electrodes. The high voltage INOX electrode and the plate or ground electrode (45 mm) are immersed in the solution during the treatment. The height of the electrodes can also be adjusted. The distance between the electrodes is adjusted according to the conductivity of the sample that will be treated, which depends on both the type of sample and the concentration of the solution. The conductivity of the solution must be measured before the treatment. The effect of water conductivity on formation of shock waves was studied by Cathignol et al. [57].

3.3. HVED Application

HVED is mostly used for the extraction of polyphenols, proteins, and pectin from waste materials [4,58,59]. In this paper, we want to show other perspectives of this technology that

will open the possibility of using HVED as a decontamination and extraction technology at the same time.

HVED can be used for inactivation of bacteria like *Sallmonella* spp., *Eschericia coli*, *Listeria*, etc. [60]. Zhao et al. [61] designed an atmospheric air dielectric barrier discharge device, which they used to uniformly decontaminate fruit surface. They reported satisfactory results for the removal of *S. aureus* without damaging the fruit surface. In addition, there are many examples of effective use of cold plasma in the removal of mycotoxins and fungi, even damaging spores [62]. These imply that HVED could also have such an effect in liquid media. Anpilov et al. [63] reported that HVED was effective in the destruction of *Escherichia coli* in water, probably because of the generation of different radicals and UV radiation. Reactive oxygen species and hydrogen peroxide produced during treatment induce oxidative stress that has a major effect on microbial inactivation. These products oxidize membrane components of microorganisms [64]. Some researchers concluded that the efficiency of HVED is due to the combination of physical, chemical, and electrical effects, and not just one factor [65]. During treatment, the formation of hydrogen peroxide occurs, which further leads to the formation of H_3O^+, which is mostly responsible for a decrease of pH. Bacteria such as *Escherichia coli* is especially sensitive to low pH, which leads to its disruption [66]. However, pH alone could not be responsible for the inactivation of these proportions [8]. The combination of UV light and H_2O_2 can lead to the mutation and damage of DNA. Ozone is an additional factor that contributes to disinfection possibilities of HVED, since it is a known disinfectant [67].

HVED treatment of cocoa shell conducted on the device described in this paper showed that this is a good procedure for the removal of 5-hydroxymethylfurfural and acrylamide [68]. It is well established that acrylamide is carcinogenic and is even regulated in the EU for different types of food products. Tessier et al. [69] reported decontamination of PAHs with corona discharge. This process was conducted in solid phase so it is not certain in which way these compounds will behave in water during discharge. Further research is needed to see if this process may successfully remove PAHs from cocoa bean shell. It is known that this technology can also be used for the removal of different organic impurities from water, possibly due to the same reasons [4]. The potential of HVED for decontamination of materials and liquids lies in the fact that species (O, OH, O_3) that are generated during discharges in liquid are very active. The radicals that are created by dissociation of water can oxidize organic compounds that are present in material or liquid that is treated, and can therefore remove them [70,71]. During electrical treatment, advanced oxidation processes are known to degrade a range of organic compounds [72]. Hydroxyl radical, ozone, and hydrogen peroxide are species that directly attack organic compounds [73]. Du et al. [74] also managed to reduce 74.4% of PAHs during arc discharge. They proposed that OH probably reacted with the aromatic ring, where further reaction with oxygen resulted in ring-cleavage products.

Plasma discharges are known for their possibility of removing fungi and mycotoxins [62]. Ouf et al. [75] eliminated ochratoxin A with cold plasma after 7.5 min. Plasma-induced reactive species are mainly responsible for decontamination, but UV light also plays an important role. Park et al. [76] managed to completely degrade aflatoxins, ochratoxins, and deoxynivalenol with cold plasma treatment. However, mycotoxins in food matrix could be a little more difficult to degrade because the matrix could slow down the effect of plasma and react with part of the reactive species [77]. It was reported that this treatment destroys the integrity of cellular structure of *Aspergillus* spores [78].

Discharge treatment proved to be effective in decreasing the content of Pb, Cd, Fe, and Mn in waste water, probably due to formation of insoluble oxides and hydroxides [79]. Rincon and Motta [80] also managed to remove zinc, copper, and nickel from waste water with an electro coagulation method. This implies that the process could be effective in the removal of metals from cocoa shell.

Since HVED technology is mostly used for extraction, this can be an additional reason for using this method. It would be economically viable to use the extract obtained after cocoa shell treatment to produce bioactive components, proteins, or pectin. Jokić et al. [81] used this method for the extraction of polyphenols and methylxanthines. In contrast to this study, where the cocoa shell was milled,

Barišić et al. [15] came to the conclusion that unmilled cocoa shell treated with HVED had a higher proportion of polyphenols and methylxanthines than control samples mixed in water. Additionally, HVED extraction of proteins from *Camellia olcifera* seed cake [82] and pectin from sugar beet pulp [83] was conducted.

When compared to other conventional extraction techniques, HVED was shown as a great process to obtain higher phenolic content in extracts from olive leaves [84] and grape seeds [85]. In the case of grape seeds, HVED also affected the size of grape seeds because of the generated shocks that can disrupt tissue and cellular structures [86]. At the same time, it was less selective regarding the amount of anthocyanins recovered during the extraction.

Due to its great potential for extraction of various biocomponents, shorter treatment, and less thermal destructiveness compared to other techniques, HVED has already proven to be an excellent extraction technique. If we also take into consideration the potential for decontamination of water and different biomaterials, this technology could replace several technologies currently present in the industry, not only for treatment of cocoa shell, but for other waste materials as well.

4. Conclusions

The cocoa industry has a large problem with the disposal of waste generated during production. One of the most interesting waste materials is cocoa bean shell. It is rich in dietary fibers, bioactive compounds, proteins, etc.—compounds that can benefit human health. However, cocoa shell may contain some undesirable compounds that need to be removed before incorporation into food products. High voltage electrical discharge treatment was shown as a promising non-thermal technology for the utilization of food by-products, which can solve the problem of harmful compounds present in cocoa shell. Different types of HVED treatment, including plasma, have been shown to be efficient in the decontamination of various food products, including the destruction of microorganisms both pathogenic and spoilage, and removal of heavy metals, PAHs, HMF, and acrylamide. Future research should focus on revealing actual mechanisms of HVED on specific compounds and microorganisms, and on the exploration of optimal conditions of treatment regarding the desired effect, but negative effects (such as potential release of metals from electrodes) should not be disregarded.

Author Contributions: All authors contributed to the conceptualization and implementation of the research study; writing—original draft preparation, V.B., A.J., I.F., and Đ.A.; writing—review and editing, B.M., D.Š., J.B., and K.D. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been supported in part by Croatian Science Foundation under the project "Application of cocoa husk in production of chocolate and chocolate-like products" (UIP 2017-05-8709).

Conflicts of Interest: The authors declare no conflict of interest.

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PRILOG 2

Received: 24 December 2018 | Revised: 6 March 2019 | Accepted: 16 March 2019

DOI: 10.1111/jfpe.13057

Journal of Food Process Engineering WILEY

ORIGINAL ARTICLE

Impact of high-voltage electric discharge treatment on cocoa shell phenolic components and methylxanthines

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Funding information
Hrvatska Zaklada za Znanost, Grant/Award Number: 2017-05-8709; Croatian Science Foundation, Grant/Award Number: 2017-05-8709

Abstract

Cocoa shell is one of the by-products obtained in chocolate industry which was considered as waste for a very long time. Recently, the presence of high-valuable bioactive components in cocoa shell was recognized, and research on the pallet of food products enriched with cocoa shell is increasing. The most abundant bioactive components of untreated cocoa shell (UCS) are theobromine (3.906 ± 0.070 mg/g), caffeine (0.646 ± 0.055 mg/g), and (+)-catechin (0.290 ± 0.005 mg/g), followed by gallic acid (0.147 ± 0.041 mg/g) and (−)-epicatechin (0.165 ± 0.099 mg/g). Furthermore, the impact of concentration (1.5 and 3% of cocoa shell in water), high-voltage electric discharge (HVED) treatment (40 and 80 Hz), and treatment time (15, 30, and 45 min, respectively) on bioactive compounds content was evaluated. Statistically significant differences between treatment conditions were obtained. Generally, water-HVED treatment at 40 Hz has lower impact on bioactive components than water, and water-HVED at 80 Hz, therefore, can have a significant effect on future treatments of materials rich in bioactive compounds.

Practical applications

HVED is an innovative nonthermal processing technique that has mechanical and electrical effect on the product resulting with lower costs and increase of functional properties. This study has shown that HVED causes lower decrease of phenolics and methylxanthines in cocoa shell which can have a significant effect on future treatments of materials rich in bioactive compounds. Because it is not recorded in scientific literature that HVED treatment was used on cocoa shell so far, it will help in future research and applications of cocoa shell in the food industry.

1 | INTRODUCTION

Solving the problem of increasing food and packaging waste has become one of the most essential assignments for today's food industry. Usage of by-products from cocoa beans production as raw materials for other industries is just one of the several good examples that fulfill both economic and environmental aspects of food waste problem (Martínez et al., 2012; Okiyama, Navarro, & Rodrigues, 2017; Panak Balentić et al., 2018). Cocoa shell is a part of the cocoa bean that is separated from cotyledon during pre-roasting or after the roasting of beans and represents up to 20% of cocoa bean (Awarikabey, Amponsah, & Woode, 2014; Okiyama et al., 2017). Several studies have shown that cocoa shell can be used as garden mulch, adsorbent, feedstuff, fuel (Fiset, Tyagi, & Bais, 2002; Bentil, Dzogbefia, & Alemawor, 2015; Panak Balentić et al., 2018) but also as an ingredient in food industry (Arlorio, Coisson, Restani, & Martelli,

2001). Due to the high content of valuable nutritive components (e.g., phenolics, dietary fibers, methylxanthines, vitamin D), production of functional products enriched with cocoa shell is constantly growing (Jozinović et al., 2017; Lecumberri et al., 2007; Nsor-Atindana, Zhong, & Mothibe, 2012). Major bioactive components of cocoa shell are methylxanthines, theobromine, and caffeine, which migrate from cotyledon to cocoa shell during fermentation and phenolics, mostly flavonols catechin and epicatechin, and condensed procyanidins (Bonvehí & Jordà, 1998; Hernández-Hernández, Viera-Alcaide, Sillero, Fernández-Bolaños, & Rodríguez-Gutiérrez, 2017). Phenolics present in cocoa shell are responsible for its antioxidant and antimicrobial activity as shown in several studies (Arlorio et al., 2001; Nsor-Atindana et al., 2012) but also are responsible for bitter and astringent taste (Bonvehí & Jordà, 1998; Okiyama et al., 2017). Methylxanthines present in cocoa bean and shell are mostly characterized as potentially harmful even poisonous components, but if consumed in small amounts, they can act as health-promoting agents due to the anticancer, antioxidant, diuretic, and smooth-muscle relaxing properties (Arlorio et al., 2001; Hartati, 2010; Panak Balentić et al., 2018).

High-voltage electric discharge (HVED) is one of the innovative techniques that are proposed as alternative for conventional processing techniques due to the shorter processing time, increased recovery yield, control of the Maillard reactions, improvement of products quality, as well as enhanced functional properties of extracts (Galanakis, 2013; Li, Fan, & Xi, 2019). HVED is used for decontamination of food, the treatment of waste products from the food industry, and extraction of bioactive components, mainly phenolics and proteins, from different foodstuffs (Parniakov, Barba, Grimi, Lebovka, & Vorobiev, 2014a; Roselló-Sotto et al., 2015; Brianceau, Turk, Vitrac, & Vorobiev, 2016; Puértolas & Barba, 2016; Puértolas, Koubaa, & Barba, 2016; Xi, He, & Yan, 2017; Li et al., 2019), but, to the best authors' knowledge, the influence of HVED on retention of bioactive components in cocoa shell is not available in scientific literature.

Therefore, the aim of this study was to evaluate the impact of high-voltage electric discharge treatment on phenolics and methylxanthines of cocoa shell retaining after the treatment with HVED in water suspension.

2 | MATERIALS AND METHODS

2.1 | Materials

Cocoa beans were roasted at 135°C for 55 min, and afterward, the cocoa shell was separated from the cotyledon. Roasted cocoa shell (UCS) was grinded in the laboratory mill (IKA, M20) and stored in freezer until analysis.

Determination of six phenolic components (gallic acid, caffeic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate) and two methylxanthines (theobromine and caffeine) in cocoa shell was performed using HPLC method with absorbance detection. All phenolics and methylxanthines standards were suitable for HPLC analysis and purchased from Sigma-Aldrich (St. Louis, MO). Methanol (J.T. Baker, Netherlands), *n*-hexane (Carlo Erba Reagenst, Spain), and formic acid (Scharlau Chemie, Spain) were HPLC grade.

2.2 | Treatments

Water solutions of unmilled cocoa shell (1.5 and 3%) were prepared and treated with HVED at 40 and 80 Hz for 15, 30, and 45 min, respectively. HVED equipment consisted of a chamber connected to a high-voltage pulse generator of 30 kV (the device was custom made by Inganiare CPTS1 for Faculty of Food Technology Osijek). Treatment chamber is equipped with a stainless steel cylindrical needle (diameter 2.5 mm) and the ground electrode in the form of a plate (diameter 45 mm). The distance between the electrodes during all treatments was 2 cm. Shearing of samples is achieved by magnetic stirrer.

To evaluate the impact of HVED on bioactive components of cocoa shell, the control samples were prepared. The control samples represented water solutions of unmilled cocoa shell at equal concentrations mixed in magnetic stirrer for 15, 30, and 45 min.

After treatments, cocoa shell was dried at 40°C in a laboratory oven (Memmert, UFE 500), grinded to obtain a fine powder, and stored in a freezer until analysis.

2.3 | Determination of phenolic components and methylxanthines using HPLC method

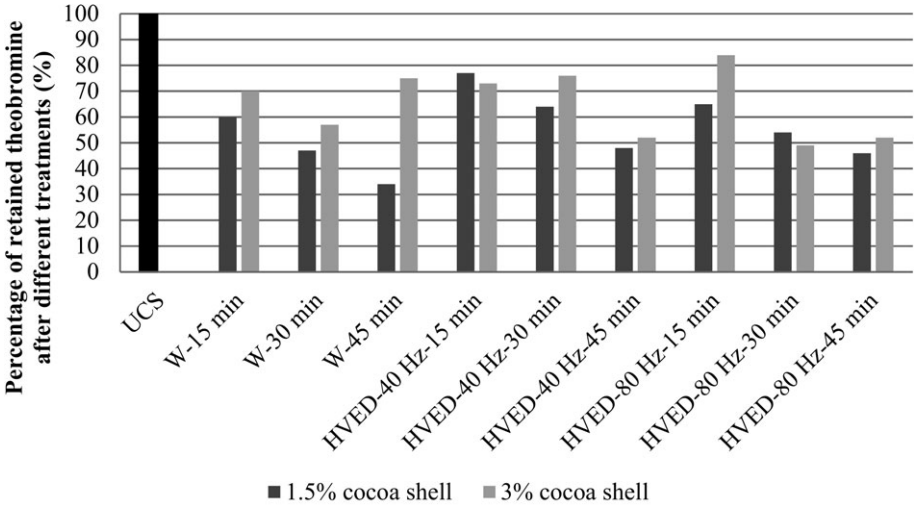
Cocoa shell samples were prepared according to the method described by Adamson et al. (1999). Two grams (± 0.01 g) of cocoa shell was extracted three times with 10 mL of HPLC grade *n*-hexane to eliminate lipids. Defatted cocoa shell samples were then air dried. The extraction of phenolic components and methylxanthines was performed by addition of 5 mL of 70% methanol, ultrasonication for 30 min, and centrifugation (10 min at 3000 rpm). The supernatant was transferred into 10 mL flask. Extraction procedure was repeated once more, the collected supernatants were combined, and the flask filled up with 70% methanol to 10 mL. Extracts were stored in freezer until analysis. Before injection, extracts were filtered through 0.45-μm nylon membrane filter.

The original method used in this study was HPLC method described by Belščak, Komes, Horžić, Kovačević-Ganić, and Karlović (2009). The chromatographic conditions were modified and optimized for applied chromatographic column and instrument. Analysis was performed on liquid chromatographic system consisting of Shimadzu LC-20AD solvent delivery module, Shimadzu CTO-20AC column oven, Shimadzu autosampler SIL-10AF, and Shimadzu SPD-M20A photodiode array detector coupled to a computer with LabSolution Lite software (Release 5.52). HPLC column Inertsil ODS-3V (GL Sciences, 250 mm × 4.6 mm, 5 μm particle size) was used for separation. Mobile phase was composed of 1% formic acid (solvent A) and HPLC grade methanol (solvent B). Mobile phase flow rate was 0.8 mL/min, and gradient elution was performed. Starting percentage of solvent B in mobile phase was 10%, followed by linear increase to 32% B at 15 min, 40% B at 20 min up to 25 min, and 60% B at 30 min. Injection volume was 20 μL. The column and detector temperatures were set at 30°C. Monitoring wavelength range was 200–400 nm, whereas the detection wavelength was set at 278 nm. Identification of phenolic components and methylxanthines was achieved based on the retention time and comparison of absorbance spectrum with those of pure components. Quantification of

TABLE 1 Average values and standard deviations of analyzed bioactive components in cocoa shell before and after treatments

		TEO (mg/g)	CAF (mg/g)	CAT (mg/g)	EPI (mg/g)	EPG (mg/g)	GA (mg/g)	CA (mg/g)	*p*-CA (mg/g)
Untreated cocoa shell (UCS)		3.906 ± 0.070	0.646 ± 0.055	0.290 ± 0.005	0.165 ± 0.099	0.009 ± 0	0.147 ± 0.041	0.004 ± 0.001	0.017 ± 0.002
Treatment conditions									
1.5% of cocoa shell	W-15 min	2.335 ± 0.721	0.296 ± 0.066	0.026 ± 0.009	0.037 ± 0.004	0.004 ± 0.001	0.011 ± 0.002	n.d.	n.d.
	W-30 min	1.832 ± 0.195	0.253 ± 0.027	0.030 ± 0.005	0.033 ± 0.003	0.004 ± 0.001	0.009 ± 0.002	n.d.	n.d.
	W-45 min	1.334 ± 0.161	0.814 ± 0.023	0.017 ± 0.002	0.029 ± 0.002	0.003 ± 0	0.004 ± 0.001	n.d.	n.d.
	HVED-40 Hz-15 min	3.008 ± 0.109	0.462 ± 0.024	0.054 ± 0.003	0.074 ± 0.003	0.008 ± 0	0.021 ± 0.002	n.d.	0.008 ± 0.007
	HVED-40 Hz-30 min	2.481 ± 0.089	0.364 ± 0.019	0.058 ± 0.003	0.055 ± 0.002	0.006 ± 0	0.014 ± 0.001	n.d.	n.d.
	HVED-40 Hz-45 min	1.868 ± 0.323	0.271 ± 0.053	0.036 ± 0.005	0.041 ± 0.006	0.004 ± 0	0.008 ± 0.003	n.d.	n.d.
	HVED-80 Hz-15 min	2.547 ± 0.389	0.366 ± 0.068	0.048 ± 0.014	0.059 ± 0.012	0.006 ± 0.001	0.015 ± 0.005	n.d.	n.d.
	HVED-80 Hz-30 min	2.122 ± 0.224	0.297 ± 0.036	0.045 ± 0.005	0.049 ± 0.004	0.006 ± 0.001	0.010 ± 0.002	n.d.	n.d.
	HVED-80 Hz-45 min	1.813 ± 0.022	0.234 ± 0.007	0.030 ± 0	0.042 ± 0.001	0.004 ± 0	0.007 ± 0	n.d.	n.d.
3% of cocoa shell	W-15 min	2.739 ± 0.185	0.388 ± 0.035	0.077 ± 0.006	0.059 ± 0.004	0.006 ± 0.001	0.027 ± 0.003	n.d.	0.014 ± 0.001
	W-30 min	2.209 ± 0.020	0.287 ± 0.005	0.053 ± 0	0.043 ± 0	0.005 ± 0	0.015 ± 0	n.d.	n.d.
	W-45 min	2.933 ± 0.051	0.434 ± 0.006	0.074 ± 0.001	0.054 ± 0.001	0.006 ± 0	0.021 ± 0.001	n.d.	0.013 ± 0
	HVED-40 Hz-15 min	2.832 ± 0.017	0.439 ± 0.009	0.064 ± 0.001	0.074 ± 0.001	0.007 ± 0	0.022 ± 0	n.d.	0.013 ± 0
	HVED-40 Hz-30 min	2.974 ± 0.031	0.467 ± 0.003	0.065 ± 0.001	0.074 ± 0.001	0.006 ± 0	0.020 ± 0	n.d.	0.013 ± 0
	HVED-40 Hz-45 min	2.032 ± 0.124	0.283 ± 0.024	0.037 ± 0.002	0.051 ± 0.001	0.004 ± 0	0.010 ± 0.002	n.d.	n.d.
	HVED-80 Hz-15 min	3.283 ± 0.020	0.549 ± 0.002	0.044 ± 0.001	0.085 ± 0	0.007 ± 0	0.027 ± 0	n.d.	0.014 ± 0
	HVED-80 Hz-30 min	1.928 ± 0.060	0.248 ± 0.011	0.037 ± 0.001	0.051 ± 0.001	0.005 ± 0	0.009 ± 0.001	n.d.	n.d.
	HVED-80 Hz-45 min	2.036 ± 0.081	0.258 ± 0.008	0.039 ± 0.002	0.050 ± 0.002	0.005 ± 0	0.011 ± 0	n.d.	n.d.

TEO, theobromine; CAF, caffeine; CAT, (+)-catechin; EPI, (−)-epicatechin; EPG, (−)-epicatechin gallate; GA, gallic acid; CA, caffeic acid; *p*-CA, *p*-coumaric acid; n.d., not determined.



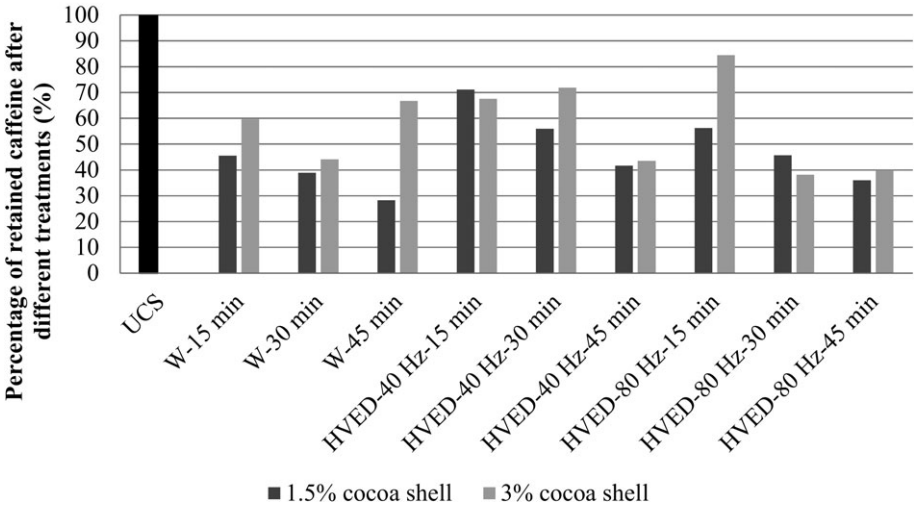


FIGURE 1 Percentage of retained methylxanthines in cocoa shell after different treatments

identified components was performed with external calibration method. All analyses were done in triplicate. The results were expressed as mg of specific component per g of cocoa shell (mg/g).

2.4 | Statistical analysis

All analyses were performed in triplicate. For each evaluated compound, average values and standard deviations were calculated. Furthermore, to evaluate statistical difference between treatment conditions, factorial analysis of variance (ANOVA) was performed using software STATISTICA® 13.3 (Dell Inc., Round Rock, TX). Differences <0.05 were considered significant.

3 | RESULTS AND DISCUSSION

The results of phenolic components (gallic acid, caffeic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate) and methylxanthines (theobromine and caffeine) in UCS, as well as the content of analyzed component after different treatments were presented in Table 1. Cocoa shell is rich with phenolic compounds and methylxanthines that migrate from cocoa cotyledons and bean during fermentation and roasting process. Further degradation of bioactive components can be observed during manipulation of cocoa shell (Hernández-Hernández et al., 2017; Kim & Keeney, 1984; Martínez et al., 2012). High-phenolic content and consequently high antioxidant activity, together with high-fiber content, reveal the potential of cocoa shell addition as a functional component to production of enriched chocolate-like and other food products. Major bioactive components of UCS are methylxanthines, theobromine with average value 3.906 ± 0.070 mg/g, and caffeine with average value 0.646 ± 0.055 mg/g. The results obtained in this study are in compliance with literature data (Bonvehí & Jordà, 1998; Hartati, 2010). Adamafio (2013) reported that theobromine in cocoa shell can be presented up to 21 g/kg, but that theobromine concentration is highly dependent on cocoa bean origin, fermentation, and roasting processing conditions. (+)-catechin was the most abundant phenolic component in cocoa shell (average 0.290 ± 0.005 mg/g) followed by (−)-epicatechin and gallic acid with average values 0.165 ± 0.099 mg/g and 0.147 ± 0.041 mg/g, respectively. Hernández-Hernández et al. (2017) reported

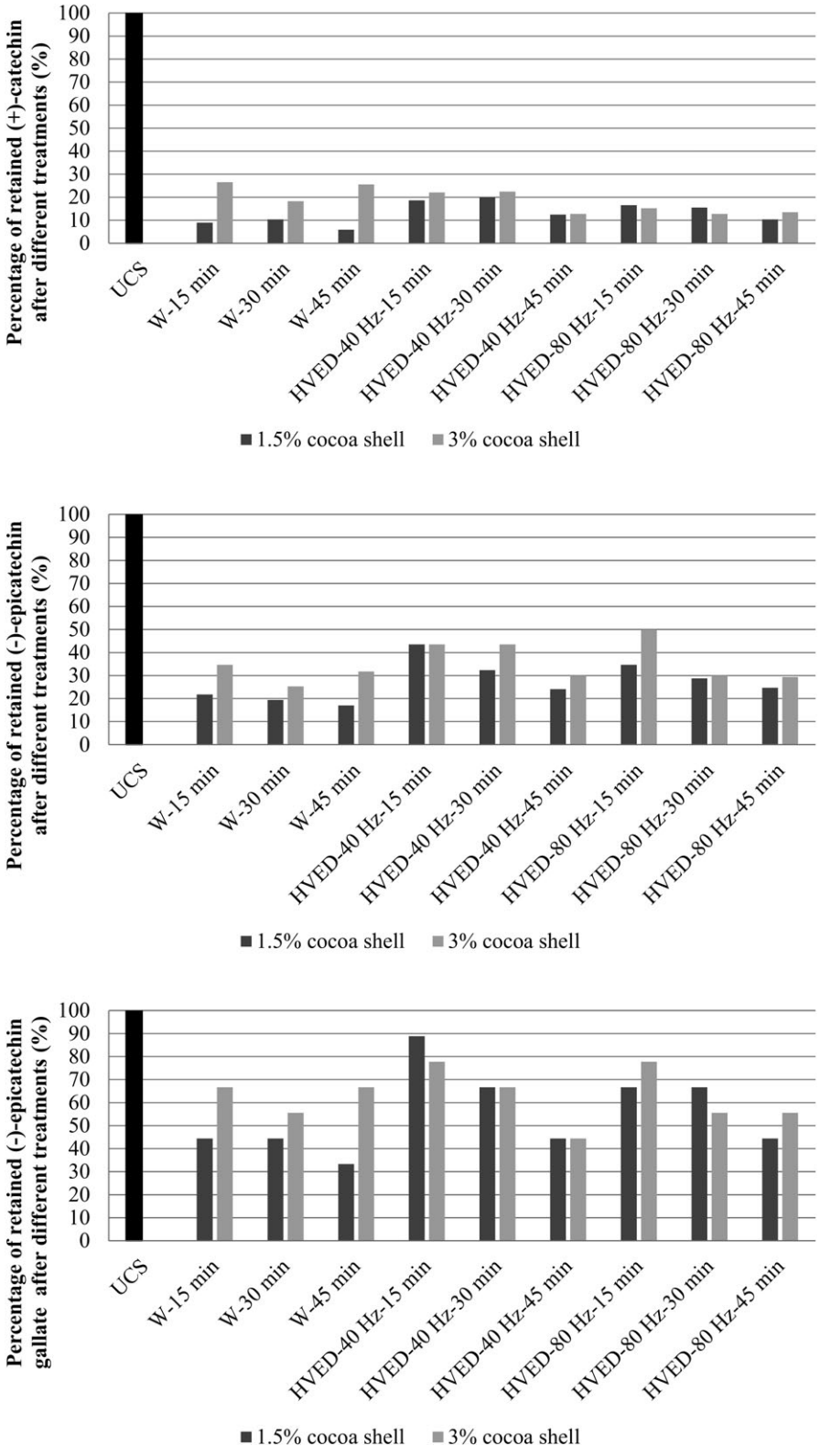
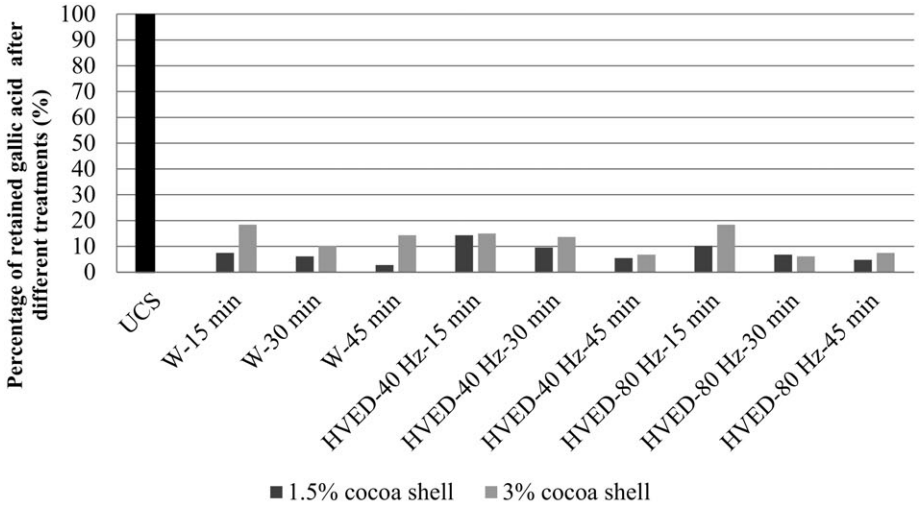
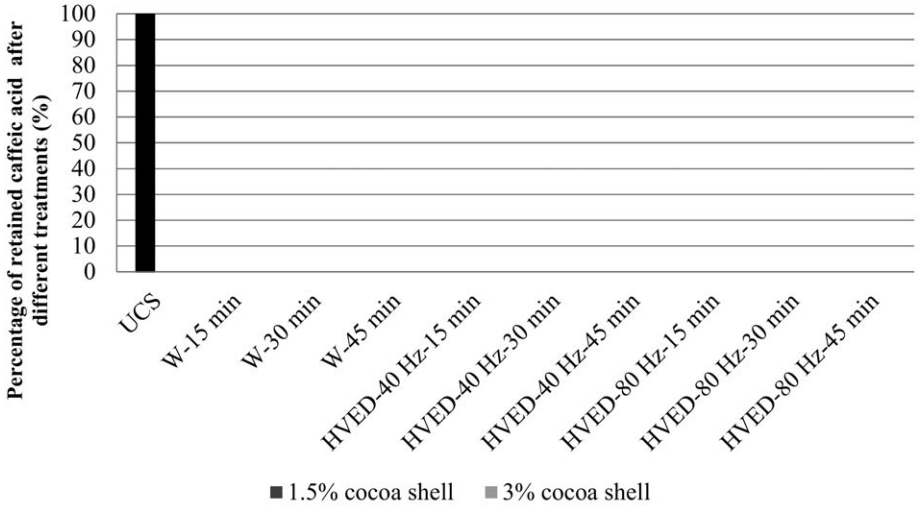


FIGURE 2 Percentage of retained catechines in cocoa shell after different treatments

higher (+)-catechin and (−)-epicatechin values in cocoa shell than obtained in this study. In both studies, cocoa shell was fermented, but in our study, cocoa shell was roasted after fermentation. Roasting can cause changes of chemical composition, increase of (+)-catechin and decrease of (−)-epicatechin content due to the isomerization (Abbe & Amin, 2008), which can partially explain the difference in phenolic contents.

Extraction of phenolics and methylxanthines from cocoa seed and its by-products is significantly dependent, first of all, on applied extraction method and later, on extraction parameters (Jokić, Gagić, Knez, Šubarić, & Škerget, 2018). Better extraction yields of bioactive components from cocoa shell were obtained using innovative methods of extraction (e.g., supercritical CO_2, pressurized ethanol, subcritical water,





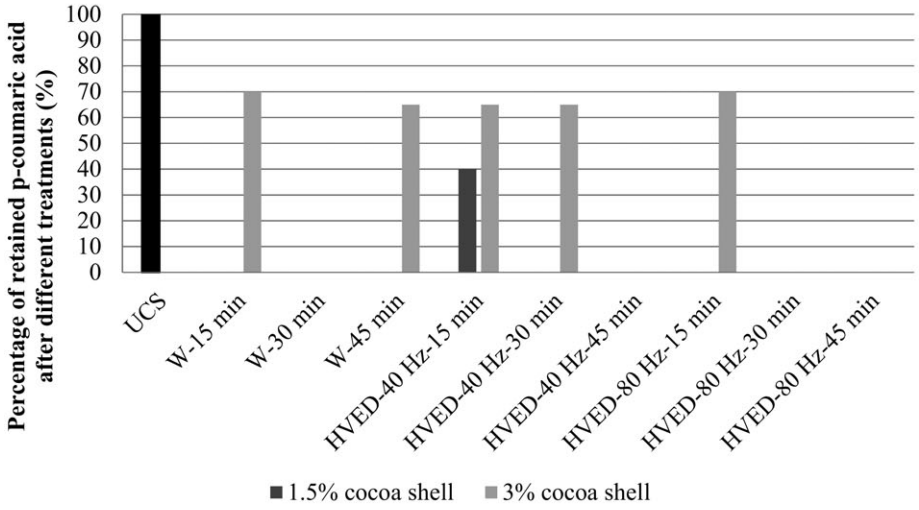


FIGURE 3 Percentage of retained phenolic acids in cocoa shell after different treatments

or extraction assisted with pulsed electric field) compared to conventional liquid extraction with various solvents (Barbosa-Pereira, Guglielmetti, & Zeppa, 2018; Jokić et al., 2018; Mazzutti, Rodrigues, Mezzomo, Venturi, & Ferreira, 2018). Application of HVED has been used for extraction of bioactive components from many food products (Barba, Bousetta, & Vorobiev, 2015; Barba, Brianceau, Turk, Bousetta, & Vorobiev, 2015; Li et al., 2019; Parniakov et al., 2014a; Parniakov, Barba, Grimi, Lebovka, & Vorobiev, 2014b). As reported by mentioned

TABLE 2 Analysis of variance (ANOVA)

Effect	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Theobromine (TEO)					
Concentration (C)	2.08	1	2.08	36.3	0.0000
Mixing time (MT)	5.63	2	2.82	49.3	0.0000
Treatment (T)	0.90	2	0.45	7.9	0.0016
C × MT	0.44	2	0.22	3.8	0.0312
C × T	0.99	2	0.49	8.7	0.0009
MT × T	1.78	4	0.44	7.8	0.0001
C × MT × T	1.93	4	0.48	8.4	0.0001
Error	1.94	34	0.06		
Total	16.05	51			
Caffeine (CAF)					
Concentration (C)	0.0619	1	0.0619	60.6	0.0000
Mixing time (MT)	0.1785	2	0.0892	87.3	0.0000
Treatment (T)	0.0517	2	0.0259	25.3	0.0000
C × MT	0.0103	2	0.0052	5.1	0.0120
C × T	0.0208	2	0.0104	10.2	0.0003
MT × T	0.0910	4	0.0227	22.3	0.0000
C × MT × T	0.0798	4	0.0199	19.5	0.0000
Error	0.0347	34	0.0010		
Total	0.5349	51			
(+)-Catechin (CAT)					
Concentration (C)	0.0034	1	0.0034	141.5	0.0000
Mixing time (MT)	0.0017	2	0.0008	34.4	0.0000
Treatment (T)	0.0012	2	0.0006	24.4	0.0000
C × MT	0.0005	2	0.0003	10.3	0.0003
C × T	0.0048	2	0.0024	98.5	0.0000
MT × T	0.0013	4	0.0003	13.0	0.0000
C × MT × T	0.0007	4	0.0002	7.2	0.0002
Error	0.0008	34	0.0000		
Total	0.0151	51			
(–)-Epicatechin (EPI)					
Concentration (C)	0.0023	1	0.0023	147.7	0.0000
Mixing time (MT)	0.0036	2	0.0018	113.7	0.0000
Treatment (T)	0.0033	2	0.0016	104.0	0.0000
C × MT	0.0001	2	0.0000	2.0	0.1532
C × T	0.0002	2	0.0001	6.0	0.0059
MT × T	0.0012	4	0.0003	19.3	0.0000
C × MT × T	0.0008	4	0.0002	12.8	0.0000
Error	0.0005	34	0.0000		
Total	0.0123	51			
(–)-Epicatechin gallate (EPG)					
Concentration (C)	0.0000	1	0.0000	12.4	0.0012
Mixing time (MT)	0.0000	2	0.0000	81.6	0.0000
Treatment (T)	0.0000	2	0.0000	18.2	0.0000
C × MT	0.0000	2	0.0000	9.8	0.0004

(Continues)

TABLE 2 (Continued)

Effect	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
C × T	0.0000	2	0.0000	25.5	0.0000
MT × T	0.0000	4	0.0000	15.2	0.0000
C × MT × T	0.0000	4	0.0000	4.4	0.0055
Error	0.0000	34	0.0000		
Total	0.0001	51			
Gallic acid (GA)					
Concentration (C)	0.0006	1	0.0006	199.5	0.0000
Mixing time (MT)	0.0010	2	0.0005	164.5	0.0000
Treatment (T)	0.0001	2	0.0000	9.7	0.0005
C × MT	0.0001	2	0.0000	12.3	0.0001
C × T	0.0002	2	0.0001	36.5	0.0000
MT × T	0.0002	4	0.0000	13.6	0.0000
C × MT × T	0.0002	4	0.0000	13.3	0.0000
Error	0.0001	34	0.0000		
Total	0.0026	51			
p-Coumaric acid (*p*-CA)					
Concentration (C)	0.0005	1	0.0005	171.26	0.0000
Mixing time (MT)	0.0004	2	0.0002	64.81	0.0000
Treatment (T)	0.0001	2	0.0001	16.52	0.0000
C × MT	0.0001	2	0.0001	18.04	0.0000
C × T	0.0000	2	0.0000	6.83	0.0032
MT × T	0.0003	4	0.0001	23.08	0.0000
C × MT × T	0.0004	4	0.0001	29.40	0.0000
Error	0.0001	34	0.0000		
Total	0.0020	51			

authors, HVED application causes electrical breakdown in water and together with different secondary phenomena (bubble cavitation, high-amplitude pressure shock waves, etc.) causes particle fragmentation and cell structure damage. The extraction conditions, like high voltage power, energy input, treatment temperature and time, considerably effect on extraction yield. The usual application of HVED is therefore as extraction technique, but the aim of this work was to evaluate the application of HVED on retention of bioactive components in cocoa shell. To the best authors' knowledge, this application of HVED is not reported yet. Phenomena of higher retaining of phenolic components in samples treated with plasma was reported by several authors (Muhammad, Liao, Cullen, Liu, & Xiang, 2018; Sarangapani, O'Toole, Bourke, & Cullen, 2017). Accurate mechanisms that lead to retaining of phenolic components after electric discharge treatment are not elucidated yet. One of the proposed mechanisms is interaction of phenolics with other food ingredients (e.g. fibers), but further studies are necessary to optimize processing conditions to insure minimal degradation of food products quality.

As can be seen in Table 1 and Figures 1–3, considerable decrease of phenolic components and methylxanthines was obtained in both control samples and samples treated with HVED. Generally, HVED treatment at 40 Hz has smaller impact on bioactive components than water and HVED at 80 Hz, but further investigations should include larger number of samples to confirm this. The highest percentage of retaining among phenolic components was obtained for (–)-epicatechin and (–)-epicatechin gallate, whereas caffeic acid was determined only in UCS. As can be seen in Figure 2, 89% and 78% of initial (–)-epicatechin gallate concentration was determined after HVED treatment at 40 Hz for 15 min in 3% and 1.5% cocoa shell, respectively. Major phenolic components in UCS, (+)-catechin, and gallic acid, showed the highest decrease of initial concentration after treatments (Figures 2 and 3). Less than 20% of gallic acid and 30% of (+)-catechin content remained after treatments, with lower decrease for 3% than 1.5% of cocoa shell. Concentration of cocoa shell in solution had also impact on retaining of *p*-coumaric acid. Namely, detectable concentration of *p*-coumaric acid was obtained only for 1.5% of cocoa shell solution after HVED treatment at 40 Hz for 15 min, whereas for 3% of cocoa shell solution, percentage of retaining *p*-coumaric acid was between 65 and 70% after short treatments regardless of treatment type (Figure 3). Theobromine and caffeine were less liable to treatments than phenolic components (Figure 1). The highest retaining percentage (84% for both components) was determined in samples treated

with HVED at 80 Hz for 15 min. Regarding impact of treatment duration, decrease trend at longer treatment was observed only in samples containing 1.5% of cocoa shell.

The differences in obtained results regarding applied treatment conditions were afterward statistically evaluated. Factorial ANOVA was performed, and the results were presented in Table 2. As can be seen in Table 2, concentration, mixing time, and applied treatment, as well as their combinations had impact on bioactive components (p value were lower than 0.05), except for (−)-epicatechin, where combination of concentration and mixing time (C × MT) was not statistically significant (p = 0.1532). The statistically significant differences between treatment conditions and results of retaining percentage of analyzed components purports the possible mechanism of interaction of phenolics with other food components in complexes which are less prone to leaching into water during extraction due to higher molecular mass, change in electrical discharge, and larger particle size. But for final confirmation of this statement, further research that involves different treatment conditions and analysis of other physicochemical parameters is necessary.

4 | CONCLUSIONS

Cocoa shell is a by-product that has a great potential for wide applications in food industry. A part of this is due to the presence of phenolic components and methylxanthines that have many health-promoting properties. The most abundant bioactive components are theobromine and caffeine, whereas catechines are major phenolics in cocoa shell. However, its safety due to high microbial load and grinding problems (very coarse particles) should be addressed. One of the solutions for these issues is application of novel nonthermal processes, such as HVED. However, any food treatment may cause changes in its chemical composition and nutritional value. In this study, influence of HVED on retention of bioactive components was evaluated, unlike other studies that focused on its use an extraction technique. The results showed higher retaining of bioactive components when compared to water treatment. Therefore, HVED treatment can be used as pretreatment step of cocoa shell processing, but future studies should include larger number of samples and different treatment conditions to optimize the process and obtain maximal quality of cocoa shell.

ACKNOWLEDGMENTS

This work has been supported in part by Croatian Science Foundation under the project 2017-05-8709. The authors are grateful to Prof. Mirta Benšić and Una Radojičić, MSc for valuable advice upon statistical analysis

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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How to cite this article: Barišić V, Flanjak I, Križić I, et al. Impact of high-voltage electric discharge treatment on cocoa shell phenolic components and methylxanthines. *J Food Process Eng*. 2019;e13057. https://doi.org/10.1111/jfpe.13057

PRILOG 3

Food Control 110 (2020) 107043

Contents lists available at ScienceDirect

Food Control

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

5-Hydroxymethylfurfural and acrylamide content of cocoa shell treated with high voltage electrical discharge

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ARTICLE INFO

Keywords:
Cocoa shell
High voltage electrical discharge
5-HMF
Acrylamide

ABSTRACT

5-hydroxymethylfurfural (5-HMF) and acrylamide, as products of Maillard's reactions, are present in roasted cocoa shell. Since the cocoa shell is increasingly being researched for use in food enrichment due to high fiber and polyphenols content, it is necessary to solve the problem of components that can be harmful to human health. We evaluated the effect of high voltage electrical discharge (HVED) on colour, moisture, 5-HMF and acrylamide content in cocoa shell obtained after roasting cocoa beans. The effects of concentration, frequency and time of cocoa shell treatment were investigated. HVED proved to be a successful method for reducing the content of acrylamide and 5-HMF. In addition, darkening of samples was observed which could mean that further reactions of those components occurred.

1. Introduction

The cocoa shell obtained before or after roasting of cocoa beans is the by-product of the food industry. Separating cocoa shell from the bean is a very important step in the chocolate industry to obtain a good quality product (Okiyama, Navarro, & Rodrigues, 2017). Since the shell is rich in fiber, bioactive components and proteins, the possibility of its application in the food, pharmaceutical or agricultural industry has been extensively investigated. There is a problem of using cocoa shell safely in food, since it is in contact with external influences, microorganisms, mycotoxins and pesticides (Panak Balentić et al., 2018) and, if roasted, contains products of Maillard's reactions.

Cocoa bean roasting is one of the most important steps in the chocolate production, because it creates the desired flavour and taste, and removes undesirable volatile compounds. Maillard's reactions are those that cause the development of flavour, taste and colour compounds characteristic to chocolate. They may also cause the development of some undesirable components such as acrylamide and 5-hydroxymethylfurfural (5-HMF) (Farah, Zaibunnisa, Misnawi, & Zainal, 2012; Sacchetti et al., 2016).

Acrylamide is produced at temperatures higher than 120 °C in various foods such as potato chips, bread, toast, biscuits, coffee, cocoa beans, etc. (Zyzelewicz et al., 2017). Numerous researches have been conducted to prevent the formation of acrylamide and for its elimination since it is known to be carcinogenic, mutagenic and reproductive toxicogenic (Pérez-Nevado, Cabrera-Bañegil, Repilado, Martillanes, & Martín-Vertedor, 2018; Capuano & Fogliano, 2011; European commission, 2002). Commission regulation (EU) 2017/2158 (European Commission, 2017) states mitigation measures for reduction of the presence of acrylamide in food like identification of critical roasting conditions, control of roasting conditions or use of asparaginase treatment. 5-HMF is a furan that can be formed at high temperatures, but it does not have to be temperature dependent because it can also occur through other chemical pathways. It is known that 5-HMF is cytotoxic in high doses (Sacchetti et al., 2016).

A high-voltage electrical discharge (HVED) is a novel non-thermal method that has been investigated for use in microbiological decontamination and as an extraction technique. According to Lukić et al. (2019), HVED is a method that generates the formation of free radicals, hydrogen peroxides, hydroxyls, etc. and may induce different changes in treated material.

In this study, the influence of HVED treatment on 5-HMF and acrylamide content in cocoa shell was determined. The treatment was applied at different times and frequencies at two different concentrations. According to our knowledge, this is the first study that was conducted to determine the influence of HVED on 5-HMF and acrylamide.

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https://doi.org/10.1016/j.foodcont.2019.107043
Received 18 October 2019; Received in revised form 5 December 2019; Accepted 6 December 2019
Available online 07 December 2019

2. Materials and methods

2.1. Chemicals and reagents

Acrylamide (AA) of 99% purity grade was acquired from Dr. Ehrenstorfer GmbH (Augsburg, Germany). $^{13}C_3$ Acrylamide (AA-IS) (isotopic purity 99%) in methanol, used as an internal standard, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The n-hexane (for GC ECD and FID analyses) was obtained from Merck (Kenilworth, USA). Acetonitrile (for UHPLC) was from ITW Reagents (Barcelona, Spain). Methanol (for UV, IR and HPLC analyses) was from Panreac AppliChem (Darmstadt, Germany). Formic acid (98%) was from Scharlau (Barcelona, Spain). Ultrapure water was used throughout the experiments (Nirosta VV System, Nirosta Water Technologies, Osijek, Croatia). Salts for QueEChERS: MgSO4 (4 g) + NaCl (0.5 g); and d-SPE salts (dispersive solid phase extraction): MgSO4 (150 g) + PSA (50 mg) were purchased from Bekolut (Haupstuhl, German).

2.2. Preparation of cocoa shell

Cocoa shell samples were obtained by winnowing cocoa beans after roasting (at 135 °C for 55 min) of fermented cocoa beans (West Africa mix supplied by Huyser, Möller B.V. Holland), and treated as previously described (Barišić et al., 2019a). Briefly, roasted cocoa shell (without further milling) was suspended in water to obtain 1.5 and 3% concentrations. The suspensions were either stirred at magnetic stirrer (control samples) or treated by HVED in custom-made system for 15, 30 and 45 min, at frequencies of 40 and 80 Hz. After separating shell from water, samples were dried in the laboratory oven (Memmert, UFE 500) at 40 °C. Dry treated samples and untreated cocoa shell were ground in the laboratory mill (IKA, M20) to obtain a fine powder. Samples were frozen (−18 °C) and stored for analysis.

2.3. Determination of acrylamide content

The preparation of samples was carried out according to Agilent application "Analyses of acrylamide in Frech fries using Agilent Bond Elut QuECHERS AOAC kit and LC-MS/MS" (Al-Taher, 2012) with some modifications. Approximately 1.00 ± 0.05 g of the homogenised sample was weighed in centrifugal assays of 50 mL. 20 μL of an internal standard solution (10 μg/mL AA-IS) was added to each vial. 5 mL of hexane was added and mixed for 1 min. 20 mL of water:acetonitrile (1:1) mixture was added and mixed for 1 min. The vials were placed on a shaker (IKA-WERKE, Staufen, Germany) (250–300 rpm) for 5 min. QueChERS ($MgSO_4$ (4 g) + NaCl (0.5 g)) were added to the mixture and mixed for 1 min. Samples were centrifuged for 15 min at 4600 rpm. The hexane layer was discarded, and 5 mL aliquot of acetonitrile extract was transferred with pipette in vial with salts (d-SPE, $MgSO_4$ (150 g) + PSA (50 mg)). The content of vial was mixed and then centrifuged for 5 min at 4600 rpm. 2 mL of an aliquot of acetonitrile extract was transferred in a glass tube and evaporated in stream of nitrogen at 45 °C. The obtained extracts were dissolved in 500 μL of water and filtered through a filter (0.2 μm, nylon membrane, Pall Life Sciences, New York, USA) in a glass vial for an autosampler. Prepared extracts were stored in the refrigerator until analysis.

The method was in-house validated and accredited in accordance with EN ISO/IEC 17025. Method validation included determination of the limit of detection (LOD), limit of quantification (LOQ), linearity, repeatability (RSDr), recovery and stability. The LOD was set at 10 μg/kg and LOQ at 20 μg/kg. The performance criteria for the LOD and LOQ are defined in the new Directive EU 2017/2158 (European Commission, 2017), where the LOD is set to be three tenths of LOQ and the LOQ is set to be two fifths of the benchmark level, for the benchmark level < 125 μg/kg. The LOD was determined from the signal-to-noise ratio (S/N = 3:1), calculated by the instrument software (MassLynx V4.1), based on data obtained by triplicate analysis of sample (n = 3) with the concentration of acrylamide corresponding to the LOD. The LOQ was determined by multiple preparation of samples (n = 6) spiked with the acrylamide stock solution at the concentration corresponding to the LOQ. The calculated coefficients of variation (CVs) was found to be 3.1%. Data for calibration curve were collected using triplicate analysis for 7 different concentrations of acrylamide stock solutions within the method working range. Linearity up to 8000 μg/kg has been achieved with a coefficient of determination of 0.998. The recoveries were determined at concentrations levels of 20, 200 and 1000 μg/kg. Each concentration level was analyzed in duplicate. The obtained recoveries ranged from 82 to 106%, with CVs less than 10. Based on the results obtained by the method validation, a measurement uncertainty of 16% was estimated. The accuracy of the results has been demonstrated in an interlaboratory comparison study organized by the Food Analysis Performance Assessment Scheme (FAPAS) program (2017). FAPAS Proficiency Test No. 3073 corresponding to vegetable crisp test material was analyzed, yielding a Z-score of 1.6.

A Waters Xevo liquid chromatograph coupled to a Waters Triple Quadrupole MS detector (Waters Corp., Milford, MA) with positive electrospray ionization and MassLynx V4.1 software was used for samples analysis. The samples were separated on a Luna C-18 column (150 × 2 mm, 3 μm; Phenomenex, California, USA) at 30 °C. The mobile phase composed of solvent (A) 0.1% (v/v) aqueous formic acid solution and solvent (B) methanol was used. The following gradient regime was applied: from 0 to 0.5 min 2% B, from 0.5 to 6 min 2–90% B, from 6 to 8 min 90% B, from 8 to 8.5 min 90-2% B, from 8.5 to 13.5 min 2% B. The injection volume was 10 μL, elution time 15 min, mobile phases had the flow of 0.2 mL/min. The detector was tandem mass spectrometer with an evaporation temperature of 550 °C, evaporation gas flow 800 L/h, the ionic source temperature was 150 °C, ionization of ESI (+) and capillary voltage: 2.8 kV. The transitions m/z 72.1 → 55.1; m/z 72.1 → 44.1 and m/z 72.1 → 27.1 were monitored for acrylamide and transitions m/z 75.1 → 58.0 for $^{13}C_3$-acrylamide, respectively. Cone voltage was set at 22 V and the collision energy at 10 V. Acrylamide content in samples was calculated according to the bracketing calibration curve. Calibration curve was created based on the peak area ratio of the acrylamide and ^{13}C labeled internal standard. Also, with each batch of samples blank and the efficiency of the extraction procedure (recovery) on the LOQ were checked. Results were expressed as μg/kg.

2.4. Determination of 5-hydroxymethylfurfural content

5-hydoxymethylfurfural (5-HMF) content was determined according to the method for determination of 5-HMF in honey described in Swiss Food Book (Schweizerisches Lebensmittelbuch, 2006). Briefly, 2.5 g of sample was weighted in a 50 mL cuvette and mixed with water on a vortex. The sample was centrifuged for 15 min at 4500 rpm. The extract was filtered through a 0.45 μm membrane filter (nylon membrane, Pall Life Sciences, New York, USA) into a vial of the automated sampler. The sample prepared for chromatographic analysis was then placed in an automatic sampler of a liquid chromatography.

5-hydroxymethylfurfural content was determined by Shimadzu chromatograph LC-10ADVP with DAD (diode array detector) using ODS C18 column (HyperClone, 250 × 4.6 mm, 5 μm) thermostated at 25 °C. The mobile phase was water:methanol (90:10) with a flow rate of 1 mL/min and injection volume 20 μL. The detector was set at 285 nm. The presence of 5-HMF was determined by comparing the retention time (RT) of the sample with the RT standard. The amount of 5-HMF present was calculated according to the calibration curve using external calibration method. The LOD was set at 0.25 mg/kg and LOQ at 1.25 mg/kg with coefficient of determination of 0.999991.

2.5. Determination of moisture content and water activity

Water activity was measured at 25 °C using HygroLab 3 (Rotronic

Switzerland). Moisture content was determined by ISO 6540:1980 (ISO 6540) and moisture content was calculated by following equation:

$$Moisture\ content\ (\%) = \frac{sample\ mass - sample\ mass\ after\ drying}{sample\ mass} \times 100 \quad (1)$$

2.6. Colour determination

To determine the colour of ground cocoa shell, chromameter Konica Minolta CR-400 with an extension for powdered samples was used (Zyzelewicz, Krysiak, Nebesny, & Budryn, 2014). The measurements were performed in a CIEL*a*b* and L*Ch° systems in 5 repetitions, where L* is value for lightness (0 is black and 100 white), a* for redness (positive values) or greenness (negative values) and b* for yellowness (positive values) or blueness (negative values). The total colour change (ΔE) was calculated using following equation (1), where L*, a*, b* represent values for the treated sample and L_0*, a_0*, b_0* values for the untreated cocoa shell:

$$\Delta E = \sqrt{(L - L_0)^2 + (b - b_0)^2 + (a - a_0)^2} \quad (2)$$

2.7. Statistical analysis

Statistical analysis was conducted using Statistica®, Version 13.4.0.14 (1984–2018 TIBCO Software Inc). To determine the statistically significant difference of treatment effects, factorial analysis of variance (ANOVA) was used ($p < 0.05$). Also, Pearson correlation analysis was conducted to determine relationship between variables ($p < 0.05$).

3. Results and discussion

3.1. Acrylamide

Acrylamide is generally formed as a part of Maillard reaction from free amino acid (predominantly asparagine) and reducing sugar. For lipid rich foods (cocoa contains app. 50% of cocoa butter) additional mechanism via acrolein and acrylic acid, produced by degradation of lipids at high temperatures, is also proposed (Zyzelewicz et al., 2017; Krishnakumar & Visvanathan, 2014). With 166 μg/kg determined in non-treated sample (Table 1), analyzed cocoa shell corresponds to values reported for chocolate products by Krishnakumar and Visvanathan (2014) and closely to values reported for coffee (200 μg/kg) and bakery products (112 μg/kg) reported by World Health Organization & Food and Agriculture Organization (2002). The concentration of acrylamide (which is formed during roasting) was the highest in the untreated cocoa shell, as can be seen in Table 1. All further treatments resulted in a significant reduction in acrylamide content, in most samples below the limit of quantification, and in some below the limit of detection. The reason for such a reduction could be its high water solubility (Farah et al., 2012). The results of acrylamide content showed a statistically significant difference when concentration, mixing, treatment, combination of concentration and treatment, and combination of all three effects was performed (Table 2). The main characteristic of HVED treatment is that it can disrupt the cell walls and is therefore used as an extraction technique. From Table 1, it can be concluded that even though the water as solvent itself had a significant effect on rate of acrylamide formation, HVED was more effective. This implies that acrylamide was much easier to extract from the treated cocoa shell because of disrupted cell walls and that HVED could be a good technique for removing acrylamide. However, to establish this relationship further research is needed to verify presence of acrylamide in the extract, and to determine the effect on other ingredients of the shell as well.

On the other hand, Table 3 shows that there is a reversed relationship between colour and acrylamide. That is, as the content of acrylamide decreased, the samples became darker. This implies that part of acrylamide may have undergone further reactions within the shell and was not fully extracted from the shell. It could have polymerized into polyacrylamide (Fig. 1) under the influence of ultraviolet light and an oxidizing agent (HVED). There are studies investigating the possibility of polymerization of acrylamide by ultraviolet light and electrical discharge (Capek, 2016; Novak, Lath, Florian, Dulaj, & Sestak, 1995) but not regarding food processing. In food, polymerization of acrylamide has been reported for high temperature (above 100 °C) and as base catalyzed process during long term storage of solutions (Adams, Hamdani, Lancker, Méjri, & De Kimpe, 2010).

Another reaction that could occur during HVED treatment is Michael addition, where acrylamide reacts as Michael acceptor and free amino acids as Michael donors (Fig. 2). The proposed hypothesis can be partially explained by the following:

- the reaction is catalyzed by alkali conditions (Adams et al., 2010) and Li, Fan, and Xi (2019) reported increase of pH by HVED treatment of water.
- free amino acids do react with acrylamide in model systems,

Table 1
Water activity, moisture, 5-hydroxymethylfurfural and acrylamide content of untreated and treated cocoa shell.

Sample	a_w	Moisture (%)	5-HMF (mg/kg)	Acrylamide (μg/kg)
UCS	0.372	5.71 ± 0.31	133.34 ± 64.37	166.00 ± 8.92
1.5%, 15 min	0.552	12.22 ± 0.00	12.02 ± 0.10	< LOQ
1.5%, 30 min	0.570	12.62 ± 0.01	8.86 ± 0.04	25.50 ± 0.50
1.5%, 45 min	0.579	13.52 ± 0.03	3.54 ± 0.05	28.00 ± 2.00
1.5%, 15 min, 40 Hz	0.557	13.12 ± 0.02	8.34 ± 0.05	< LOQ
1.5%, 30 min, 40 Hz	0.532	11.36 ± 0.11	10.22 ± 0.14	< LOQ
1.5%, 45 min, 40 Hz	0.545	12.09 ± 0.01	9.29 ± 0.08	< LOQ
1.5%, 15 min, 80 Hz	0.577	13.08 ± 0.02	11.47 ± 0.01	27.50 ± 0.50
1.5%, 30 min, 80 Hz	0.547	12.28 ± 0.08	10.18 ± 0.16	< LOQ
1.5%, 45 min, 80 Hz	0.567	12.57 ± 0.07	5.91 ± 0.13	< LOQ
3%, 15 min	0.559	14.06 ± 0.07	36.94 ± 0.12	21.50 ± 0.50
3%, 30 min	0.561	14.03 ± 0.04	24.99 ± 0.10	< LOQ
3%, 45 min	0.572	15.03 ± 0.05	25.32 ± 0.29	< LOD
3%, 15 min, 40 Hz	0.580	12.78 ± 0.03	25.56 ± 0.16	< LOQ
3%, 30 min, 40 Hz	0.591	13.23 ± 0.02	24.50 ± 0.24	< LOQ
3%, 45 min, 40 Hz	0.579	13.16 ± 0.08	24.59 ± 0.09	< LOQ
3%, 15 min, 80 Hz	0.597	12.80 ± 0.01	32.75 ± 0.08	< LOQ
3%, 30 min, 80 Hz	0.583	13.75 ± 0.00	25.61 ± 0.22	< LOD
3%, 45 min, 80 Hz	0.780	13.84 ± 0.02	22.05 ± 0.22	< LOD

UCS: untreated cocoa shell; 5-HMF: 5- hydroxymethylfurfural; ± Standard deviations (n = 2); LOQ: 20 μg/kg; LOD: 10 μg/kg.

Table 2
Factorial analysis of variance.

		Sum of squares	DF	Mean Square	F – Value	p - Value
L*	Intercept	177855.1	1	177855.1	174272844	< 0.001*
	Concentration (C)	157.3	1	157.3	154098	< 0.001*
	Mixing (M)	11.0	2	5.5	5369	< 0.001*
	Treatment (T)	14.9	2	7.4	7289	< 0.001*
	C*M	4.0	2	2.0	1957	< 0.001*
	C*T	14.9	2	7.5	7320	< 0.001*
	M*T	3.9	4	1.0	950	< 0.001*
	C*M*T	8.4	4	2.1	2057	< 0.001*
	Error	0.1	72	0.0		
a*	Intercept	6896.751	1	6896.751	1960542	< 0.001*
	Concentration (C)	0.166	1	0.166	47	< 0.001*
	Mixing (M)	0.282	2	0.141	40	< 0.001*
	Treatment (T)	0.248	2	0.124	35	< 0.001*
	C*M	0.018	2	0.009	3	< 0.001*
	C*T	0.769	2	0.385	109	< 0.001*
	M*T	0.110	4	0.027	8	< 0.001*
	C*M*T	0.339	4	0.085	24	< 0.001*
	Error	0.253	72	0.004		
b*	Intercept	22379.84	1	22379.84	11806480	< 0.001*
	Concentration (C)	0.05	1	0.05	29	< 0.001*
	Mixing (M)	6.62	2	3.31	1747	< 0.001*
	Treatment (T)	3.43	2	1.72	905	< 0.001*
	C*M	0.45	2	0.23	120	< 0.001*
	C*T	3.27	2	1.63	862	< 0.001*
	M*T	0.56	4	0.14	74	< 0.001*
	C*M*T	9.08	4	2.27	1198	< 0.001*
	Error	0.14	72	0.00		
C	Intercept	29278.92	1	29278.92	40415688	< 0.001*
	Concentration (C)	0.16	1	0.16	224	< 0.001*
	Mixing (M)	6.28	2	3.14	4336	< 0.001*
	Treatment (T)	3.49	2	1.74	2406	< 0.001*
	C*M	0.42	2	0.21	287	< 0.001*
	C*T	3.91	2	1.95	2698	< 0.001*
	M*T	0.63	4	0.16	218	< 0.001*
	C*M*T	8.37	4	2.09	2888	< 0.001*
	Error	0.05	72	0.00		
h°	Intercept	334297.3	1	334297.3	8314246	< 0.001*
	Concentration (C)	0.5	1	0.5	13	< 0.001*
	Mixing (M)	6.3	2	3.1	78	< 0.001*
	Treatment (T)	2.4	2	1.2	30	< 0.001*
	C*M	0.5	2	0.3	6	< 0.001*
	C*T	1.3	2	0.6	16	< 0.001*
	M*T	0.5	4	0.1	3	0.025837*
	C*M*T	10.5	4	2.6	65	< 0.001*
	Error	2.9	72	0.0		
ΔE	Intercept	1576.425	1	1576.425	990799.2	< 0.001*
	Concentration (C)	99.747	1	99.747	62692.3	< 0.001*
	Mixing (M)	17.060	2	8.530	5361.1	< 0.001*
	Treatment (T)	22.596	2	11.298	7100.9	< 0.001*
	C*M	4.121	2	2.061	1295.1	< 0.001*
	C*T	7.049	2	3.525	2215.3	< 0.001*
	M*T	1.169	4	0.292	183.7	< 0.001*
	C*M*T	15.719	4	3.930	2469.8	< 0.001*
	Error	0.115	72	0.002		
Moisture (%)	Intercept	6164.344	1	6164.334	1242533	< 0.001*
	Concentration (C)	10.802	1	10.802	2177	< 0.001*
	Mixing (M)	1.543	2	0.772	156	< 0.001*
	Treatment (T)	5.482	2	2.741	553	< 0.001*
	C*M	2.229	2	1.115	225	< 0.001*
	C*T	1.110	2	0.555	112	< 0.001*
	M*T	2.411	4	0.603	121	< 0.001*
	C*M*T	2.217	4	0.554	112	< 0.001*
	Error	0.089	18	0.005		

(*continued on next page*)

Table 2 (*continued*)

		Sum of squares	DF	Mean Square	F – Value	p - Value
Acrylamide	Intercept	2584.028	1	2584.028	70.63402	< 0.001*
	Concentration (C)	521.361	1	521.361	14.25133	0.001386*
	Mixing (M)	737.556	2	368.778	10.08049	0.001156*
	Treatment (T)	977.056	2	488.528	13.35383	< 0.001*
	C*M	14.222	2	7.111	0.19438	0.825050
	C*T	447.722	2	223.861	6.11921	0.009385*
	M*T	402.611	4	100.653	2.75133	0.060265
	C*M*T	983.944	4	245.986	6.72399	0.001711*
	Error	658.500	18	36.583		
5-HMF	Intercept	11528.92	1	11528.92	261974.1	< 0.001*
	Concentration (C)	2933.97	1	2933.97	66669.2	< 0.001*
	Mixing (M)	224.91	2	112.45	2555.3	< 0.001*
	Treatment (T)	14.16	2	7.08	160.9	< 0.001*
	C*M	51.92	2	25.96	589.9	< 0.001*
	C*T	43.76	2	21.88	497.2	< 0.001*
	M*T	126.62	4	31.65	719.3	< 0.001*
	C*M*T	12.55	4	3.14	71.3	< 0.001*
	Error	0.79	18	0.04		

DF: degree of freedom; *p < 0.05 statistically significant.

producing Michael adduct (Adams et al., 2010; Zamora, Delgado, & Hidalgo, 2010) and cocoa bean contains sufficient quantities of free amino acids (leucine, valine, alanine, isoleucine, phenylalanine) (Beckett, Fowler, & Ziegler, 2017). Adeyeye, Oyarekua, and Adesina (2014) reported that, among others, cocoa shell contains glycine, methionine and lysine, all of which have been proven to interact with acrylamide. Although amino acids are considered as main aroma precursor in cocoa, only 25% of them will react to produce aroma compounds (Beckett et al., 2017). Part of them will undergo Maillard reactions and give Amadori compounds, however, there is possibility that part of them will interact with acrylamide.

- reaction is reversible, although activation energy is higher for reverse reaction (Adams et al., 2010) which could explain phenomenon observed in this research – for water-treated samples in 1.5% suspension, prolongation of treatment raised amount of acrylamide detected in shell from < LOQ for 15 min treatment to 25.5 μg/kg and 28.0 μg/kg for 30 min and 45 min, respectively.
- Adams et al. (2010) stated that aqueous medium benefits reduction of acrylamide content through Michael addition with amino acids and hence, treatments in this research were done in aqueous suspensions.

After all this has been said, authors remark that this hypothesis requires additional research in order to reveal amount and profile of free amino acids in cocoa shell, exact pH change during this treatment and reveal possible reaction mechanisms through investigations in model solutions of identified free amino acids and acrylamide, in relative ratios as in cocoa shell.

Recently, presence of melanoidins has been reported for roasted coffee (Oracz & Nebesny, 2019; Pastoriza, Rufián-Henares, & Morales, 2012). There are implications that these final products of Maillard reactions could be formed in cocoa as well (Barišić et al., 2019b). If this is true, melanoidins can also act as Michael donor in reaction with acrylamide (Pastoriza et al., 2012) during HVED treatment. The reaction of acrylamide with melanoidins would be supported by results of colour change and darkening of samples (Table 4).

To sum up, food and food by-products, regardless the origin and composition, are always a complex material, with many substances, including macro- and micro-molecules, which may interact, depending on conditions the food is subjected to. In addition, electric fields increase energy of particles resulting in vibration, excitation, association, dissociation, and ionization. Ions may be involved in reaction mechanisms such as ion-ion (neutralization), ion-molecule, Penning ionization, quenching etc. Furthermore, photo-catalyzed reactions: emissions, absorption and ionization may occur (Misra, Pankaj, Segat, & Ishikawa, 2016). However, not all species formed by high voltage electronic discharge interact with food, they may recombine or diffuse into the liquid phase (Gavahian, Chu, Mousavi Khaneghah, Barba, & Misra, 2018). Hence, with such complexity within the system, it is very difficult to reveal actual mechanisms.

Pérez-Andrés, Charoux, Cullen, and Tiwari (2018) in their review state that cold plasma may induce oxidation of lipids and proteins and changes of secondary structure of proteins, as well as inhibition of enzymes. Changes in protein structure and conformation influence their reactivity, including asparagine reactivity towards formation of acrylamide. Kalum and Hendriksen (2016) reported reduction of acrylamide formation in fried potato by pulsed electric field treatment prior to frying (*cited from* Dourado et al., 2019).

Table 3
Pearson's correlation coefficients.

Variable	L	a*	b*	C	h°	ΔE	5-HMF	AA	a_w	Moisture
L	1.000									
a*	0.141	1.000								
b*	0.455	**0.897**	1.000							
C	0.421	**0.921**	**0.998**	1.000						
h°	**0.619**	**0.707**	**0.945**	**0.926**	1.000					
ΔE	**−0.968**	−0.372	**−0.661**	**−0.632**	**−0.779**	1.000				
5-HMF	0.196	**0.670**	**0.708**	**0.711**	**0.616**	−0.360	1.000			
AA	**0.475**	**0.507**	**0.630**	**0.622**	**0.601**	**−0.575**	**0.881**	1.000		
a_w	**−0.558**	**−0.535**	**−0.665**	**−0.655**	**−0.670**	**0.638**	**−0.577**	**−0.668**	1.000	
Moisture	**−0.736**	**−0.469**	**-0–661**	**−0.644**	**−0.692**	**0.805**	**−0.757**	**−0.843**	**0.723**	1.000

Bold values were considered significant at p < 0.05.

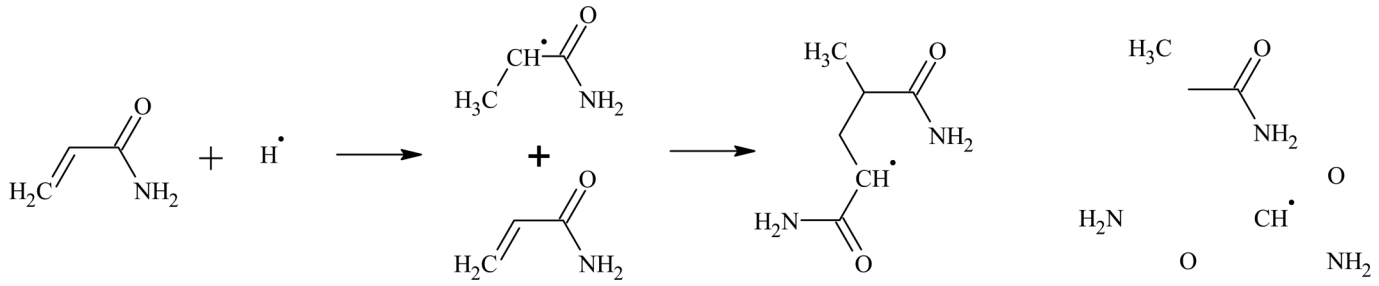

Fig. 1. Polymerization of acrylamide.

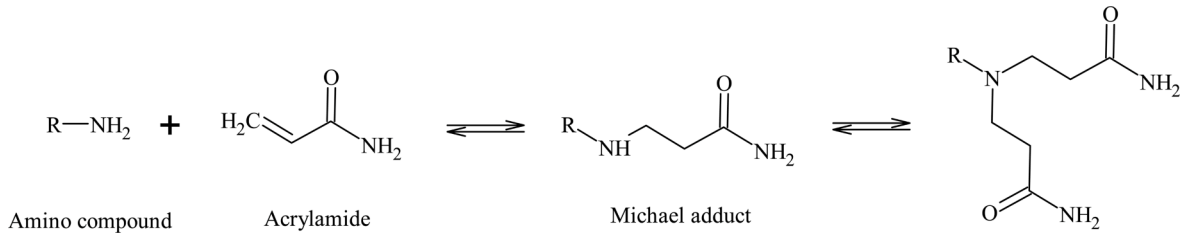

Fig. 2. Michael addition.

Table 4
Colour of grinded samples of cocoa shell.

Sample	L*	a*	b*	C	h°	ΔE
UCS	47.856 ± 3.08	9.23 ± 0.44	17.98 ± 0.08	20.21 ± 0.13	62.84 ± 1.20	
1.5%, 15 min	46.51 ± 0.02	8.51 ± 0.08	15.48 ± 0.07	17.66 ± 0.03	61.19 ± 0.32	2.93 ± 0.05
1.5%, 30 min	45.76 ± 0.02	8.59 ± 0.12	15.44 ± 0.06	17.67 ± 0.02	60.77 ± 0.25	3.36 ± 0.03
1.5%, 45 min	45.27 ± 0.01	8.45 ± 0.03	15.02 ± 0.04	17.24 ± 0.03	60.64 ± 0.14	4.01 ± 0.03
1.5%, 15 min, 40 Hz	46.06 ± 0.03	9.00 ± 0.06	16.97 ± 0.02	19.22 ± 0.01	62.07 ± 0.20	2.08 ± 0.03
1.5%, 30 min, 40 Hz	45.71 ± 0.02	8.70 ± 0.04	15.45 ± 0.03	17.73 ± 0.03	60.64 ± 0.15	3.36 ± 0.03
1.5%, 45 min, 40 Hz	45.79 ± 0.04	8.77 ± 0.04	15.58 ± 0.04	17.88 ± 0.02	60.62 ± 0.15	3.20 ± 0.05
1.5%, 15 min, 80 Hz	45.26 ± 0.03	8.80 ± 0.05	15.65 ± 0.03	17.96 ± 0.02	60.64 ± 0.17	3.51 ± 0.03
1.5%, 30 min, 80 Hz	46.15 ± 0.02	8.74 ± 0.04	15.94 ± 0.02	18.18 ± 0.01	61.27 ± 0.13	2.71 ± 0.02
1.5%, 45 min, 80 Hz	45.47 ± 0.02	8.83 ± 0.02	16.16 ± 0.03	18.42 ± 0.01	61.35 ± 0.08	3.03 ± 0.02
3%, 15 min	43.12 ± 0.02	8.89 ± 0.02	16.21 ± 0.03	18.49 ± 0.03	61.25 ± 0.04	5.07 ± 0.03
3%, 30 min	41.73 ± 0.06	8.79 ± 0.04	15.60 ± 0.05	17.91 ± 0.02	60.62 ± 0.18	6.59 ± 0.07
3%, 45 min	41.53 ± 0.02	8.91 ± 0.04	15.65 ± 0.03	18.01 ± 0.04	60.35 ± 0.10	6.75 ± 0.03
3%, 15 min, 40 Hz	44.13 ± 0.03	8.88 ± 0.05	16.14 ± 0.03	18.42 ± 0.02	61.19 ± 0.18	4.17 ± 0.04
3%, 30 min, 40 Hz	44.45 ± 0.03	8.85 ± 0.03	16.21 ± 0.03	18.47 ± 0.02	61.36 ± 0.10	3.86 ± 0.04
3%, 45 min, 40 Hz	43.73 ± 0.03	8.72 ± 0.03	15.85 ± 0.05	18.09 ± 0.03	61.18 ± 0.15	4.67 ± 0.04
3%, 15 min, 80 Hz	44.42 ± 0.02	8.91 ± 0.05	16.44 ± 0.05	18.70 ± 0.03	61.53 ± 0.18	3.78 ± 0.03
3%, 30 min, 80 Hz	42.40 ± 0.02	8.63 ± 0.05	15.15 ± 0.03	17.44 ± 0.01	60.32 ± 0.18	6.18 ± 0.03
3%, 45 min, 80 Hz	42.67 ± 0.01	8.59 ± 0.07	14.90 ± 0.04	17.20 ± 0.01	60.04 ± 0.28	6.07 ± 0.02

UCS: untreated cocoa shell; ΔE: total colour change; ± Standard deviations (n = 5).

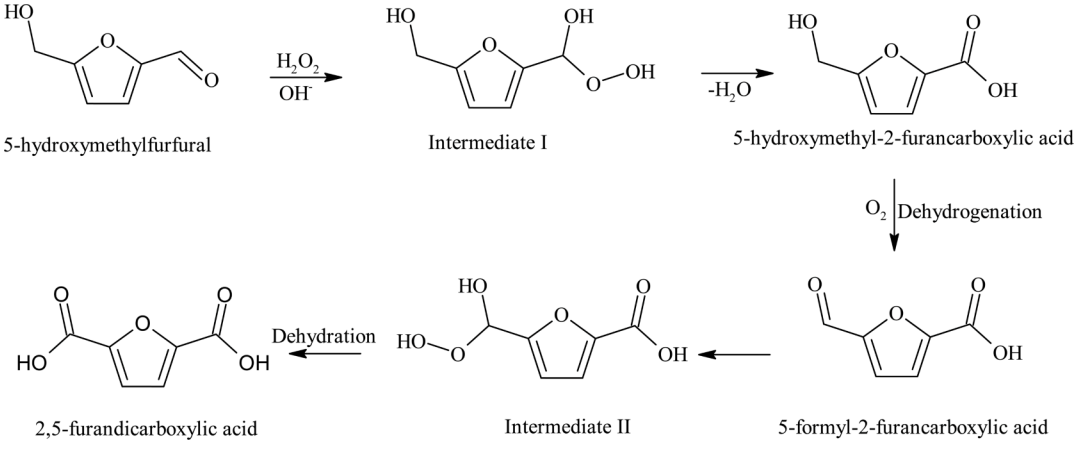

Fig. 3. 5-HMF to FDCA.

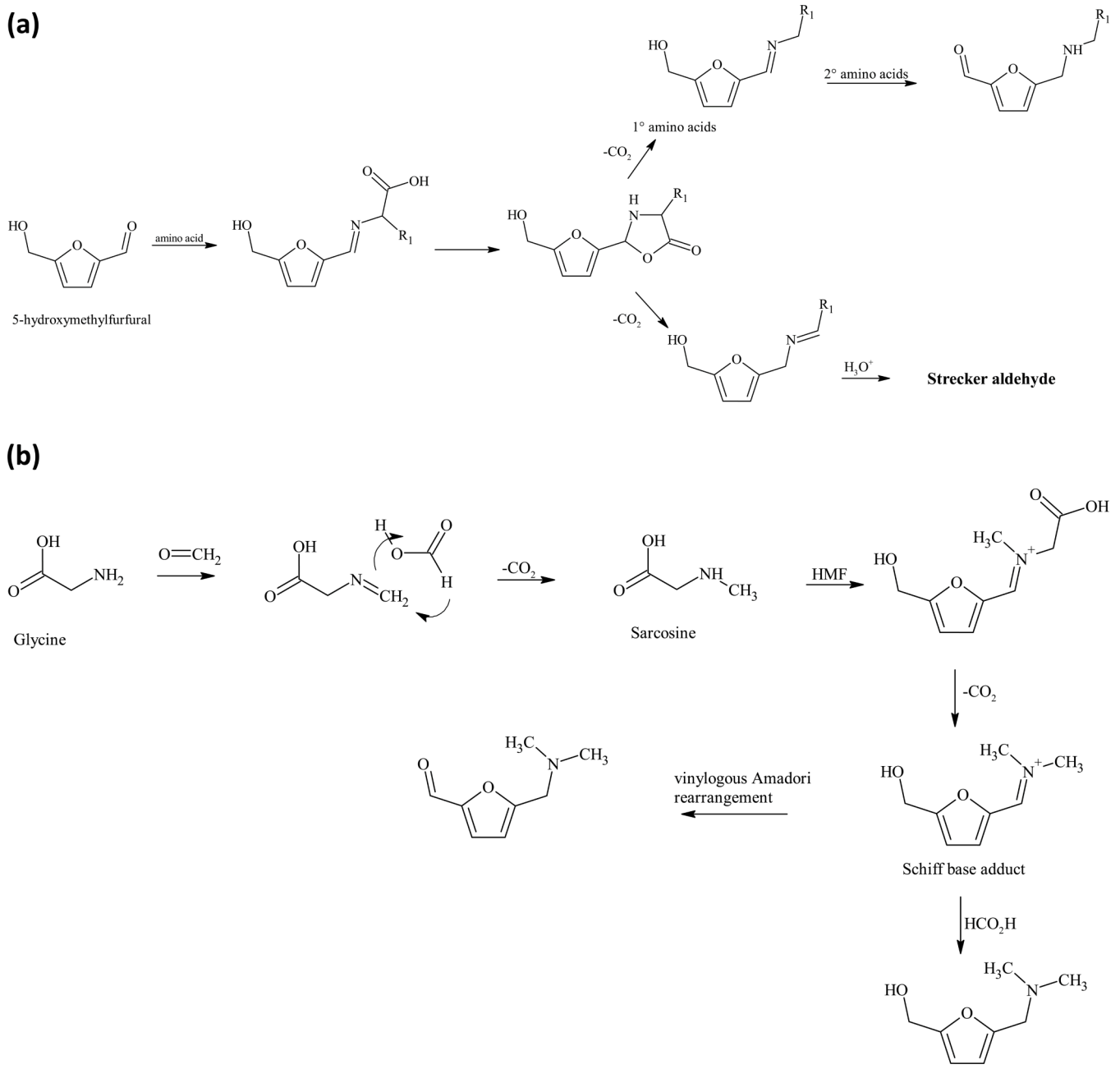

Fig. 4. a) Reaction with primary and secondary amino acids. b) Formation of Shiff base with glycine.

3.2. 5-hydroxymethylfurfural

This compound also forms during roasting by Maillard reactions. Table 1 shows that the untreated cocoa shell had a highest concentration of 5-HMF. All applied treatments and their combinations resulted in a significant decrease in 5-HMF concentration in cocoa shells. The most significant reduction was in samples that were treated only in water probably because 5-HMF diffuses into the water due to its high water solubility (Gomes, Pereira, Ribeiro, & Souza, 2015). This is supported by the fact that more significant 5-HMF reduction was observed in suspension concentrations of 1.5%, since more 5-HMF would have to leach into water to reach equilibrium. Furthermore, the sample with the lowest concentration of 5-HMF (3.54 ± 0.05 mg/kg) was treated in water for 45 min at 1.5% concentration. In HVED-treated samples, 5-HMF reduction occurred to a lower extent.

Although this research showed the reduction in content of 5-HMF in cocoa shell, Jokić et al. (2019) did not report any presence of 5-HMF in the HVED extract obtained from cocoa shell. This, along with decrease of L* values in present research (Table 4), which shows darkening of samples after treatments, may be indicative of advancement of Maillard reaction and conversion of 5-HMF to further Maillard products, induced by HVED. Since the HVED is oxidation method it could result in conversion of 5-HMF to 2,5-furandicarboxylic acid (FDCA) in aqueous media (Fig. 3). FDCA is a very stable component that is poorly soluble in most common solvents (Lewkowski, 2001). Recently, many studies deal with 5-HMF conversion into FDCA because FDCA is a promising substitute for terephthalate in polyesters (Koopman, Wierckx, de Winde, & Ruijssenaars, 2010).

In addition, 5-HMF undergoes reactions with primary and secondary amino acids in advanced Maillard reactions (Fig. 4a), and it has been reported that it forms Shiff base with glycine (Fig. 4b) (Nikolov & Yaylayan, 2011). Although these are considered thermally induced

reactions, energy released by HVED treatment could also trigger them.

The results of 5-HMF content analysis showed a statistically significant difference when concentration, mixing, treatment, combination of concentration and treatment, and combination of all three effects was performed (Table 2).

In the future, it is necessary to explore the possibility of utilizing HVED in the FDCA synthesis. This method has the potential to become a new "green" technique for removing high concentrations of 5-HMF from food but also for the production of FDCA that is increasingly used in the industry.

3.3. Colour

Colour change of treated samples is shown in Table 4. Decrease of L* values indicates that all the treated samples had darkened. Such an effect can be attributed to the drying of the samples upon which the Maillard reactions can occur (Lario et al., 2004). Yaylayan (2003) reported that Amadori rearrangement of amino acids with α-hydroxycarbonyl compounds may lead the Maillard reaction mainly towards chromogenic pathways and melanoidization.

Another reason for darkening of samples may be increased moisture and a_w compared to non-treated sample (Table 1). Namely, Romano, Argyropoulos, Gottschalk, Cerruto, and Müller (2010) reported that increased moisture of the sample causes better propagation of light in the sample, resulting in lower reflection. A greater total colour change (ΔE) and reduction of L* value were observed in water-treated samples than in samples treated with HVED, which is in correlation with a_w and moisture content of the samples. However, a part of the protein could be extracted during the HVED process, reducing the extent of Maillard reactions and darkening of the samples (Parniakov, Barba, Grimi, Lebovka, & Vorobiev, 2014; Sarkis et al., 2015). The slight decrease in values a*, b*, C and h° in all treated samples is also noticeable. The positive a* and b* values indicate that all samples are in the domain of red and yellow colour. Statistical analysis showed that there are statistically significant differences in values L*, b*, C, h° and ΔE of all effects and their combinations in treated samples (Table 2).

4. Conclusions

HVED proved to be a good procedure for reduction of 5-HMF and acrylamide content in the cocoa shell. However, it is yet to be established whether the treatment favours further reactions of these compounds and their conversion to other products or the extraction into the water. Since HVED is a highly complex method in which different radicals are created, it is necessary to examine more thoroughly the effect of electrical discharge on these components in cocoa shell, but in other food products and by-products as well.

Author contribution

Veronika Barišić: Investigation, Data Curation, Writing - Original Draft, Writing – Review & Editing. Flanjak Ivana: Investigation, Writing - Original Draft, Writing – Review & Editing. Ana Tot: Methodology, Validation, Investigation. Maja Budeč: Methodology, Validation, Investigation. Mirta Benšić: Formal analysis. Antun Jozinović: Writing – Review & Editing, Visualization. Jurislav Babić: Resources, Project administration, Funding acquisition. Drago Šubarić: Writing – Review & Editing, Supervision. Borislav Miličević: Writing – Review & Editing. Đurđica Ačkar: Conceptualization, Resources, Writing - Original Draft, Visualization, Supervision, Project administration, Funding acquisition.

Funding

This work has been supported in part by Croatian Science Foundation under the project UIP 2017-05-8709.

Declaration of competing interest

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

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Does High Voltage Electrical Discharge Treatment Induce Changes in Tannin and Fiber Properties of Cocoa Shell?

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Received: 4 June 2020; Accepted: 18 June 2020; Published: 19 June 2020

Abstract: Cocoa shell is a by-product of the chocolate industry that is rich in dietary fiber and bioactive components. In this research, the influence of high voltage electric discharge (HVED) treatment on chemical and physical characteristics of the cocoa shell, i.e., the effects of applied time and frequencies on grinding ability, water binding capacity (WBC), dietary fibers and tannin content was investigated. HVED had a significant influence on the chemical and physical properties of cocoa shell, all of which could be linked to changes in fiber properties. Along with the fiber content, grinding ability and water binding capacity were increased. These properties have already been linked to fiber content and soluble/insoluble fiber ratio. However, this research implies that change in fiber properties could be linked to tannin formation via complexation of other polyphenolic components. Additional research is needed to verify this effect and to establish mechanisms of tannin formation induced by HVED and its influence on fiber quantification.

Keywords: cocoa shell; high voltage electrical discharge; tannin; dietary fiber; water binding capacity; grindability

1. Introduction

Cocoa shell is the major by-product of the cocoa processing industry. It is a part of the cocoa bean that is separated from cotyledon during pre-roasting or after the roasting of beans [1]. It has been reported that several tons of cocoa shell need to be disposed annually, which poses a large problem [2,3]. Cocoa beans are rich in bioactive compounds, which are stored in the cotyledon. During fermentation, these components diffuse into cocoa shell, which becomes rich in bioactive compounds [4]. In addition, cocoa shell is rich in dietary fiber, mainly consisting of cellulose, carbohydrates and pectic polysaccharides [2] and presents great material for use in food industry and enrichment of food poor in dietary fibers. In the last few years, cocoa shell has been used as a raw biomass material, feedstuff, adsorbent, soil conditioner, garden mulch or burnt for fuel [5,6].

High voltage electric discharge (HVED) is a non-thermal process that has been used for the treatment of waste products from the food industry in the last few years [7]. It is also used as an extraction method, because it can disrupt the cellular walls and increase the overall mass transfer of the cellular content [8]. HVED is an innovative technique that interjects energy directly in aqueous solution between electrodes that are submerged. Electric discharge in water consists of two phases: corona streamer discharge process and arc discharge process. For the streamer discharge process weak shock waves are characteristic, as well as small number of bubbles and active radicals. When transiting to arc discharge process, number of bubbles is increased, shock waves become stronger, turbulence and concentration of free radicals are increased [9]. These shock waves and explosions of cavitation bubbles can affect particle size by fragmentation of cell membranes [10]. Electric discharge directly in water leads to production of molecular oxygen and hydrogen, hydrogen peroxide, hydroxyl radicals and oxygen radical ion, all of which are very reactive species.

Since HVED can disrupt cellular walls, which are in cocoa shell predominantly composed of cellulose with lesser amounts of hemicellulose and pectin [3], the aim of this study was to evaluate HVED influence on cocoa shell dietary fiber content and properties related to it. For use of cocoa shell in food industry, dietary fiber content, grindability, water and oil binding capacity and content of bioactive components are very important.

2. Materials and Methods

2.1. Preparation of Cocoa Shell

Cocoa shell samples were obtained after roasting fermented cocoa beans (West Africa mix supplied by Huyser, Möller B.V., Edam, Holland) at 135 °C for 55 min in custom made roaster (Metal workshop "ILMA", Požega, Croatia). After that, the cocoa shell was easily separated by hand from the cotyledon.

Untreated cocoa shell (UCS) sample was obtained by grinding cocoa shell attained after separation from the cotyledon. Control samples were obtained by mixing the unmilled cocoa shell in water for 15, 30 and 45 min at concentrations of 1.5% and 3.0%. After mixing, the shell was separated from water and dried in the laboratory oven (Memmert, UFE 500, Schwabach, Germany) at 40 °C. Dry samples were ground in the laboratory mill (IKA, M20, Staufen, Germany) (25 g for 2 min with cooling) to obtain a fine powder (composite sample obtained by repeated grinding) and as such were frozen and stored for analyses. The grinded untreated cocoa shell was also frozen and stored for analysis in the same way as a cocoa shell mixed in water.

2.2. HVED Treatment

High-voltage electrical discharge equipment which was described by Barišić et al. [11] includes a chamber connected to a high-voltage pulse generator of 30 kV (the device was custom made by Inganiare CPTS1, Osijek, Croatia for the Faculty of Food Technology Osijek). Treatment chamber contains a stainless steel cylindrical needle (diameter 2.5 mm), and the ground electrode in the form of a plate (diameter 45 mm). Mixing of samples is achieved by magnetic stirrer. The distance between the electrodes during all treatments was 2 cm. Electric field density was 15 kV/cm during all treatments. HVED energy input ranged between 13.11–79.80 kJ/kg.

Unmilled cocoa shell (same as control samples prepared in water) was treated in HVED device at concentrations of 1.5% (6 g in 400 mL of distilled water) and 3.0% (12 g in 400 mL of distilled water). The treatment time was 15, 30 and 45 min, and the used frequencies were 40 and 80 Hz. Each sample (HVED, control or untreated) was treated until 200 g of sample was gained which gave us uniform sample for all analyses. The cocoa shell treated with HVED was dried, grind and stored until analyses in the same way as the control samples. Control samples (mixed in water) and HVED treated samples were dried to a dry matter content of 86.00 ± 0.85%.

2.3. Tannin Content

2.3.1. Extraction

Each sample was weighed (2 g) and extracted three times with 10 mL of *n*-hexane (Carlo Erba Reagents, Val de Reuil, France) to remove lipids. Samples were dried at air over night and extracted with 5 mL 70% methanol (J. T. Baker, Deventer, Netherland) in ultrasound bath. After that, samples were centrifuged for 10 min at 3000 rpm (Sigma 2-16, Sigma, Osterode, Germany). Supernatant was decanted in 10 mL volumetric flask. That procedure was repeated twice after which flask with supernatant was filled up with 70% methanol.

2.3.2. Spectrophotometric Analysis

Tannin content was determined by method described by Amorim et al. [12]. Method is based on binding of tannins with casein. Calibration curve was created with the standard solutions of tannic acid (Sigma-Aldrich, St. Louis, USA) in the range of concentrations from 0.5 to 3 mg/mL ($y = 0.9011x + 0.0095$; $R^2 = 0.9993$). Total phenol content and residual phenol content (obtained after complexation of tannin and casein) were determined spectrophotometrically at 760 nm according to the method of Singleton et al. [13]. Tannin content in prepared extracts was calculated Equation (1) as the difference between total phenol content and residual phenol content. Results are presented as mg of tannic acid per g of defatted sample (mg TA/g) and as a percentage of tannin in total phenol content (%).

$$\text{Tannin}\left(\frac{\text{mg TA}}{\text{g}}\right) = \text{total phenol content} - \text{residual phenol content} \quad (1)$$

2.4. Determination of Dietary Fibers

Dietary fibers were determined according to gravimetric AOAC method 991.43 [14]. Samples were treated with thermostable α-amylase, protease and amyloglucosidase (Megazyme Total Dietary Fiber Assay Kit, Megazyme Ltd., Bray, Ireland). The share of insoluble dietary fibers (IDF, %) was determined gravimetrically after filtration, and soluble dietary fibers (SDF, %) were determined by precipitation from the obtained filtrate. After correction for undigested protein (Kjeldahl method) and ash (mineralization at 525 °C), total dietary fibers were calculated Equations (2) and (3) as a sum of IDF and SDF. The values were calculated on the dry matter of the sample.

$$\text{Total Dietary Fibre } (\%) = \frac{\frac{R_1+R_2}{2} - p - A - B}{\frac{m_1+m_2}{2}} \times 100 \quad (2)$$

$$B = \frac{BR_1 + BR_2}{2} - BP - BA \quad (3)$$

where: R_1 = residue weight 1 from m_1; R_2 = residue weight 2 from m_2; m_1 = sample weight 1; m_2 = sample weight 2; A = ash weight from R_1; P = protein weight from R_2; B = blank; BR = blank residue; BP = blank protein from BR_1; BA = blank ash from BR_2.

2.5. Grindability of Cocoa Shell

The grindability of cocoa shell was determined by sieving the powdered cocoa shell samples on analytical sieve shaker (Retsch GmbH, AS200, Haan, Germany) and measurement of mass of obtained fractions. A total of 50 g of the sample was sieved through six sieves (50, 71, 100, 125, 200 and 315 μm) during 15 min. After weighing each fraction, results were expressed as percentages of cocoa shell mass that was weighted on each sieve (%).

2.6. Water Binding Capacity (WBC) and Oil Binding Capacity (OBC)

For determination of WBC standard AACC Method 88-04 [15], was used. To 2.5 g of cocoa shell sample 30 mL of water was added. These solutions were left to stand at room temperature with periodic mixing. After that, the samples were centrifuged at 3000 rpm for 15 min (Centra-MP4R, IEC, Mumbai, India). The supernatant was decanted, and the remaining residue was weighted. The analysis was performed in two repetitions. The results were calculated Equation (4) and were expressed as grams of H_2O absorbed per gram of cocoa shell (g/g).

$$\mathrm{WBC}\left(\frac{\mathrm{g}}{\mathrm{g}}\right) = \frac{\text{gel mass}}{\text{dry matter mass in the initial sample}} \quad (4)$$

For determination of OBC same procedure was used. The only difference was that for OBC instead of water cold pressed rapeseed oil was used. Results were expressed as grams of oil absorbed per gram of cocoa shell (%) obtained by formula Equation (5):

$$\mathrm{OBC}\left(\frac{\mathrm{g}}{\mathrm{g}}\right) = \frac{\text{gel mass}}{\text{dry matter mass in the initial sample}} \quad (5)$$

2.7. Fourier Transform Infrared Spectroscopy with Attenuated Total Reflection (FTIR-ATR) Analysis

FTIR–ATR spectra were recorded with a Cary 630 spectrometer (Agilent, Santa Clara, CA, USA) in wavenumber range from 4000 to 650 cm^{-1}. For each sample, 32 scans were recorded and averaged with a spectral resolution of 16 cm^{-1}.

2.8. Statistical Analysis

Statistical analysis was conducted using Statistica®, Version 13.4.0.14 (1984–2018 TIBCO Software Inc, Palo Alto, CA, USA). To determine the statistically significant difference of treatment effects, main effects and factorial analysis of variance (ANOVA) were used. P-value that was considered significant was 0.05. In addition, Pearson's correlation coefficients was determined ($p < 0.05$).

3. Results and Discussion

3.1. Tannin Content

Tannin content of untreated cocoa shell and treated samples are shown in Figure 1 where results for tannin content (mg TA/g of defatted sample) and percentages of tannins in total phenols (%) are presented. It can be seen that the untreated shell had the lowest content of tannins, and the tannin content increased with all treatments. In samples treated with HVED, share of tannins in total phenols ranged from 45.03 to 65.09%. In our previous research [16], we have measured the decrease of content of all major polyphenolic compounds in cocoa shell treated with HVED (catechin, epicatechin, epicatechin gallate, gallic acid, caffeic acid and *p*-coumaric acid). These components are extractable by water, and the decrease in treated shell may have been the consequence of extraction, as reported by Jokić et al. [17], however, they are also prone to reactions of condensation in suitable conditions (Figure 2). Since the aim of this study was not to establish the effect of HVED treatment on extraction of bioactive compounds, cocoa shell was not milled before treatment, unlike in research of Jokić et al. [17]. Extraction yield is also dependent on electric field intensity, contact surface between material and solvent, liquid to solid ratio, etc. Considering above mentioned, HVED conditions applied in this research are not favorable for extraction [9]. Thus, extraction of polyphenolic compounds was aggravated. HVED generates different reactive species, which may have induced polymerization. Hence, HVED is a source of radicals that can easily oxidize tannins, which leads to an increase in their rigidity. Contrary to our results, Delsart et al. [18] and Lukić et al. [19] reported decrease of total tannin content in red wine treated by HVED and cold plasma, respectively, ascribing it to oxidation of tannins during treatment.

However, one has to bear in mind that cold plasma and HVED treatment differ in that cold plasma includes gas introduction into liquid, and that there are major differences in chemical composition, mainly polyphenolic profile, of the treated samples. In addition, since treatment time in this research was significantly longer, oxidized tannins and other phenols might have been involved in mutual reactions, mainly because the oxidized phenols are hydrophobic. It has been reported that hydrophobic reactions can occur among polyphenols and induce their aggregation [20].

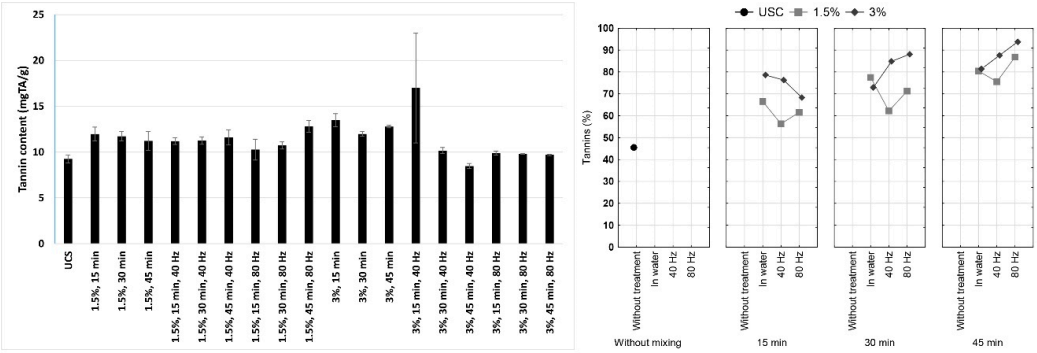

Figure 1. Tannin content (expressed on defatted sample weight) in cocoa shell before and after the high voltage electric discharge (HVED) treatment and percent of tannin in total phenols.

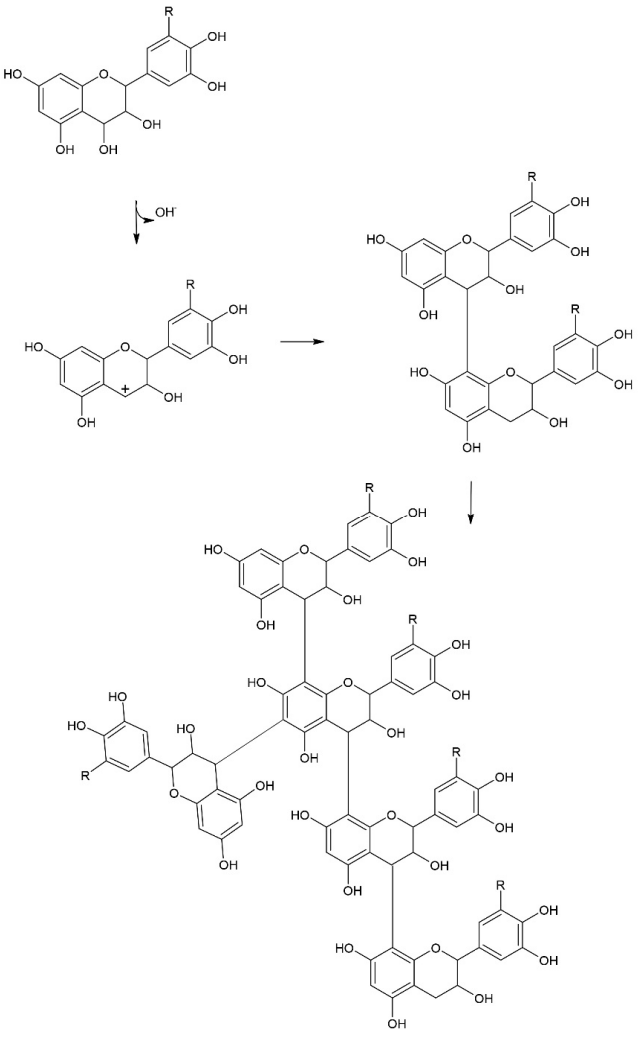

Figure 2. Condensation of polyphenols.

Results of tannin content, both in sample and in total phenols show that tannins are very resistant to HVED treatment. With the exception of 3.0% sample treated at 40 Hz, where significant reduction of tannin content occurred with the increase of treatment time, a slight increase of their content after treatment was observed (Figure 1), possibly due to oxidation and aggregation of tannins, but also due to the loss of a portion of soluble substances during treatment, which led to a change in the ratio of components in the samples.

Statistical analysis confirmed that tannins are very resistant to HVED treatment, since statistical significance was not established. Furthermore, factorial analysis of variance showed that influence of concentration and mixing time on percent of tannin in total phenols is statistically significant (Table 1). Coefficient of correlation is showing that tannin (%) is in relation with the smallest and largest particles, insoluble and total fibers. This may indicate that tannins have an impact on the proportion of fibers in cocoa shells, since the content of insoluble and total fibers increase as the proportion of tannin increases.

Table 1. Factorial analysis of variance.

		Sum of Squares	DF	Mean Square	F-Value	*p*-Value
OBC (g/g)	Intercept	392.4578	1	392.4578	533,103.9	<0.001 *
	Concentration (C)	0.2885	1	0.2885	391.8	<0.001 *
	Mixing (M)	0.1274	2	0.0637	86.5	<0.001 *
	Treatment (T)	0.1614	2	0.0807	109.6	<0.001 *
	C*M	0.0200	2	0.0100	13.6	<0.001 *
	C*T	0.0474	2	0.0237	32.2	<0.001 *
	M*T	0.0516	4	0.0129	17.5	<0.001 *
	C*M*T	0.0821	4	0.0205	27.9	<0.001 *
	Error	0.0133	18	0.0007		
WBC (g/g)	Intercept	2384.278	1	2384.278	313,752.4	<0.001 *
	Concentration (C)	15.149	1	15.149	1993.6	<0.001 *
	Mixing (M)	3.762	2	1.881	247.6	<0.001 *
	Treatment (T)	0.040	2	0.020	2.6	0.101638
	C*M	0.130	2	0.065	8.5	0.002470 *
	C*T	0.177	2	0.088	11.6	<0.001 *
	M*T	0.266	4	0.066	8.8	<0.001 *
	C*M*T	0.516	4	0.129	17.0	<0.001 *
	Error	0.137	18	0.008		
Tannin (mg TA/g of defatted sample)	Intercept	4714.410	1	4714.410	1021.198	<0.001 *
	Concentration (C)	0.030	1	0.030	0.006	0.936726
	Mixing (M)	13.343	2	6.671	1.445	0.261805
	Treatment (T)	17.066	2	8.533	1.848	0.186168
	C*M	24.240	2	12.120	2.625	0.099895
	C*T	10.946	2	5.473	1.186	0.328333
	M*T	29.441	4	7.360	1.594	0.218875
	C*M*T	24.668	4	6.167	1.336	0.294923
	Error	83.098	18	4.617		
Tannin (% of total polyphenols)	Intercept	114,239.9	1	114,239.9	3614.463	<0.001 *
	Concentration (C)	202.5	1	202.5	6.406	0.020914 *
	Mixing (M)	453.1	2	226.5	7.167	0.005134 *
	Treatment (T)	84.5	2	42.3	1.337	0.287391
	C*M	75.8	2	37.9	1.200	0.324306
	C*T	49.7	2	24.9	0.786	0.470508
	M*T	146.0	4	36.5	1.155	0.363192
	C*M*T	102.3	4	25.6	0.809	0.535434
	Error	568.9	18	31.6		

OBC: oil binding capacity; WBC: water binding capacity; DF: degree of freedom; * $p < 0.05$ statistically significant.

3.2. Dietary Fibers

Proportions of soluble, insoluble and total fibers of cocoa shell samples are shown in Figure 3. It can be seen that content of insoluble and total fibers in treated samples is higher than in untreated cocoa shell. The effect of HVED on soluble dietary fibers was not statistically significant (Table 2). An increase of insoluble fiber share after treatment had statistical significance for mixing time and there is a visible trend. A greater effect on increase of insoluble fiber content was in HVED treated samples at 1.5% concentration than at 3.0% due to greater energy input at lower sample concentration.

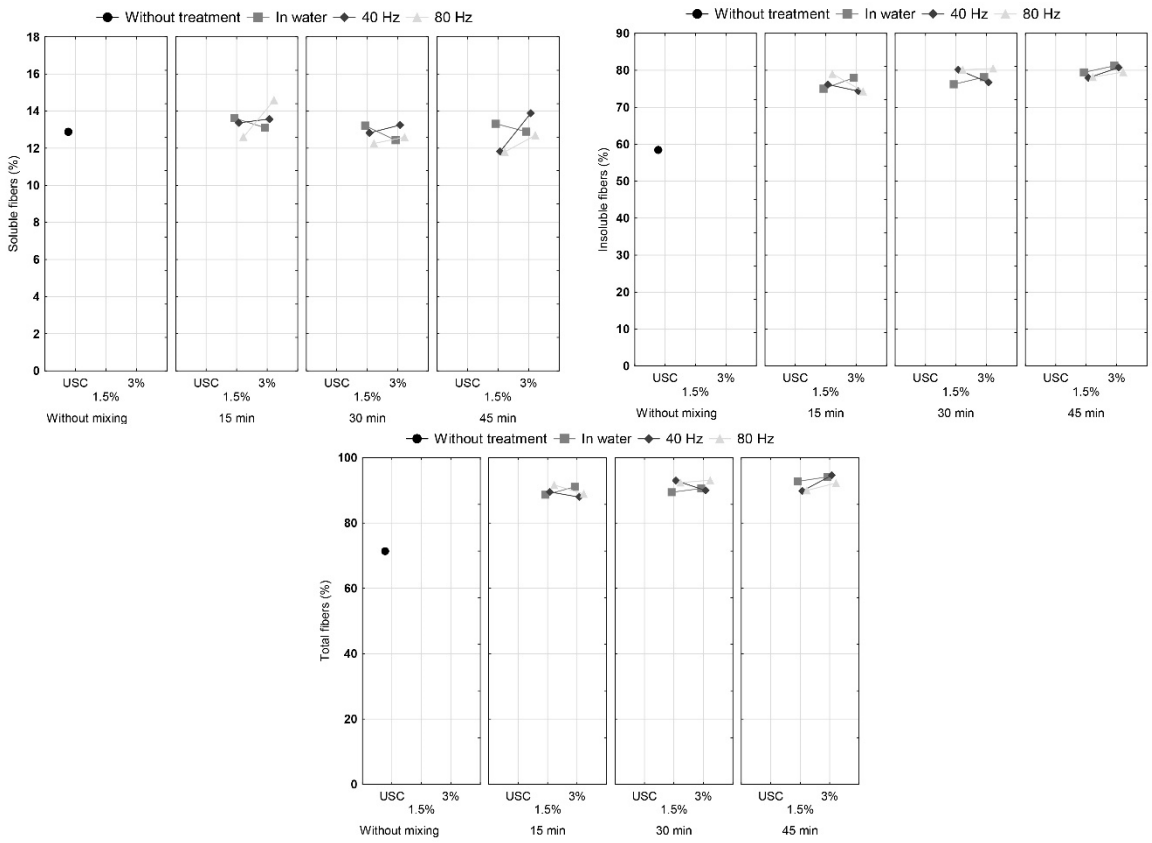

Figure 3. Content of insoluble, soluble and total fibers in cocoa shell before and after the HVED treatment.

Increasing the fiber content in treated cocoa shells can be explained by the fact that during various treatments, fiber probably bonded with other components of the cocoa shell. However, some researches have shown that results obtained by gravimetric determination of fiber may be increased due to presence of insoluble proteins and condensed tannins [21–23]. The method used in this research has a step to exclude undigested proteins from the results for fiber content, however, condensed tannins may have an effect on the observed increase.

Condensed tannins are, along with resistant protein and Maillard reaction products, part of so-called Klason lignin [21], which is not always considered as a fiber. As shown by Perez et al. [24], roasted husk contains large amounts of free amino-acids and sugars, and our previous research [16] showed significant amounts of catechins. HVED generates free radicals and charged particles and highly reactive species (H^+, OH^-, H_2O_2), which may have induced advanced Maillard reactions and reactions of condensation of catechins to condensed tannins (Figure 2).

Our previous research showed that most likely 5-HMF and acrylamide are reacting with free radicals created by HVED and generating new compounds, which could be a part of Klason lignin [25]. This could contribute to increase of insoluble dietary fibers especially because condensed tannins and products of advanced Maillard reactions are insoluble in water.

In addition, we noticed that HVED treated samples had more undigested proteins than non-treated samples (results not shown). Decreased digestibility of proteins can be result of complex formation with tannins especially because HVED treatment generates change in pH and surface charge, which could be favorable conditions for complexation. Reduced in vitro and in vivo digestibility of proteins due to formation of complexes with tannins has already been reported for sorghum and several Acacia species. In addition, protein-protein complexation induced by tannins, and enzyme inhibition by tannins were also reported [26]. Although corrections for proteins were made, the other components that were bonded to them were not included here.

Table 2. Main effects analysis of variance.

	Effect	Sum of Squares	DF	Mean Square	F-Value	*p*-Value
0–50 µm	Intercept	169.1845	1	169.1845	59.27547	0.000006 *
	Concentration	20.7446	1	20.7446	7.26806	0.019455 *
	Mixing	2.4918	2	1.2459	0.43652	0.656146
	Treatment	2.8862	2	1.4431	0.50561	0.615433
	Error	34.2505	12	2.8542		
51–71 µm	Intercept	2025.733	1	2025.733	451.2232	<0.001 *
	Concentration	54.266	1	54.266	12.0875	0.004574 *
	Mixing	12.171	2	6.085	1.3555	0.294609
	Treatment	1.952	2	0.976	0.2174	0.807671
	Error	53.873	12	4.489		
72–100 µm	Intercept	1371.127	1	1371.127	705.1271	<0.001 *
	Concentration	18.601	1	18.601	9.5659	0.009312 *
	Mixing	0.303	2	0.152	0.0780	0.925440
	Treatment	1.729	2	0.864	0.4446	0.651247
	Error	23.334	12	1.945		
101–125 µm	Intercept	525.4466	1	525.4466	970.2258	<0.001 *
	Concentration	0.9016	1	0.9016	1.6648	0.221262
	Mixing	0.5083	2	0.2542	0.4693	0.636443
	Treatment	0.8918	2	0.4459	0.8234	0.462285
	Error	6.4989	12	0.5416		
126–200 µm	Intercept	3189.472	1	3189.472	1644.420	<0.001 *
	Concentration	1.189	1	1.189	0.613	0.448736
	Mixing	2.527	2	1.264	0.651	0.538775
	Treatment	2.636	2	1.318	0.679	0.525389
	Error	23.275	12	1.940		
201–315 µm	Intercept	5128.784	1	5128.784	3745.356	<0.001 *
	Concentration	0.147	1	0.147	0.107	0.748864
	Mixing	3.778	2	1.889	1.379	0.288933
	Treatment	4.432	2	2.216	1.618	0.238628
	Error	16.432	12	1.369		
>315 µm	Intercept	31,757.57	1	31,757.57	1505.873	<0.001 *
	Concentration	26.88	1	26.88	1.275	0.280959
	Mixing	45.68	2	22.84	1.083	0.369462
	Treatment	52.88	2	26.44	1.254	0.320301
	Error	253.07	12	21.09		
Insoluble fibers	Intercept	109,774.9	1	109,774.9	30,704.36	<0.001
	Concentration	0.1	1	0.1	0.02	0.883234
	Mixing	36.8	2	18.4	5.15	0.024326 *
	Treatment	2.7	2	1.4	0.38	0.691002
	Error	42.9	12	3.6		
Soluble fibers	Intercept	3038.191	1	3038.191	7378.913	<0.001 *
	Concentration	0.962	1	0.962	2.336	0.152303
	Mixing	2.116	2	1.058	2.570	0.117785
	Treatment	0.501	2	0.250	0.608	0.560488
	Error	4.941	12	0.412		
Total fibers	Intercept	149,338.0	1	149,338.0	43,452.47	<0.001 *
	Concentration	1.6	1	1.6	0.47	0.508137
	Mixing	21.7	2	10.9	3.16	0.079032
	Treatment	1.0	2	0.5	0.14	0.867489
	Error	41.2	12	3.4		

DF: degree of freedom; * $p < 0.05$ statistically significant.

There are already some researches investigating the effect of electrical discharge on fibers. Yuan et al. [27] concluded that plasma improves the tensile strength and surface roughness, which leads to higher interfacial contact. In addition, during the treatment, it came to oxidation of fibers. Sinha and Panigrahi [28] observed increased hydrophobicity of jute fibers after plasma treatment, probably

because of oxidation or decrease of phenolic and secondary alcoholic groups. Improved flexural strength of fibers occurred because of better adhesion between fibers and matrix. Bozaci et al. [29] and Karahan and Özdoğan [30] came to the conclusion that fibers have increased hydrophilicity, rougher surface and higher proportion of damaged fibers after plasma treatment.

Additional research is needed to reveal whether proposed mechanisms may be applicable to influence of HVED on fibers in cocoa shell.

3.3. Grindability of Cocoa Shell

The largest change in share of particles after HVED treatment was in the particle size ranges 0–50 μm and >315 μm (Table 3). Untreated cocoa shell had the largest percentage of particles between 0 and 50 μm and the smallest percentage of particles larger than 315 μm compared to treated and control samples. Any treatment, either only in water or with HVED, has led to an increase in the share of particles with size greater than 315 μm and a reduction in the share of particles with size less than 50 μm which was proven by coefficient of correlation (Table 4). There is a relation between the smallest and the largest particles. Main effect analysis of variance showed that there was a statistically significant difference between different sample concentrations during treatment for particle sizes of 0–50 μm, 51–71 μm and 72–100 μm (Table 2). In all treated samples decrease in the percentage of smaller particles and an increase in the percentage of larger particles was observed. The minimum change occurred in the sample 1.5%, 30 min, 40 Hz. Statistical analysis shows that there was a correlation between particle sizes and dietary fibers implying that difficulty to grind HVED treated cocoa shell can be caused by increased content of fibers.

Table 3. Grindability of cocoa shell samples before and after the HVED treatment.

Sample	0–50 μm (%)	51–71 μm (%)	72–100 μm (%)	101–125 μm (%)	126–200 μm (%)	201–315 μm (%)	>315 μm (%)
UCS	15.19	21.89	11.83	7.94	18.24	14.10	10.81
1.5%, 15 min	3.63	14.70	8.27	5.29	14.66	17.87	35.58
1.5%, 30 min	3.12	12.64	7.83	5.07	13.12	16.76	41.47
1.5%, 45 min	1.71	10.64	7.33	4.74	12.11	15.44	48.03
1.5%, 15 min, 40 Hz	5.64	13.78	7.42	5.52	13.62	17.42	36.61
1.5%, 30 min, 40 Hz	8.39	13.47	7.33	5.16	13.35	17.51	34.80
1.5%, 45 min, 40 Hz	3.67	13.67	7.42	5.15	12.65	16.94	40.50
1.5%, 15 min, 80 Hz	5.48	10.55	6.28	4.66	11.59	15.75	45.69
1.5%, 30 min, 80 Hz	2.64	11.71	8.59	5.50	13.33	16.75	41.48
1.5%, 45 min, 80 Hz	2.98	9.95	8.93	5.54	13.07	16.67	42.87
3.0%, 15 min	3.52	9.98	7.22	4.64	11.72	15.36	47.56
3.0%, 30 min	2.28	9.19	9.33	5.77	13.10	16.40	43.93
3.0%, 45 min	2.16	9.26	10.98	6.40	15.05	17.31	38.84
3.0%, 15 min, 40 Hz	1.36	8.89	11.45	5.79	15.12	18.76	38.64
3.0%, 30 min, 40 Hz	0.64	4.04	11.23	7.40	15.23	18.69	42.77
3.0%, 45 min, 40 Hz	2.08	8.84	10.14	5.24	12.78	16.18	44.75
3.0%, 15 min, 80 Hz	1.50	11.73	10.83	6.07	15.48	19.18	35.20
3.0%, 30 min, 80 Hz	1.32	6.51	9.05	4.92	12.47	15.72	50.01
3.0%, 45 min, 80 Hz	3.07	11.41	7.46	4.41	11.17	15.14	47.33

UCS: untreated cocoa shell.

Table 4. Pearson's coefficients of correlation.

Variable	0–50 μm	51–71 μm	72–100 μm	101–125 μm	126–200 μm	201–315 μm	>315 μm	WBC (g/g)	OBC (g/g)	Insoluble Fibers (%)	Soluble Fibers (%)	Total Fibers (%)	Tannin (mg TA/g)	Tannin (%)
0–50 μm	1.000													
51–71 μm	0.839	1.000												
72–100 μm	0.006	−0.083	1.000											
101–125 μm	0.364	0.199	0.819	1.000										
126–200 μm	0.430	0.402	0.803	0.908	1.000									
201–315 μm	−0.456	−0.329	0.312	0.210	0.303	1.000								
>315 μm	−0.809	−0.795	−0.467	−0.717	−0.851	0.018	1.000							
WBC (g/g)	−0.252	−0.106	−0.473	−0.422	−0.391	0.096	0.349	1.000						
OBC (g/g)	−0.827	−0.838	−0.103	−0.479	−0.600	0.114	−0.862	0.233	1.000					
Insoluble fibers (%)	−0.765	−0.714	−0.455	−0.674	−0.751	0.264	0.883	0.450	0.776	1.000				
Soluble fibers (%)	−0.519	−0.351	0.098	−0.179	−0.023	0.582	0.268	0.306	0.334	0.334	1.000			
Total fibers (%)	−0.791	−0.723	−0.425	−0.666	−0.722	0.318	0.875	0.464	0.780	0.994	0.435	1.000		
Tannin (mg TA/g)	−0.244	−0.154	0.058	−0.089	0.035	0.357	0.097	−0.118	−0.100	0.123	0.065	0.127	1.000	
Tannin (%)	−0.768	−0.747	0.004	−0.365	−0.511	0.018	0.762	−0.014	0.844	0.635	0.167	0.627	0.067	1.000

Bold values were considered significant at $p < 0.05$.

3.4. Water and Oil Binding Capacity

WBC and OBC are important parameters for processing of food and any change in these properties influences production process. Water binding capacity (WBC) and oil binding capacity (OBC) of cocoa shell samples are shown in Figure 4. It is visible that the sample of untreated cocoa shell had the lowest WBC and OBC. Samples treated at a concentration of 1.5% had the higher WBC compared to samples treated at a concentration of 3.0%. OBC showed the opposite trend, where samples treated at 3.0% had higher capacity for binding oil than samples treated at 1.5%. The largest increases can be observed in samples treated for 45 min in water and with HVED. Statistical analysis showed that there was a statistically significant difference between tested concentrations and shearing time but treatment (with or without HVED) did not show statistical significance. All combinations of these effects have proven to be significant (Table 1).

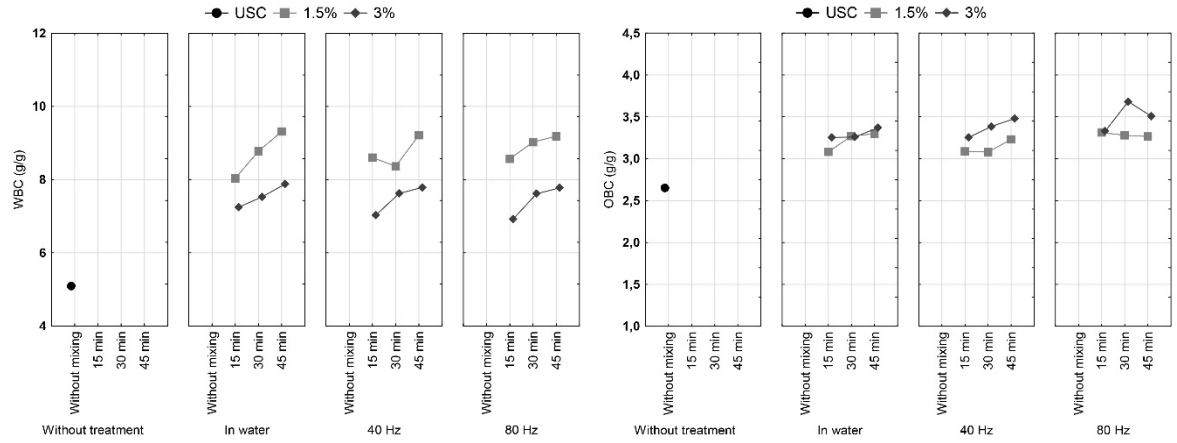

Figure 4. Oil and water binding capacity of cocoa shell before and after the HVED treatment.

According to Sangnark and Noomhorm [31], water and oil binding capacity are correlated to particle size. This research also revealed correlation of OBC with particle sizes (Table 4). Additionally, porosity, overall charge density and hydrophobic properties of fibers, all of which may be changed by HVED treatment, can greatly affect WBC and OBC [31,32]. This may also be substantiated by correlation of OBC with total fiber, insoluble fiber and tannin content in this research.

3.5. FTIR-ATR

The changes in chemical composition by HVED treatment were supported by FTIR-ATR analysis. All the treatments had similar trend so only representative spectra are shown in Figure 5.

In untreated cocoa shell C=O stretching at 1737 cm^{-1} is presented only with a shoulder, and there is a peak at 1602.8 cm^{-1}. After the treatment, a small peak appears at 1737 cm^{-1}. Karahan and Özdoğan [30] ascribed this peak to ester groups of pectin. This is implying that increased content of soluble fibers may be linked to the appearance of this peak after the treatment. However, according to Günzler and Gremlich [33] and Grillo et al. [34], this is also C=O stretch in unconjugated esters, carboxylic acids, aldehydes and ketones.

C-H asymmetric deformation vibrations in untreated shell are presented through a shoulder at 1410 cm^{-1}, whereas after the treatments peak appears at 1431.3 cm^{-1}.

In the untreated cocoa shell there is a peak at 1028.7 cm^{-1} with two shoulders at 1096 cm^{-1} and 1148 cm^{-1}. Treatments did not change shoulder at 1096 cm^{-1}, unlike the other one that has shifted to 1155 cm^{-1} (C-H deformation) and a small peak appears there. This is also close to peak (1152 cm^{-1}) of C-O-C asymmetric vibration in carbohydrates and glucosides according to Grillo et al. [34].

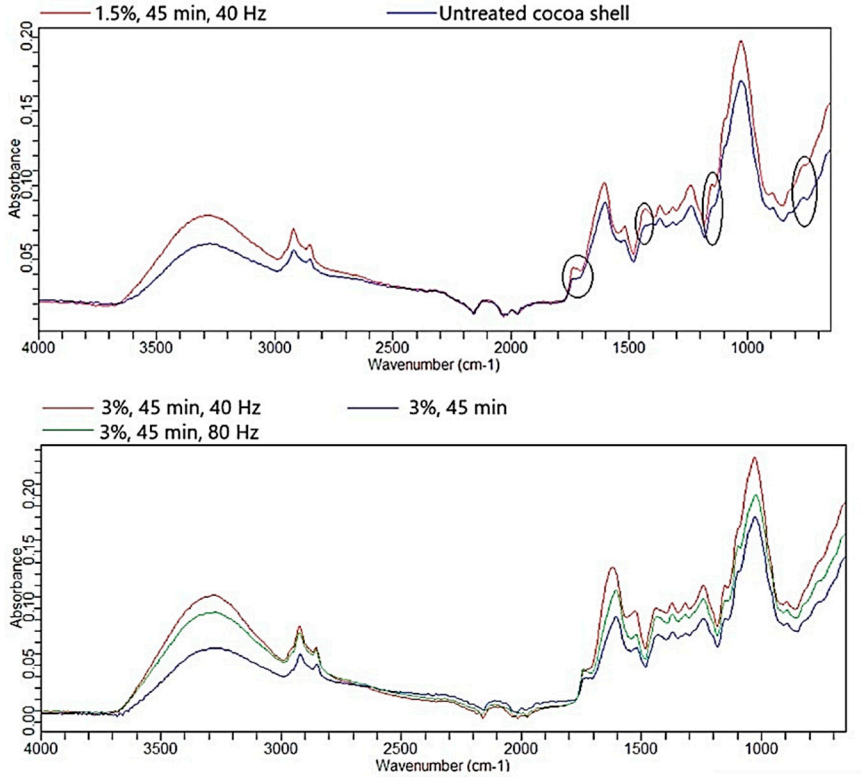

Figure 5. Representative Fourier transform infrared spectroscopy with attenuated total reflection (FTIR-ATR) spectra of cocoa shell before and after the HVED treatment.

Untreated cocoa shell had small peak at 760 cm^{-1} (ring deformation vibrations). Treatments transfers this to shoulder.

These changes in spectra are the result of combined effect of changes in fiber composition (insoluble:soluble ratio) and phenol changes. Bozaci et al. [29] also observed shift of the bands after cold plasma treatment of jute fibers. They assigned this to reaction of fibers with active species from the plasma.

4. Conclusions

In essence, our study showed influence of HVED on fiber properties (soluble, insoluble and total fiber content) and related physical properties—occurrence of larger particle size and increase of water and oil binding capacity. In addition, it has been established that changes in fiber properties correlate to changes in tannin content. It is evident that HVED has a significant influence on the physical and chemical characteristics of cocoa shells due to formation of large number of reactive species, including free radicals and ions, and the reactions occurring during treatment need to be further examined in order to see why such changes are taking place and to reveal actual mechanisms that are involved. Other chemical characteristics of the modified cocoa shell in future studies should be considered as well. In addition, the effect of change in physical and chemical properties of shell on its applicability in different foods needs to be examined because its properties such as grinding, taste, color etc. are the main reasons for its non-use in food production. This research is valuable for future applications of untreated and cocoa shells treated with HVED in the food industry.

Author Contributions: Conceptualization, V.B. and Đ.A.; methodology, I.F., M.K.; investigation, V.B., I.F., M.B., A.J.; writing—original draft preparation, V.B., Đ.A.; writing—review and editing, A.J., J.B., D.Š., B.M., Đ.A.; visualization, A.J., K.D., M.J.; funding acquisition, Đ.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been supported in part by Croatian Science Foundation under the project UIP 2017-05-8709.

Conflicts of Interest: The authors declare no conflicts of interest.

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PRILOG 5

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Izvorni znanstveni rad

Mikrobiološka kvaliteta kakaove ljuske

Sažetak

U ovom radu određena je mikrobiološka kvaliteta kakaove ljuske koja se dobiva kao nusproizvod u proizvodnji čokolade, nakon prženja kakaovog zrna. Kakaova ljuska je tretirana postupkom visokonaponskog električnog pražnjenja (VNEP) pri 40 i 80 Hz tijekom 15, 30 i 45 min u vodenoj suspenziji (pri koncentraciji kakaove ljuske 1,5 i 3,0 %). Istraživanje je pokazalo da je kakaova ljuska, iako je prije analize prošla termički tretman (prženje) jako opterećena mikroorganizmima, ali da se primjenom VNEP može reducirati broj enterobakterija. Naknadna obrada kakaove ljuske sušenjem pri 40 °C, međutim, uzrokuje oporavak mikroorganizama.

Ključne riječi: *kakaova ljuska, visokonaponsko električno pražnjenje, aerobne mezofilne bakterije, kvasci i plijesni, enterobakterije*

Uvod

Kakaova ljuska je nusproizvod u proizvodnji čokolade. Odvaja se od kakaovog zrna najčešće nakon prženja jer se prženjem olabavi veza ljuska – kotiledon te su manji gubici na endospermu koji se koristi u daljnjoj proizvodnji čokolade.

Uobičajeno se kakaova ljuska koristi kao sredstvo za malčiranje, kao stočna hrana i u proizvodnji biogoriva (Panak Balentić i sur., 2018), no kako je iznimno bogata vlaknima i polifenolima, a sadrži i metilksantine i vitamin D (Nsor-Atindana i sur., 2012a i b; Martinez i sur., 2012), ima veliki potencijal i za primjenu u prehrambenoj industriji. Tako su do sad istraživane mogućnosti ekstrakcije polifenolnih tvari (Arlorio i sur., 2005), teobromina (Hartati, 2010), dijetalnih vlakana (Nsor-Atindana i sur., 2012b) i pektina (Mollea i sur., 2008; Vriesmann i sur., 2011), kakaova ljuska korištena je kao adsorbens za pročišćavanje voda (de Luna i sur., 2017) i supstrat u proizvodnji bioplina (Ward-Doria i sur., 2016) i enzimskih preparata (Yusof i sur., 2016; Khanamadi i sur., 2016).

Kakaovom ljuskom obogaćivani su keksi (Karlina i sur., 2012), ekstrudirani snack proizvodi (Jozinović i sur., 2016), vlaknima kakaove ljuske obogaćeni su kruh (Collar i sur., 2009) i muffini (Martinez-Cervera i sur., 2011), polifenoli kakaove ljuske korišteni su kao antioksidansi u ulju za prženje (Manzano i sur., 2017) i kuhanoj govedini (Ismail i Lee, 2006).

Međutim, veliko ograničenje u primjeni kakaove ljuske mogla bi biti njena mikrobiološka kvaliteta jer je ljuska svojevrsna zaštita zrna od utjecaja okolišnih faktora, uključujući i mikroorganizme. Stoga je cilj ovoga rada bio ispitati mikrobiološku kvalitetu kakaove ljuske uzete nakon prženja i utjecaj naknadne obrade ljuske na nju.

Materijali i metode

Kakaovo zrno prženo je na 135 °C tijekom 55 min. Nakon toga, odvojena je ljuska koja je uzeta za daljnja istraživanja.

Kao nulti uzorak (netretirana kakaova ljuska) uzeta je ljuska nakon prženja. Nadalje, pripravljene su 1,5 % i 3 % suspenzije kakaove ljuske u destiliranoj vodi koje su tretirane na sljedeći način:

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-Miješanjem na magnetnoj miješalici pri sobnoj temperaturi tijekom 15 min, 30 min i 45 min

-Tretiranjem visokonaponskim električnim pražnjenjem (VNEP) pri 40 Hz i 80 Hz tijekom 15 min, 30 min i 45 min.

Uređaj za VNEP konstruiran je na Prehrambeno-tehnološkom fakultetu, a izrađen je u suradnji s firmom Inganiare CPTS1. Tretiranje uzoraka u komori uređaja radi se „pin-to-plate" (razmak između elektroda 2 cm), a miješanje uzoraka je pomoću magnetske miješalice.

Nakon tretmana ljuska je filtracijom odvojena od tekućeg dijela te osušena na 40 °C u laboratorijskom sušioniku (Memmert UFE 500) i samljevena u laboratorijskom mlinu (IKA Universal mill M20 S000) tako da prolazi kroz sito 2 mm.

Svi uzorci su odmah nakon tretmana zamrznuti (-18 °C) i tako čuvani do analize.

Udio suhe tvari određen je sušenjem na 105 °C do konstantne mase, a aktivitet vode pomoću uređaja za mjerenje aktiviteta vode (HygroLab 3, Rotronic). Mikrobiološka analiza provedena je prema standardnim metodama: EN ISO 4833-1:2013, EN ISO 6579:2005, EN ISO 6579/Cor2:2010, EN ISO 21528-2:2013 i EN ISO 21527:2009. Svaka metoda je rađena iz posebnih razrjeđenja, tj. za svaku metodu po uzorku je vršena posebna odvaga 25 g za *Salmonella* spp., a za ostale tri metode posebno po 10 g kakaove ljuske.

Rezultati i rasprava

Cilj ovoga rada bio je ispitati mikrobiološku kvalitetu kakaove ljuske koja se dobije kao nusproizvod nakon prženja kakaovog zrna prije i nakon tretmana visokonaponskim električnim pražnjenjem (VNEP) i sušenja pri 40 °C. Temperatura sušenja odabrana je u skladu s preporukama za termički tretman sirovina bogatih polifenolima, kako bi se smanjila toplinska degradacija ovih bioaktivnih komponenti.

U Tablici 1 prikazane su vrijednosti udjela suhe tvari i aktiviteta vode u analiziranim uzorcima. Iz dobivenih rezultata vidljivo je da je miješanjem u vodi, bez obzira na to je li primijenjen tretman VNEP ili ne, porastao aktivitet vode u uzorcima, ali je on i dalje značajno niži od 0,95.

Tablica 1. Udio suhe tvari i aktivitet vode (a_w) u prženoj kakaovoj ljusci prije i nakon tretmana suspenzije neusitnjene ljuske koncentracije 1,5 % i 3,0 % (miješanje – u vodi pri sobnoj temperaturi; 40 Hz i 80 Hz – tretman visokonaponskim električnim pražnjenjem u vodi uz miješanje pri navedenoj frekvenciji)

Table 1. Dry matter content and water activity (a_w) in roasted cocoa husk before and after the treatment in water suspension 1,5% and 3,0% (shearing – in water at room temperature; 40 Hz and 80 Hz – treatment by high-voltage electric discharge in water with shearing at the appropriate frequency)

Tretman / Treatment	1,5 %		3,0 %	
	Suha tvar / Dry matter (%)	a_w	Suha tvar / Dry matter (%)	a_w
Netretirana ljuska / untreated cocoa husk	94,29	0,372	94,29	0,372
15 min, miješanje / shearing	87,78	0,552	85,93	0,559
30 min, miješanje / shearing	87,38	0,570	85,96	0,561
45 min, miješanje / shearing	86,45	0,579	84,97	0,572
15 min, 40 Hz	86,87	0,557	87,22	0,58
30 min, 40 Hz	88,63	0,532	86,76	0,591
45 min, 40 Hz	87,90	0,545	86,83	0,579
15 min, 80 Hz	86,92	0,577	87,19	0,597
30 min, 80 Hz	87,72	0,547	86,24	0,583
45 min, 80 Hz	87,43	0,567	86,15	0,78

U Tablicama 2 i 3 prikazani su rezultati mikrobiološke analize kakaove ljuske. Napravljena „dvostruka slijepa provjera" – za usporedbu su, osim netretirane ljuske i ljuske tretirane VNEP pri dvije frekvencije, pripravljeni i uzorci koji su samo miješani u vodi tijekom odgovarajućeg vremena kako bi se razlučilo ima li na mikroorganizme utjecaja VNEP ili samo voda.

Tablica 2. Mikrobiološka kvaliteta pržene kakaove ljuske prije i nakon tretmana suspenzije neusitnjene ljuske koncentracije 1,5 % (miješanje – u vodi pri sobnoj temperaturi; 40 Hz i 80 Hz – tretman visokonaponskim električnim pražnjenjem u vodi uz miješanje pri navedenoj frekvenciji)

Table 2. Microbial quality of roasted cocoa husk before and after the treatment in water suspension 1,5% (shearing – in water at room temperature; 40 Hz and 80 Hz – treatment by high-voltage electric discharge in water with shearing at the appropriate frequency)

Uzorak / Sample	Metode i kriteriji prihvatljivosti / Methods and criteria			
	Salmonella spp. (n.n.* u 25 g)	*Enterobacteriaceae* (m=10 cfu/g M=10^2cfu/g)	Aerobne mezofilne bakterije / Aerobic mesophilic bacteria cfu (m=10^4cfu/g M=$5x10^4$cfu/g)	Kvasci i plijesni / Yeasts and molds (m=10^2cfu/g M=10^3cfu/g)
Netretirana kakao ljuska / untreated cocoa husk	n.n. u 25 g	$2,7x10^3$cfu/g	$1,4x10^6$cfu/g	<10 cfu/g
1,5 %, 15 min, miješanje / shearing	n.n. u 25 g	$1,6x10^5$ cfu/g	$3,2x10^6$cfu/g	$1,8x10^3$cfu/g
1,5 %, 30 min, miješanje / shearing	n.n. u 25 g	$3,5x10^6$cfu/g	$2,4x10^7$cfu/g	$2,4x10^4$cfu/g
1,5 %, 45 min, miješanje / shearing	n.n. u 25 g	$1,2x10^6$ cfu/g	$2,3x10^7$cfu/g	$2,6x10^4$cfu/g
1,5 %, 15 min, 40 Hz	n.n. u 25 g	$1,5x10^6$cfu/g	$1,6x10^7$cfu/g	$1,5x10^4$cfu/g
1,5 %, 30 min, 40 Hz	n.n. u 25 g	$1,5x10^5$ cfu/g	$2,2x10^6$cfu/g	$3,6x10^3$cfu/g
1,5 %, 45 min, 40 Hz	n.n. u 25 g	$1,4x10^4$ cfu/g	$2,8x10^5$cfu/g	$1,8x10^3$cfu/g
1,5 %, 15 min, 80 Hz	n.n. u 25 g	$5,1x10^4$cfu/g	$6,8x10^6$cfu/g	$1,8x10^3$cfu/g
1,5 %, 30 min, 80 Hz	n.n. u 25 g	$5,5x10^4$cfu/g	$1,7x10^7$cfu/g	$9,0x10^3$cfu/g
1,5 %, 45 min, 80 Hz	n.n. u 25 g	$7,6x10^5$cfu/g	$2,6x10^7$cfu/g	$1,9x10^4$cfu/g

**odsutnost / not detected*

Niti u jednom uzorku nije detektirana prisutnost *Salmonella* spp. Svi uzorci imali su veliki broj aerobnih mezofilnih bakterija (od $2,8 \times 10^5$ cfu/g u ljusci tretiranoj u 1,5 % suspenziji 45 min pri 40 Hz do $2,8 \times 10^7$ cfu/g u ljusci tretiranoj u 3 %-tnoj suspenziji tijekom 30 min na 80 Hz) i enterobakterija (od $1,9 \times 10^3$ cfu/g u ljusci tretiranoj u 3 %-tnoj suspenziji na 80 Hz tijekom 15 min do $3,5 \times 10^6$ cfu/g kod ljuske miješane u vodi u 1,5 %-tnoj suspenziji tijekom 30 min). Pri tome su svi uzorci tretirani u 1,5 %-tnoj suspenziji imali veći broj enterobakterija od netretirane kakaove ljuske, a u 3 %-tnoj suspenziji samo je kod jednog uzorka došlo do smanjenja njihovog broja. Nakon svih tretmana došlo je do porasta broja plijesni i kvasaca u odnosu na netretiranu ljusku, pri čemu je on nešto manji kod uzoraka na kojima je primijenjeno VNEP u 1,5 %-tnoj suspenziji, dok je u 3 %-tnoj suspenziji kod uzoraka tretiranih VNEP taj broj čak veći nego kod uzoraka koji su samo miješani u vodi.

Ranija istraživanja, međutim, pokazala su da se VNEP može koristiti u cilju smanjenja mikrobiološke kontaminacije. Tako su Ahmed i sur. (2017) objavili da su primjenom plazme u vodi inaktivirali *Escherichia coli*. Pri tome su utvrdili da i reaktivne čestice koje se generiraju i/ili do-

daju tijekom tretmana (O_2, H_2O_2) imaju utjecaja na sterilizirajući efekt plazme, koji se zadržao i 72 h nakon tretmana. Ragni et al. (2016) ispitali su utjecaj materijala od kojeg su izrađene elektrode za plinsku plazmu koja se generira dielektričnim pražnjenjem na *Escherichia coli* i *Listeria monocytogenes*. Utvrdili su da napon, jakost struje i aktivna snaga te reaktivne čestice (u ovom slučaju nitrati i nitriti) nemaju značajnog utjecaja na dekontaminaciju vode, ali su srebro i mesing imali značajan utjecaj. Butscher i sur. (2016) utvrdili su i da priroda tretiranog materijala značajno utječe na uspješnost procesa, pri čemu je smanjenje broja endospora *Geobacillus stearothermophilus* na polipropilenskim glatkim površinama bilo značajno veće u odnosu na zrna pšenice, koja imaju neravnu površinu i duboku brazdicu.

Delsart i sur. (2105) primijenili su VNEP u inaktivaciji mikroorganizama u vinu te su postigli značajno smanjenje broja plijesni i bakterija, ali tretman nije bio uspješan kao pulsirajuće električno polje, a došlo je i do sniženja udjela polifenola.

Tablica 3. Mikrobiološka kvaliteta pržene kakaove ljuske prije i nakon tretmana suspenzije neusitnjene ljuske koncentracije 3,0 % (miješanje – u vodi pri sobnoj temperaturi; 40 Hz i 80 Hz – tretman visokonaponskim električnim pražnjenjem u vodi uz miješanje pri navedenoj frekvenciji)

Table 3. Microbial quality of roasted cocoa husk before and after the treatment in water suspension 3% (shearing – in water at room temperature; 40 Hz and 80 Hz – treatment by high-voltage electric discharge in water with shearing at the appropriate frequency)

Uzorak / Sample	Metode i kriteriji prihvatljivosti /Methods and criteria			
	Salmonella spp. (n.n.* u 25 g)	*Enterobacteriaceae* (m=10 cfu/g M=10^2cfu/g)	Aerobne mezofilne bakterije / Aerobic mesophilic bacteria (m=10^4cfu/g M=$5x10^4$cfu/g)	Kvasci i plijesni / Yeasts and molds (m=10^2cfu/g M=10^3cfu/g)
Netretirana kakao ljuska / untreated cocoa husk	n.n. u 25 g	$2,7x10^3$cfu/g	$1,4x10^6$cfu/g	<10 cfu/g
3 %, 15 min, miješanje / shearing	n.n. u 25 g	$1,0x10^4$cfu/g	$7,4x10^5$cfu/g	$2,7x10^3$cfu/g
3 %, 30 min, miješanje / shearing	n.n. u 25 g	$6,7x10^5$cfu/g	$3,4x10^6$cfu/g	$9,1x10^2$cfu/g
3 %, 45 min, miješanje / shearing	n.n. u 25 g	$1,8x10^5$cfu/g	$3,0x10^6$cfu/g	$9,1x10^2$cfu/g
3 %, 15 min, 40 Hz	n.n. u 25 g	$7,9x10^3$ cfu/g	$1,7x10^6$cfu/g	$1,3x10^5$cfu/g
3 %, 30 min, 40Hz	n.n. u 25 g	$9,1x10^3$cfu/g	$6,0x10^5$cfu/g	$1,1x10^4$cfu/g
3 %, 45 min, 40 Hz	n.n. u 25 g	$2,9x10^4$cfu/g	$8,0x10^5$cfu/g	$9,1x10^3$cfu/g
3 %, 15 min, 80 Hz	n.n. u 25 g	$1,9x10^3$cfu/g	$1,0x10^6$cfu/g	$3,2x10^4$cfu/g
3 %, 30 min, 80 Hz	n.n. u 25 g	$1,3x10^5$cfu/g	$2,8x10^7$cfu/g	$9,5x10^4$cfu/g
3 %, 45 min, 80 Hz	n.n. u 25 g	$3,1x10^4$cfu/g	$1,2x10^6$cfu/g	$3,2x10^4$cfu/g

**odsutnost / not detected*

Do porasta broja mikroorganizama u ovom istraživanju, međutim, najvjerojatnije nije došlo uslijed tretmana VNEP, nego tijekom sušenja. Naime, temperatura sušenja bila je 40 °C, što nije značajno više od optimalne temperature za razvoj mikroorganizama te je tijekom sušenja mo-

glo doći do oporavka preživjelih stanica i njihovog razmnožavanja. Do sličnih rezultata došlo se u radu Mandure (2016), gdje je utvrđeno da je nakon 18 sati na 30 °C došlo do rekuperacije stanica *E. coli*, iako je tretman plazmom pri 60, 90 i 120 Hz uzrokovao oksidacijski stres i odumiranje bakterija.

Kako bi se provjerila ova hipoteza, odabran je uzorak ljuske tretiran u 3 %-tnoj suspenziji 15 min na 40 Hz. Tretman je ponovljen u istim uvjetima, ali je odmah po završetku tretmana dekantiran višak vode, a tretirana ljuska zamrznuta i liofilizirana u laboratorijskom liofilizatoru Alpha LSC Plus, Christ, Njemačka (početna temperatura proizvoda: -11 °C; vakuum postignut nakon 4 min; temperaturna rampa: -20 °C, 2 h/-15 °C, 2 h/-10 °C, 2,5 h/-5 °C, 2 h/0 °C, 3 h/final drying: 25 °C, 2 h). Liofiliziranoj ljusci također je određena mikrobiološka kvaliteta te je utvrđeno da sadrži < 10 cfu/g enterobakterija, $2{,}4 \times 10^6$ cfu/g aerobnih mezofilnih bakterija i 10^2 cfu/g kvasaca i plijesni što ukazuje na uspješnost VNEP u redukciji broja enterobakterija.

Zaključak

Iako je kakaova ljuska dobar izvor prehrambenih vlakana i polifenola te ima veliki potencijal za primjenu u proizvodnji prehrambenih proizvoda u okviru kružne ekonomije, pri tome je potrebno voditi računa i o njenoj sigurnosti za potrošača. S obzirom da se radi o dijelu zrna koji je u velikoj mjeri izložen okolišnim utjecajima, posebnu pozornost treba obratiti na njenu mikrobiološku kvalitetu i mogućnosti poboljšanja iste. Primjenom visokonaponskog električnog pražnjenja moguće je reducirati broj enterobakterija na kakaovoj ljusci, ali je potrebno dodatno istražiti optimalne uvjete tretiranja i utjecaj naknadne manipulacije ljuskom na rekuperaciju mikroorganizama.

Zahvala

Ovaj rad je sufinancirala Hrvatska zaklada za znanost projektom UIP-2017-05-8709.

Zahvaljujemo se Javnoj ustanovi "Veterinarski Zavod Hercegovačko-neretvanske županije/kantona", Mostar, BiH na pomoći u provedbi analiza, posebno doc. dr. sc. Maji Arapović i Anki Čerkez.

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Prispjelo/Received: 9.12.2018. Prihvaćeno/Accepted: 1.3.2019.

Original scientific paper

Microbial Quality of Cocoa Husk

Abstract

In this paper, microbial quality of cocoa husk, a by-product of chocolate production after roasting cocoa bean, was evaluated. Cocoa husk was treated by high-voltage electrical discharge (HVED) at 40 and 80 Hz during 15, 30 and 45 min in water suspension (at concentration of 1,5 and 3,0%). The research showed that cocoa husk, although it has been subjected to thermal treatment during roasting, is heavily loaded with microorganisms, but the number of enterobacteria may be reduced by HVED. Subsequent drying at 40 °C, on the contrary, enables revival of microorganisms.
Keywords: *cocoa shell, high-voltage electrical discharge, aerobic mesophilic bacteria, yeasts and moulds, enterobacteria*

PRILOG 6

Received: 17 July 2021 | Revised: 9 December 2021 | Accepted: 1 January 2022

DOI: 10.1111/jfpp.16308

Journal of Food Processing and Preservation | Institute of Food Science + Technology ifst | WILEY

ORIGINAL ARTICLE

Valorization of cocoa shell: Impact of high voltage electrical discharge and drying technology on properties of cocoa shell

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Funding information
This work has been supported in part by the Croatian Science Foundation under the project "Application of cocoa husk in production of chocolate and chocolate-like products" 2017-05-8709

Abstract

There is a growing interest for use of cocoa shell for the enrichment of different food products and for resolving problem of disposal of shell. High voltage electrical discharge (HVED) is a non-thermal technology that could solve problems linked to use of cocoa shell (contaminants and undesirable components). This paper investigated the broader impact of HVED on the properties of cocoa shells and how much influence drying (freeze- and oven- drying), which needs to be conducted, actually has on these properties. After the treatments, oil binding capacity increased in freeze-dried samples from 1.598 to 2.054 g/g. Also, water was easier to remove from HVED-treated oven-dried samples (by 1.75%). HPLC analysis showed that HVED caused better preservation of (–)-epicatechin and (–)-epicatechin gallate after oven-drying. Klason lignin contents slightly increased and differential scanning calorimetry showed higher thermostability of cocoa shell especially after HVED and freeze-drying because peak shifted for 11.54°C.

Practical applications

Cocoa shell is a valuable by-product of the chocolate industry, and it presents a problem for the environment. High voltage electrical discharge (HVED) would be a sustainable solution for this problem. After the HVED treatment, drying process is needed, and this study presents insights in a combination of these two technologies on cocoa shell properties. These results show that cocoa shells can be modified with these processes and are suitable for application in different food products.

1 | INTRODUCTION

Cocoa shell is a by-product of the chocolate industry, which is generated in large quantities (700 thousand tons worldwide every year) (Rojo-Poveda et al., 2020). This by-product is rich in insoluble and soluble fibers, proteins, and fat, with a similar fatty acid composition as cocoa butter (Okiyama et al., 2019). In addition, it has a high content of methylxanthines and phenolic components (Barišić, Flanjak, Križić, et al., 2020). Many scientists are investigating the possibilities of using the cocoa shell in food production. There are already studies of the addition of milled cocoa shell in snack products (Jozinović et al., 2019) and functional beverage (Rojo-Poveda et al., 2019) and the addition of soluble dietary fibers obtained from the cocoa shell with the enzymatic process in muffins (Martínez-Cervera et al., 2011) and bread (Collar et al., 2009).

The main obstacle to using the cocoa shell in the production of food products is that it contains undesirable components. These components are present because of cocoa bean processing where the cocoa shell is in contact with external pollution. Because of that, it can contain different *Enterobacteriaceae* (Nascimento et al., 2015), ochratoxin A (Copetti et al., 2013) and other aflatoxins (Copetti et al., 2012), heavy metals (Assa et al., 2018), etc. In our previous

study (Barišić, Jozinović, et al., 2020) we described problems of the use of the cocoa shell in food production and presented high voltage electrical discharge (HVED) as a possible solution.

HVED is a novel non-thermal technology based on the creation of electric discharges in water (Boussetta et al., 2013). It is reported that it can be used as a water cleaning technology, for microorganism inactivation, as an extraction technology, etc. (Boussetta & Vorobiev, 2014). During this treatment, when streamer propagation occurs between electrodes, UV-light and different radicals are created (Boussetta et al., 2011). This could lead to the poration of the cell membrane (electroporation) of treated materials (Li et al., 2019). After such treatment in water, drying of treated materials is needed so that it can be used in food production. If by-products of the food industry are to be used in food production, the goal is to produce a material in which as many bioactive components as possible are preserved. Freeze-drying is one of the most desirable technologies for accomplishing that. This process is conducted at low temperatures during which sublimation of water occurs, unlike oven-drying where water is removed by evaporation (Chaloeichitratham et al., 2018).

The aim of this study was to investigate the effect of drying technology (freeze- and oven-drying) and HVED on the physicochemical characteristics of the roasted cocoa shell. The main issue in this paper was to evaluate to which extent those drying technologies after HVED treatment actually affect the physicochemical modifications of the cocoa shell previously reported in our studies. The aim was also to get a better insight into the changes that are happening and to connect these changes with the previously published results. We determined water content, water activity, water- and oil-binding capacity (OBC), color, specific volume, apparent density, bulk density, thermostability, and content of phenols, methylxanthines, tannins, and Klason lignin of cocoa shell samples. All these parameters are important for getting insight into material's possible behavior in food systems and the content of bioactive components with which food could be enriched.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

Cocoa shell was collected after the roasting of fermented cocoa beans (West Africa mix, Huyser, Möller B.V., Edam, Holland). Beans were roasted at 135°C for 55 min after which the shell was separated from the cotyledon. Schematic presentation of sample preparation is presented in Figure 1. The control sample (untreated cocoa shell [UCS] sample) of the cocoa shell was prepared by grinding cocoa shell collected after separation from cotyledon. For the preparation of all samples, unmilled cocoa shell was used to decrease the contact area of cocoa shell and water and thus decrease a loss of bioactive components. Control water samples were prepared by mixing unmilled cocoa shell in demineralized water (0.5%) for 10 min. HVED-treated samples were prepared by HVED treatment of unmilled cocoa shell in demineralized water. HVED generator consisted of 30 kV high-voltage pulse generator described by Barišić, Jozinović, et al. (2020). Treatment was conducted at 70 Hz, for 10 min in a water suspension with a concentration of 0.5% and with 0.5 cm distance between the needle and ground electrode. Water used for treatment had pH 5.86 ± 0.00 and conductivity 12.05 ± 0.30 μS/cm. Before treatment mixtures with cocoa shell had pH 5.14 ± 0.09 and conductivity 86.00 ± 4.00 μS/cm. Mixtures after the treatment had pH 5.38 ± 0.10 and conductivity 247.50 ± 4.50 μS/cm.

Treated samples were divided into two subsamples. One part of water control (WDCS sample) and HVED-treated (HDCS sample) samples were dried in a laboratory oven (Memmert, UFE 500) at

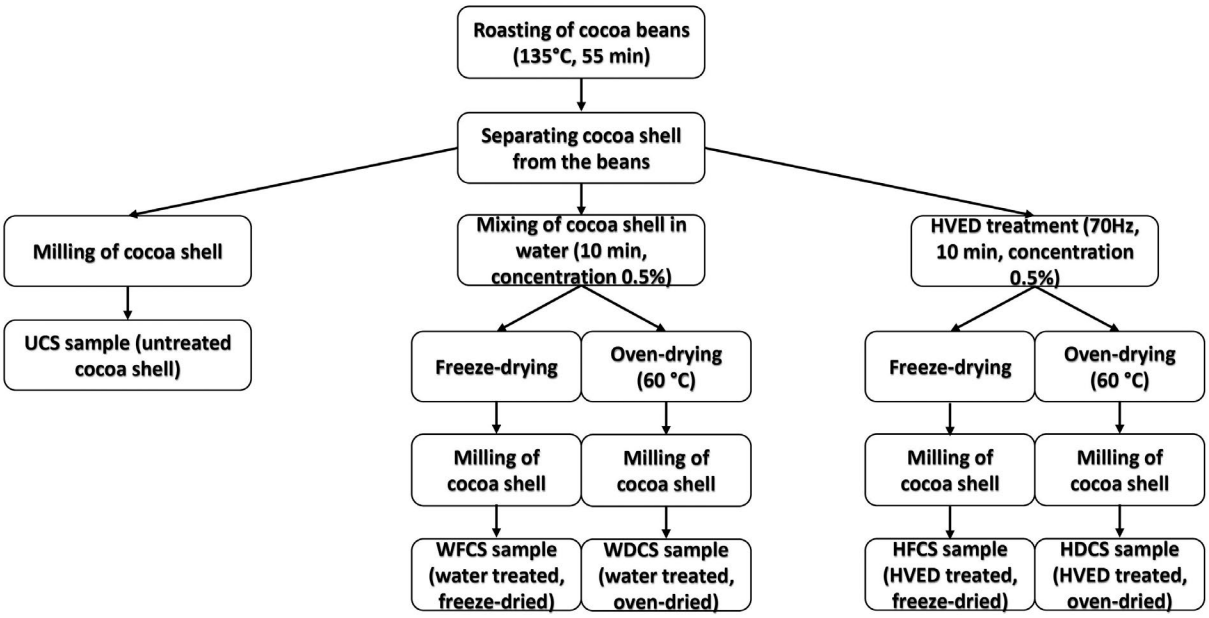


FIGURE 1 Schematic presentation of sample preparation

60°C until constant mass, and another part by freeze-drying (WFCS and HFCS samples).

Prior to freeze-drying, samples were frozen at −80°C. Pressure during the main drying was 0.250 mbar and during final drying 0.050 mbar. All samples (25 g) were milled in a laboratory mill (IKA, M20) for 2 min. As such, samples were stored until analyses.

2.2 | Water content

The water content of cocoa shell samples was determined according to ISO 6540 method (ISO, 1980). Samples (3 ± 0.1 mg) were dried in a laboratory oven (Memmert, UFE 500) at 105°C until a constant mass was achieved. Measurements were conducted in two repetitions and water content was calculated according to (1):

$$w\,[\%] = \frac{m_2}{m_1} \times 100 \tag{1}$$

where w (%)—water content, m_1 (g)—sample mass before drying, and m_2 (g)—sample mass after drying.

2.3 | Water activity

Water activity was determined using HydroLab 3 (Rotronic, Loveland, USA) which is calibrated in 0.000 to 1.000 a_w range. Measurements were conducted after drying in two repetitions and were presented as average results ± standard deviation.

2.4 | Water and OBC

Water-binding capacity (WBC) and OBC were determined using AACC Method 88–04 (AACC, 1983). 30 ml of water (for WBC) and 30 ml of cold-pressed linseed oil (for OBC) were added to 2.5 g of the cocoa shell. Mixtures were left to stand for 30 min with periodic mixing. After that, mixtures were centrifuged for 15 min at 3,000 rpm (Centra-MP4R, IEC). The supernatant was decanted and the remaining residue was weighted. Results were calculated according to (2) and were expressed as grams of water (for WBC) and oil (for OBC) absorbed per gram of cocoa shell (g/g). Analyses were performed in two repetitions from which average result and standard deviation were calculated.

$$\text{WBC/OBC}\left[\frac{\text{g}}{\text{g}}\right] = \frac{\text{gell mass}}{\text{dry matter mass in the initial sample}} \tag{2}$$

2.5 | Color

The color of cocoa shell samples was determined with chromameter Konica Minolta CR-400 (Tokyo, Japan). Measurements were conducted five times for every sample and were performed in a CIE $L^*a^*b^*$ and $L^*Ch°$ systems. L^* value is for lightness (0 is black and 100 white), a^* for redness (positive values) or greenness (negative values) and b^* for yellowness (positive values) or blueness (negative values). Using the following Equation (3) total color change (ΔE) was calculated. In the equation L_0^*, a_0^*, and b_0^* represent UCS, while L^*, a^*, and b^* represent values for the treated samples.

$$\Delta E = \sqrt{(L-L_0)^2 + (b-b_0)^2 + (a-a_0)^2} \tag{3}$$

2.6 | Specific volume, apparent, and bulk density

Specific volume, apparent, and bulk density were measured according to the method described by de Escalada Pla et al. (2012). Apparent density was determined by measuring the volume that 3 g of cocoa shell occupies in 10 ml graduated cylinder. The cylinder was gently taped until there was no more decrease in sample level. Specific volume was calculated as the inverse of apparent density (ρa^{-1}). Bulk density (ρb) was determined by pouring ground cocoa shell in a graduated cylinder up to the mark for 10 ml. Cylinder with samples was weighed at analytical balance.

For every sample, measurements were done in triplicate. Mean values and standard deviation were calculated for each sample.

2.7 | Total phenolic content

Milled samples were weighed (1 g) and lipids were removed by extracting three times with 10 ml n-hexane (mass of samples after defatting 0.9381–0.9778 g). Samples were left to dry overnight and after that 5 ml of 70% methanol was added for the extraction in an ultrasound bath (30 min, 80/320 W, 35 kHz). After the extraction, samples were centrifuged at 3,000 rpm for 10 min. The supernatant was transferred in 10 ml volumetric flask. Extraction was repeated one more time, and a flask with supernatant was filled up with 70% methanol (Belščak et al., 2009).

For the determination of total phenolic content (TPC), Folin–Ciocalteu method was used (Singleton et al., 1999). In a volumetric flask (10 ml), 100 µl of extract, 6 ml of water, and 500 µl of undiluted Folin–Ciocalteu reagent were added. After 6 min 1.5 ml of 20% Na_2CO_3 was added and the flask was filled up with water. Mixtures were left in dark for 2 hr after which absorbance was measured at 760 nm (1 ml of mixture) against blank (spectrophotometer Shimadzu, UV-1800). Analyses were performed in triplicate for every sample. TPC was expressed as mg of gallic acid (0.02–0.5 mg/ml gallic acid used for calibration curve) per g of defatted sample.

2.8 | Tannin content

The method was described by Amorim et al. (2008). Sample extracts for the determination of tannin content in cocoa shell were prepared in the same way as for TPC. Tannin content was calculated as the difference between TPC and residual phenolic content (determined

as described above) obtained after tannin complexation with casein. In extract with residual phenolic content tannins were removed from the medium through complexation with casein. The calibration curve was done with tannic acid (0.5–3 mg/ml) and results are expressed as mg of tannic acid per g of defatted sample and as a percentage of tannins in TPC.

2.9 | Klason lignin

Klason lignin content was determined according to the method described by Kirk and Obst (1988). Cocoa shell samples were digested with 72% sulfuric acid, after which mixtures were diluted and secondary hydrolysis was conducted in the autoclave at 120°C for 1 hr. Mixtures were filtered, dried and residues were ashed. Results are expressed as a percentage of lignin insoluble in 72% H_2SO_4.

2.10 | Determination of methylxanthines and phenolic components by HPLC method

Extracts prepared for TPC were used for HPLC determination of methylxanthines and phenolic components. Extracts were filtered through 0.45 μm nylon-membrane filter before the analysis. Chromatographic conditions used in this study were described by Barišić, Flanjak, Križić, et al. (2020). Mobile phase: solvent A was 1% formic acid and solvent B was HPLC grade methanol. Gradient elution was performed and the flow rate of the mobile phase was 0.8 ml/min. In the beginning, solvent B accounted for 10% in the mobile phase, after that solvent B was linearly increased to 32% at 15 min, 40% at 20 min up to 25 min, and 60% at 30 min. Injection volume was 20 μl and column and detector temperatures were set at 30°C. The monitoring wavelength range was between 200 and 400 nm, while the detection wavelength was set at 278 nm. Based on the absorbance spectrum and retention time of pure components, phenolic components and methylxanthines were identified. For quantification of identified components, external calibration method was performed. Concentrations of compounds used for calibration: gallic, caffeic, and *p*-coumaric acid, (−)-epicatechin, 0.03–0.16 mg/ml, (−)-epicatechin gallate, and (+)-catechin 0.02–0.09 mg/ml, caffein 0.01–0.26 mg/ml, theobromine 0.02–0.50 mg/ml. Analysis was done in duplicate for each sample and the results were expressed as mg of specific component per g of a defatted cocoa shell (mg/g).

2.11 | Differential scanning calorimetry

Differential scanning calorimetry (DSC 822^e, Mettler Toledo, Switzerland) was used to determine the thermal properties of cocoa shell. The DSC was calibrated with indium before use. The cocoa shell samples were weighed (4–5 mg) in 40 μl aluminum pans. An empty aluminum pan was used as a reference. Samples were heated from 25 to 420°C with a constant heating rate at 20°C/min. Phase properties as the onset (T_{go}), midpoint (T_{gm}), and endpoint (T_{ge}) of T_g and specific heat changes (ΔH), were analyzed by DSC thermogram using a STAR^e Evaluation V6_V12 Conversation software. Each sample was analyzed in duplicate and mean values and standard deviations were calculated.

2.12 | Statistical analysis

To determine the effect of drying and HVED-treatment on the properties of cocoa shell, one-way ANOVA correction with Welch *F*-test was used because it was determined that groups had unequal variances. Statistical analysis was conducted using Statistica, Version 13.4.0.14 (1984–2018 TIBCO Software Inc., California, USA). Results were considered significant at a *p*-value of .05. In this analysis, we determined the statistical significance of treatment (HVED treated and water control compared) and drying (freeze-dried and oven-dried compared).

3 | RESULTS AND DISCUSSION

3.1 | Water content and water activity

Table 1 shows that UCS had the highest water content. HVED-treated oven-dried samples (HDCS) had a lower water content than oven-dried samples mixed with water (WDCS). This shows that HVED could have disturbed the structure of the cocoa shell due to electroporation and thus cause easier water removal than

TABLE 1 Water content and water activity of cocoa shell samples

Sample	Water content (%)	a_w	WBC (g/g)	OBC (g/g)
UCS	9.651 ± 0.048	0.471 ± 0.002	5.288 ± 0.038	1.598 ± 0.013
WDCS	6.687 ± 0.027	0.153 ± 0.001	6.237 ± 0.056	1.578 ± 0.000
HDCS	4.936 ± 0.023	0.057 ± 0.001	6.575 ± 0.086	1.523 ± 0.072
WFCS	3.100 ± 0.005	0	6.047 ± 0.062	2.054 ± 0.043
HFCS	3.780 ± 0.013	0	6.136 ± 0.012	2.031 ± 0.085

Abbreviations: HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried.

in the sample that was not treated with HVED. Brahim et al. (2017) concluded that high-energy shock waves produced during HVED treatment had induced fragmentation of cellulose. Freeze-dried samples had the lowest water content, but there was no significant difference between cocoa shell mixed with water (WFCS) and HVED-treated cocoa shell (HFCS) (Table 2) because freeze-drying is much more efficient for the removal of bound and free water (Trelea et al., 2015). Borchani et al. (2011) also concluded that freeze-drying gives samples with lower water content compared to oven-drying.

The water activity of all samples showed the same trend as the water content. Although the UCS had the highest a_w, all other samples had sufficiently low values to prevent microbial growth. Water activity shows that freeze-dried samples did not have water that would be available for chemical and biochemical reactions. Although WFCS and HFCS had the same water activity, HFCS had slightly higher water content. This indicates that HVED caused the entrapment of water in the cocoa shell matrix. HVED affected the easier release of free water during drying, but it also caused a part of the water to be more bound within the cocoa shell matrix which can be seen from the water content of WFCS and HFCS samples.

3.2 | Water and oil binding capacities

Water- and oil-binding capacities are physical properties that provide information regarding pore volume and behavior of materials in the food matrix (de Escalada Pla et al., 2012). Freeze-dried and oven-dried samples had higher values of WBC compared to the UCS, but the increase was slightly more pronounced in oven-dried samples. Guillon and Champ (2000) stated that production processes could modify the physical properties of fiber matrix, which affects hydration properties. HVED generates reactive species that could react with the matrix of cocoa shell and make it more susceptible to water uptake. In previous research by Macedo et al. (2020) where the cold plasma was used for modification of fibers, results showed that OBC was increased in modified fibers. This phenomenon can be the result of surface erosion of fibers and rearrangement of hydrophobic groups on surface which then have increased affinity for nonpolar liquids. Results in the present research show that HVED treatment caused increased affinity for polar liquids like water, indicating that plasma created in water probably induces the number of hydrophilic groups on fiber surfaces.

Oil-binding capacity (OBC) depends on material composition, surface properties, and hydrophobic nature of particles

TABLE 2 One-way ANOVA correction with the Welch *F*-test

	Grouping variable	Welch *p*-value		Grouping variable	Welch *p*-value
Dry matter	Drying	**.012761**	Bulk density	Drying	**.000010**
	Treatment	.650726		Treatment	.377573
a_w	Drying	–	Theobromine	Drying	.672998
	Treatment	.366180		Treatment	**.015449**
WBC	Drying	**.053271**	Caffeine	Drying	.892681
	Treatment	.214023		Treatment	**.018278**
OBC	Drying	**.000091**	(+)-Catechin	Drying	**.024334**
	Treatment	.858107		Treatment	.162426
L^*	Drying	**.016322**	(−)-Epicatechin	Drying	**.003978**
	Treatment	.355509		Treatment	.923919
a^*	Drying	**.004862**	(−)-Epicatechin gallate	Drying	**.063741**
	Treatment	.612508		Treatment	.393741
b^*	Drying	**.055428**	Gallic acid	Drying	–
	Treatment	**.000424**		Treatment	.889654
C	Drying	.141808	Caffeic acid	Drying	.371941
	Treatment	**.000005**		Treatment	**.006793**
$h°$	Drying	**.013098**	*p*-Coumaric acid	Drying	.813538
	Treatment	**.048762**		Treatment	**.000550**
ΔE	Drying	.083833	Total phenolic content	Drying	**.003429**
	Treatment	**.000173**		Treatment	.445195
Specific volume	Drying	**<.000001**	Klason lignin	Drying	.470626
	Treatment	.901643		Treatment	.426702
Apparent density	Drying	**<.000001**	Tannins (%)	Drying	**.005559**
	Treatment	.975265		Treatment	.007030

Note: Bold values are considered significant at $p < .05$.

TABLE 3 Color parameters of cocoa shell samples

Sample	L^*	a^*	b^*	C	$h°$	ΔE
UCS	46.72 ± 0.00	9.59 ± 0.03	18.72 ± 0.02	21.03 ± 0.02	62.86 ± 0.09	
WDCS	47.03 ± 0.01	8.48 ± 0.01	17.15 ± 0.03	19.13 ± 0.03	63.68 ± 0.04	1.94 ± 0.02
HDCS	44.18 ± 0.01	8.77 ± 0.03	16.47 ± 0.03	18.66 ± 0.03	61.96 ± 0.11	3.49 ± 0.02
WFCS	46.28 ± 0.09	8.53 ± 0.07	17.17 ± 0.04	19.18 ± 0.01	63.58 ± 0.22	1.93 ± 0.02
HFCS	47.91 ± 0.02	8.33 ± 0.05	16.96 ± 0.03	18.90 ± 0.03	63.85 ± 0.16	2.47 ± 0.03

Abbreviations: HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried.

(Borchani et al., 2011). Table 2 shows that freeze-dried samples had the highest OBC. De Escalada Pla et al. (2012) also reported larger OBC when freeze-drying was applied than in natural and air-dried samples. They stated that material can uptake more oil when it has a higher specific volume. Our results also showed the same correlation. These two properties are very important to determine the behavior of particular material in food systems.

3.3 | Color

Materials that are incorporated in food systems affect organoleptic parameters and color is one of them that is very important to consumers. The color parameters of cocoa shell samples are presented in Table 3. HVED-treated samples had lower L^* values after oven-drying (60°C) and higher L^* values after freeze-drying (−40 to 20°C), showing that oven-drying produced a darker sample and freeze-drying gave a lighter sample of HVED-treated samples. All samples were in the domain of red and yellow color which can be seen from positive a^* and b^* values. It is known that high temperatures can cause non-enzymatic browning which leads to darker samples (Borchani et al., 2011). The darkening of samples after heating can be partially explained with non-enzymatic oligomerization of catechins that results in the creation of yellow to brown complex components (Gadkari & Balaraman, 2015). In this research, the cocoa shell had been thermally treated (roasted) prior to treatments applied here, so enzymatic browning may be excluded as the reason. However, non-enzymatic browning reactions that had started during roasting are advanced by the subsequent thermal treatment in the present research (drying). Since freeze-drying is conducted at much lower temperatures it is expected that it would result in higher L^* values (brighter color).

HDCS sample had the lowest L^* value which implies that HVED treatment contributed to the creation of color precursors, which reacted during oven-drying and resulted in a darker sample. In our previous study (Barišić, Flanjak, Tot, et al., 2020) where 5-hydroxymethylfurfural (5-HMF) and acrylamide content of HVED-treated cocoa shell was determined, it was stated that treatment could favor further reaction of these components. Drying had a statistically significant effect on L^* and a^* parameters, while HVED treatment had a statistically significant effect on C and ΔE. Also, Table 3 shows that b^* and $h°$ were affected by both drying and HVED treatment. After all treatments samples had a less pronounced red and yellow color, shown by lower a^* and b^* parameters. Also, HVED had a larger effect on the decrease in yellow color than water control samples. Samples WDCS, WFCS, and HFCS had ΔE values which indicate that color change can be easily noticed by trained analyst and ΔE values of HDCS had shown that color change could be noticed by the untrained eye (Jukić et al., 2007).

TABLE 4 Specific volume, apparent, and bulk density of powdered cocoa shell samples

Sample	Specific volume (cm^3/g)	Apparent density (g/cm^3)	Bulk density (g/cm^3)
UCS	2.300 ± 0.054	0.435 ± 0.010	0.481 ± 0.014
WDCS	2.022 ± 0.031	0.495 ± 0.008	0.438 ± 0.009
HDCS	2.067 ± 0.047	0.484 ± 0.011	0.411 ± 0.011
WFCS	2.922 ± 0.068	0.342 ± 0.008	0.346 ± 0.013
HFCS	2.811 ± 0.016	0.356 ± 0.002	0.319 ± 0.012

Abbreviations: HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried.

3.4 | Specific volume, apparent, and bulk density

Physical characteristics (specific volume, apparent density, and bulk density) of the cocoa shell are presented in Table 4. These parameters are important for the transportation, storage, and packaging of powdered materials and foods (Chaloeichitratham et al., 2018). Main changes are visible for samples that were freeze-dried, as a result of water sublimation during freeze-drying which produces high porosity of samples (Chaloeichitratham et al., 2018). Bulk density was slightly lower for HVED-treated samples. This parameter depends on water content and pore volume, which is affected by matrix structure and to what extent it is collapsed (de Escalada Pla et al., 2012). HVED-treated samples had lower water content, and HVED could have influenced the pore volume of the cocoa shell, as it has been proven for inorganic compounds (De Coste et al., 2015) and organic cells (Moreau et al., 2008). Statistical analysis showed that drying had a significant effect on these parameters while HVED did not. Therefore, it can be concluded that the amount of water in

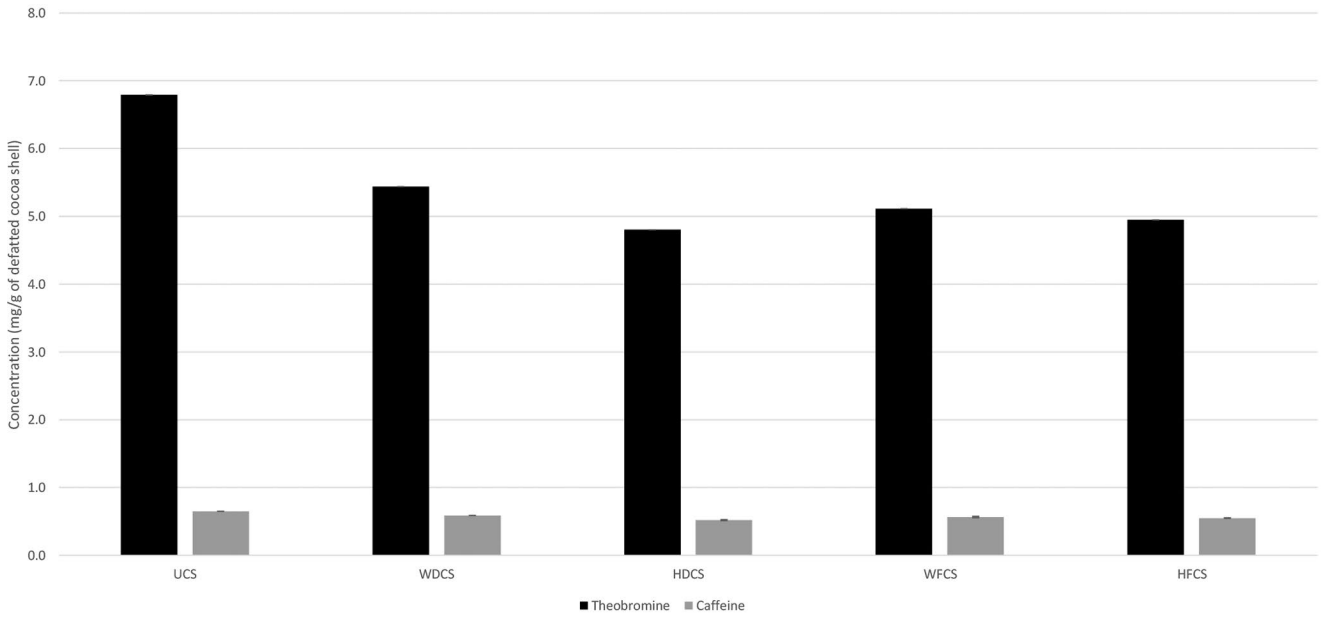


FIGURE 2 Content of methylxanthines in cocoa shell samples. HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried

the samples and the way in which water exits from material, that is, consequences that occur and affect the structure of material have the largest effect on these parameters.

3.5 | Methylxanthines, phenolic components, total phenolic, and tannin contents

The UCS had the highest content of theobromine and caffeine (Figure 2). HVED treatment had a statistically significant effect on the content of these methylxanthines while the drying method did not. This shows that methylxanthines are stable at a temperature of 60°C while HVED causes a decrease in methylxanthine content. In case of freeze-drying, there was no big difference in methylxanthines content between HVED-treated and control. This could be because of HVED induced extraction of these compounds in water, oxidation induced by radicals created during HVED treatment, or a combination of both phenomena where higher temperature (60°C) caused further oxidation. The possible oxidation reaction of caffeine induced by Fenton and/or Fenton-like reagent, as shown by de Oliveira et al. (2015), is supported by the reduction of ^{56}Fe and ^{57}Fe content in cocoa shell after the HVED treatment (results not presented in this paper) and the fact that H_2O_2 is formed in water during HVED treatment, and Dalmazio et al. (2005) proposed mechanisms of caffeine degradation through oxidation, reaction with OH· radicals and water molecules, all present in HVED-treated water. Additionally, Stadler et al. (1996) have shown that catechins, which are present in cocoa shell, improve oxidation of caffeine. Jokić et al. (2019) reported that HVED had induced extraction of theobromine and caffeine, however, the yield of these compounds in water extracts depended on the conditions of treatment (solvent:solid ratio, frequency, and time of HVED treatment). Due to complexity of possible reactions further investigation with other instrumental techniques is needed in order to confirm which of the proposed mechanism prevails.

UCS had the highest content of (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate (Figure 3). Also, freeze-dried samples had a higher content of these compounds than oven-dried samples. Drying showed a statistically significant effect on the content of these compounds, while treatment did not. One of the reasons for this situation if the fact that catechins are highly susceptible to high temperatures, oxidation, light, and alkaline medium. During the heat treatment, many different reactions occur between catechins and other cocoa shell components (e.g., proteins, enzymes, and caffeine) but also between catechins themselves (Gadkari & Balamaran, 2015). Fan et al. (2016) have conducted research on thermal stability of different catechins and showed that epicatechin was the least stable to high temperatures (60 and 90°C) among evaluated catechins. The lowest stability of epicatechin was attributed to epimerization reaction. (−)-epicatechin showed the lowest stability toward heating in this study as well (Figure 3). HDCS sample had a higher content of (−)-epicatechin and (−)-epicatechin gallate than the WDCS sample. Freeze-dried samples had the opposite trend. In our previous study (Barišić, Flanjak, Križić, et al., 2020) HVED-treated and oven-dried samples had a higher content of phenolic components than control samples treated only in water, but the applied temperature of oven drying was lower (40°C). (−)-Epicatechin and (−)-epicatechin gallate also had the largest percentage of retaining. As it was stated in the previous paper, HVED treatment could induce interactions

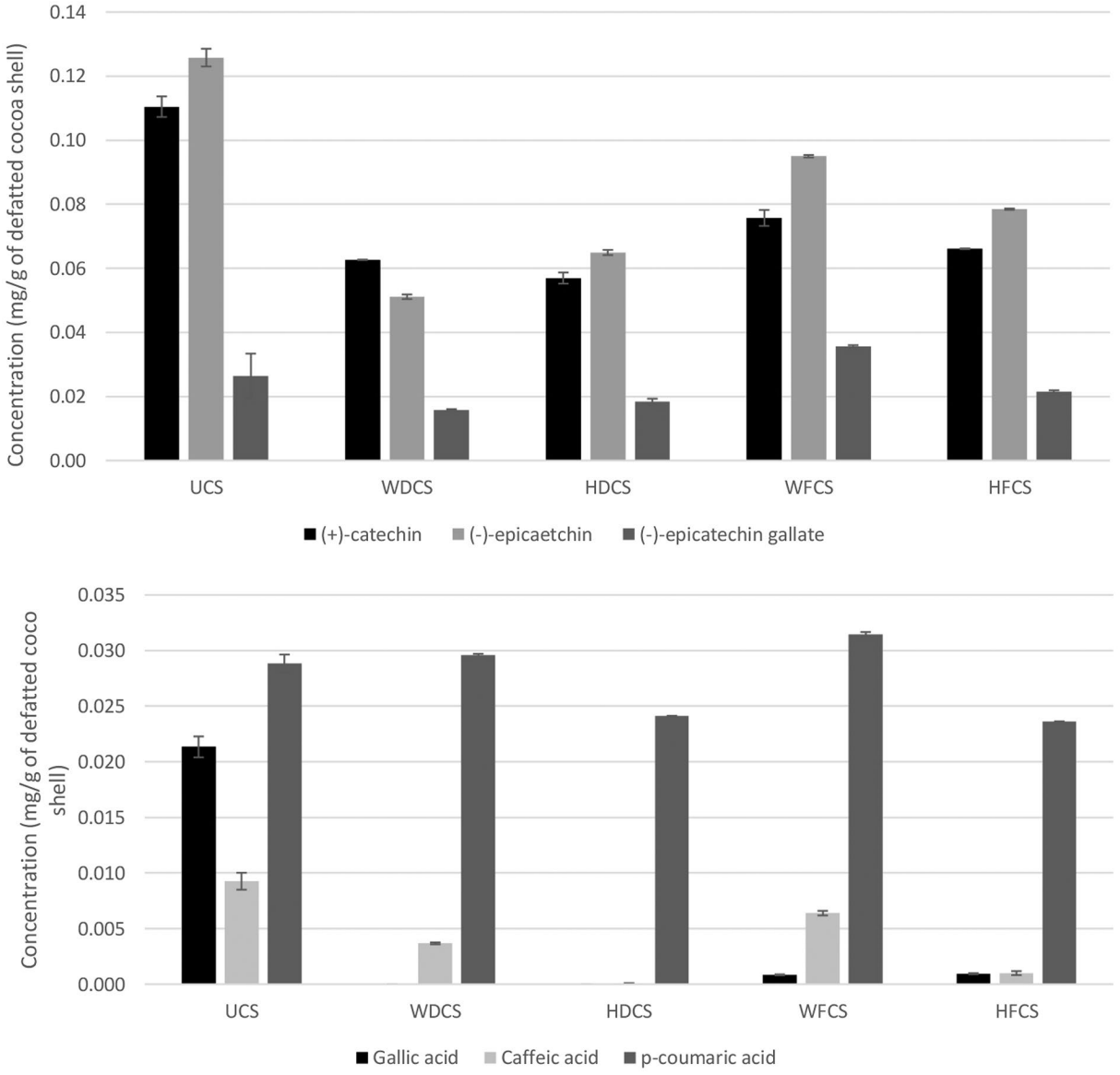


FIGURE 3 Contents of phenolic components in cocoa shell samples. HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried

of phenolic compounds with fibers and protect phenols during oven-drying.

Gallic acid showed the lowest stability after the treatment and different drying technologies. It was not detected after the oven-drying and very low concentrations were detected after the freeze-drying (Figure 3). The results of phenolic acid degradation can be partially explained by differences in the thermal stability of phenolic acid. Namely, as reported Setyaningsih et al. (2016) degradation of gallic acid was observed at 60°C while p-coumaric acid and caffeic acid remained stable at that temperature. Statistical analysis (Table 2) showed that the treatment had a statistically significant effect on caffeic and *p*-coumaric acids. Figure 3 shows that the WFCS sample had the highest content of *p*-coumaric acid. There is a possibility that this component was easier to extract after freeze-drying because of pores created during sublimation.

UCS had the largest and WDCS had the lowest TPC among all cocoa shell samples (Figure 4). Statistical analysis showed that treatment had a significant effect on TPC (Table 2). Freeze-dried samples had a higher content of total phenols than oven-dried samples. This is in the agreement with the study of Borchani et al. (2011) where freeze-dried samples had the highest TPC compared to oven- and sun-dried samples. Food industry by-products are known to have a certain percent of phenolic components, which is important for the reuse of such materials due to the positive effect of phenolic compounds on human health and the prevention of diseases (Borchani et al., 2011). Orphanides et al. (2013) and Roslan et al. (2020) also reported lower degradation of phenolic components and TPC in freeze-dried samples compared to the conventional heated samples.

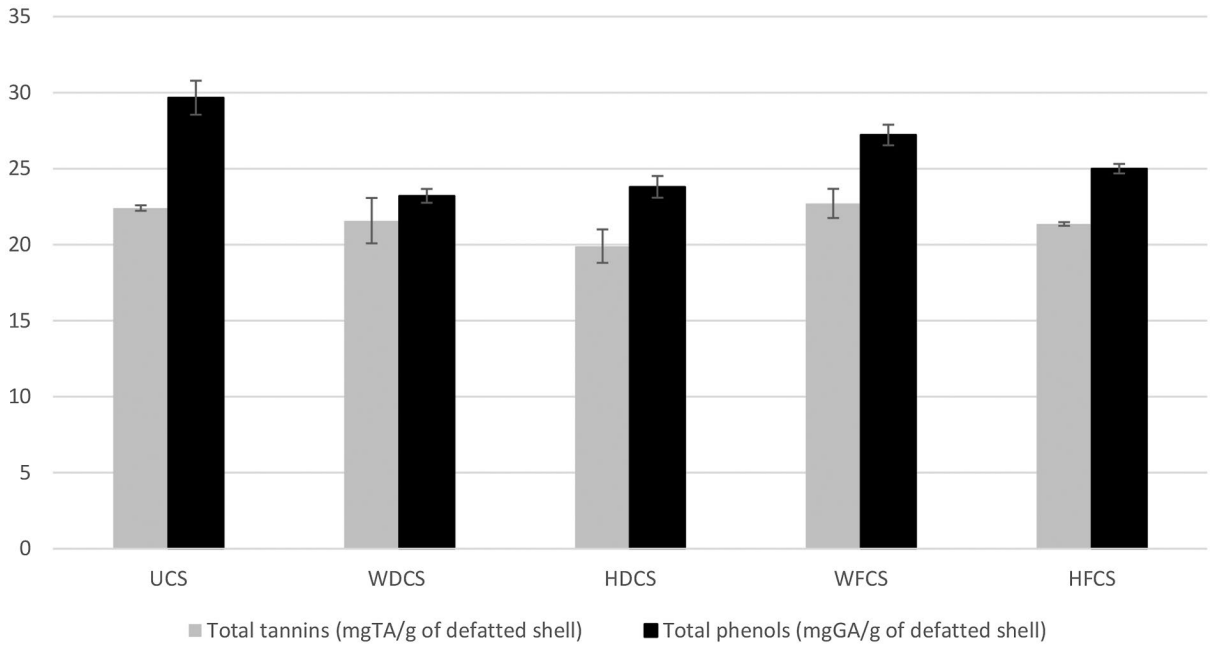
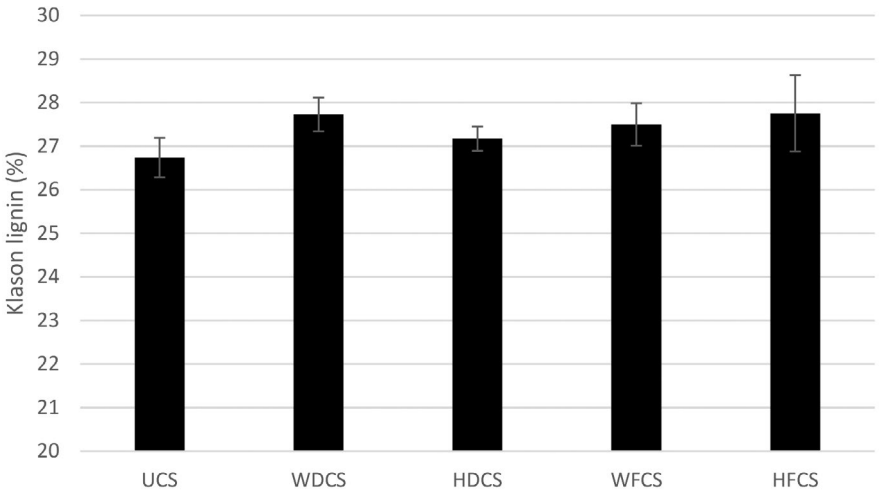


FIGURE 4 Total phenolic and tannin content of cocoa shell samples. HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried

FIGURE 5 Klason lignin content of cocoa shell samples. HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried



The tannin content of cocoa shell samples showed a similar trend as the TPC, although tannins were more resistant to HVED-treatment and drying technologies (Figure 4). As for the results of phenolic components, it can be concluded that larger molecules are more resistant to HVED treatment and drying conditions. This is also supported by the results for gallic acid, which was not detected after oven-drying at 60°C.

3.6 | Klason lignin

In our previous paper where cocoa shell was treated with HVED, analysis of dietary fiber content showed that treatment increased the content of insoluble dietary fibers (Barišić, Flanjak, Kopjar, et al., 2020). Since it was already reported that the content of insoluble dietary fiber of cocoa shell is overrated because of the high contents of Klason lignin (Redgwell et al., 2003) in this study we determined that fraction to determine if the increase of insoluble dietary fibers after HVED is caused by Klason lignin fraction. Klason lignin represents lignin that is not soluble in 72% sulfuric acid. It is well established that acid-insoluble fraction does not have to be just lignin. In research by Li et al. (2007) results showed that approximately 50% of Klason lignin in hydrothermally treated samples was lignin. This phenomenon is also seen in acid-treated materials and is termed as pseudo-lignin. Our results of Klason lignin show that all treated cocoa shells had a higher content of Klason lignin than the UCS. HVED treatment and drying technology could both have an effect (Figure 5). The lignin-like structure can be created through a reaction of substitution on lignin aromatic rings (Shinde et al., 2018).

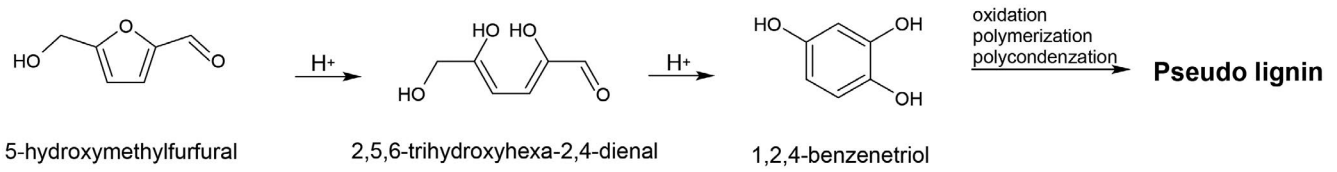


FIGURE 6 Proposed mechanism for pseudo lignin formation

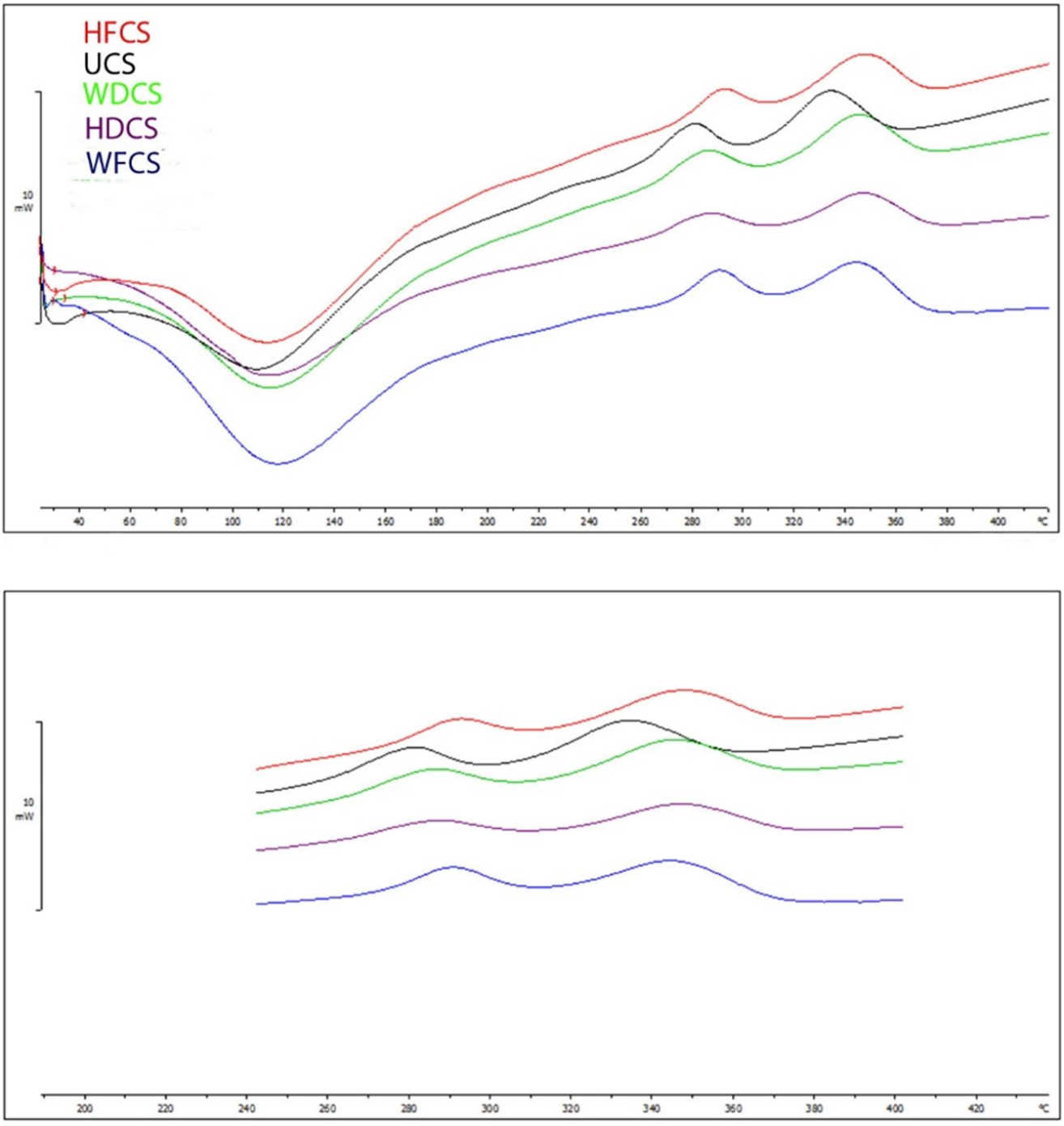


FIGURE 7 Differential scanning calorimetry curves of cocoa shell samples. HDCS, HVED-treated cocoa shell and oven-dried; HFCS, HVED-treated cocoa shell and freeze-dried; UCS, untreated cocoa shell; WDCS, cocoa shell mixed with water and oven-dried; WFCS, cocoa shell mixed with water and freeze-dried

In our previous research on the effect of HVED on 5-HMF, the mechanism for the further reaction of this compound and the formation of new organic compounds was proposed (Barišić, Flanjak, Tot, et al., 2020). One of the proposed formations of pseudo-lignin is the conversion of 5-HMF to other aromatics. These key intermediates can polymerize to a three-dimensional polymer or can be bound to exist lignin, which then acts as Klason lignin. Previous results of cocoa shell treated with HVED showed the formation of the shoulder at FTIR-ATR spectra which is characteristic of aromatic C–H vibrations of lignin (Barišić, Flanjak, Kopjar, et al., 2020). Since lignin is an aromatic biopolymer this formation of the mentioned shoulder could be one of the indicators for increased Klason lignin content. One of the proposed mechanisms for the formation of pseudo-lignin is presented in Figure 6. The proposed reaction can be conducted in water medium with present H^+, which is highly possible in this research, with electrical discharges created directly in water. Normally, during the treatment of lignocellulosic materials, this reaction has been described to start from the carbohydrates from which 5-HMF is formed (Shinde et al., 2018), but in our case, since 5-HMF is already present in cocoa shell, reaction would occur with fewer steps and over a shorter period of time.

3.7 | Differential scanning calorimetry

Differential scanning calorimetry showed an endothermic peak from 110 to 120°C and two exothermic peaks in area 280–347°C for all samples of cocoa shell. The exothermic peaks that appeared after 280°C are characteristic of hemicellulose, cellulose, and lignin (Bargougui et al., 2018). Figure 7 shows that the peaks shifted to higher temperatures in all treated samples, showing that they are more thermostable. The greatest thermostability at the first peak was observed for freeze-dried HVED-treated (HFCS) sample, where the peak appeared around 292.39°C, while in the UCS that peak was around 280.86°C. The second peak was also shifted greatly in HVED-treated samples (HFCS and HDCS) and it was around 347°C, while in the UCS the same peak was at 335.27°C. In the study conducted by Bargougui et al. (2018) where the cocoa shell was modified, these two peaks were also shifted to higher temperatures. They concluded that this was due to morphological changes in the material. Our previous study of HVED-treated cocoa shell showed that a larger peak, characteristic for C–H deformation, vibration of CH_2 and CH_3 groups of carbohydrates appeared at FTIR-ATR spectra (Barišić, Flanjak, Kopjar, et al., 2020). These results, combined with DSC analysis of HVED-treated cocoa shell show that, clearly, HVED causes some modification of carbohydrates present in the cocoa shell. Also, one of the reasons for the increased thermostability of cocoa shell after treatments could be because of the increased content of lignin. In the study conducted by Fu et al. (2020) lignocellulose nanofibrils with larger content of lignin showed higher temperatures of degradation.

4 | CONCLUSIONS

HVED and drying by two different methods (freeze- and oven-drying) induced changes in physical properties of cocoa shell. HVED treatment and freeze-drying most probably modified matrix structure and disturbed fibers, which are the most abundant component of the cocoa shell. Oven-drying affected color of samples more than freeze-drying. Also, water content was higher for oven-dried samples. Phenolic components and methylxanthines were more preserved in freeze-dried samples, although HVED treatment decreased the content of these components. This shows that cocoa shell could be modified by a combination of different treatments and drying technologies to make it more suitable for addition to a particular type of food to which such characteristics correspond. In addition, the part of bioactive components, which are desirable components of food, can be preserved after different treatments. Tannins showed high resistance to treatment and drying conditions. Also, this study shows that some modifications of lignin, cellulose, and hemicellulose occur during HVED treatment, which needs to be further studied. This study could bring a new perspective for the use of HVED and various drying technologies, not only for cocoa shells but also for treating other by-products of the food industry. HVED treatment and its modification of cocoa shell components could bring a new perspective to the pre-treatment of the cocoa shell for use as an adsorbent and for the production of biofuels.

ACKNOWLEDGMENTS

This work has been supported in part by the Croatian Science Foundation under the project “Application of cocoa husk in production of chocolate and chocolate-like products” 2017-05-8709.

CONFLICT OF INTERESTS

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Veronika Barišić: Conceptualization; Formal analysis; Writing – original draft. **Ivana Flanjak:** Formal analysis; Writing – original draft. **Ante Lončarić:** Investigation; Methodology. **Anita Pichler:** Methodology; Writing – review & editing. **Antun Jozinović:** Visualization; Writing – review & editing. **Jurislav Babić:** Writing – review & editing. **Drago Šubarić:** Writing – review & editing. **Borislav Miličević:** Writing – review & editing. **Đurđica Ačkar:** Conceptualization; Project administration; Writing – original draft.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Barišić, V., Flanjak, I., Lončarić, A., Pichler, A., Jozinović, A., Babić, J., Šubarić, D., Miličević, B., & Ačkar, Đ. (2022). Valorization of cocoa shell: Impact of high voltage electrical discharge and drying technology on properties of cocoa shell. *Journal of Food Processing and Preservation*, 00, e16308. https://doi.org/10.1111/jfpp.16308